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Influence of dispersive agent on nanomaterial agglomeration and implications for biological effects in vivo or in vitro

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A B S T R A C T
Comparing the dispersing properties of the porcine lung surfactant Curosurf® to bovine serum albumin (BSA), the present study investigated how a more close simulation of the in vivo situation influences nanomaterial dispersion and hence the proportion of the administered dose that will reach the in vitro test system, i.e. the ‘effective dose’. Dispersions of 16 OECD reference nanomaterials (ZnO, Ag, TiO2, CeO2, SiO2, and multi-walled carbon nanotubes (MWCNTs)) were assessed. Overall, the NMs were better dispersed in the BSA-supplemented medium. BSA-addition combined with ultrasonication proved most effective in deagglomerating MWCNTs, but also reduced agglomeration for most metal oxide nanomaterials as compared to the Curosurf® dispersions. However, all materials were at least partially agglomerated in either dispersing agent. For the different nanomaterials, the calculated effective dosage upon 12- or 24-h test substance incubation differed considerably (and to different extents) depending on the applied dispersing agent. When testing nanomaterial effects in vitro, selection of the type of cell culture medium and its additives should take into account what the system is intended to mimic. Study protocols should address whether they aim at best-possible dispersion of the nanomaterials or at simulating more realistically in vivo tissue uptake and distribution.

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1. Introduction

Integrated approaches for the testing and assessment of nanomaterials (NMs) that include in vitro methods are under discussion to streamline animal testing (Oomen et al., 2014). To date, however, in vitro--in vivo correlations of NM toxic potency are unsatisfactory. The complexity of NM dosimetry is addressed as contributing to this problem (Cohen et al., 2014; DeLoid et al., 2014; Landsiedel et al., 2014). Applied NM dosages are not identical with effective dosages reaching cells (in vitro or in vivo; Cohen et al., 2014). The effective dosage is influenced by the sedimentation and diffusion properties that different NMs exhibit under the given cell culture conditions, which largely depend upon the effective densities and diameters of the suspended NM agglomerates (Teeguarden et al., 2007; Schaefer et al., 2012; DeLoid et al., 2014).

In culture media or other biological fluids, nanoparticles (NPs) interact with proteins or phospholipids thereby forming a characteristic ‘corona’ on their surface (Lundqvist et al., 2011; Monopoli et al., 2011, 2012). An NM’s tendency to agglomerate is governed by its surface properties that can change spontaneously due to protein adsorption (Schaefer et al., 2012). Consequently, corona formation directly affects the type and extent of NM agglomeration, and the type of NM dispersing agent used influences in vitro particokinetics (Ruge et al., 2012; DeLoid et al., 2014). Different NM dispersants have been investigated, including natural lung surfactants (Wang et al., 2010a, 2010b), phospholipids (Sager et al., 2007; Vippola et al., 2009), organic solvents (Soto et al., 2005), and serum or albumin additives (Bihari et al., 2008; Vippola et al., 2009; Schulze et al., 2011).

Comparing the dispersing properties of the porcine lung surfactant Curosurf® to bovine serum albumin (BSA), we assessed how more closely simulating the in vivo situation upon inhalation influences NM dispersion. Sixteen OECD reference nanomaterials (OECD, 2010) covering metal and metal oxide NMs and multi-walled carbon nanotubes (MWCNTs) were prepared in BSA- or Curosurf®-supplemented suspensions using otherwise identical

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dispersion protocols. Applied in pediatrics, Curosurf® is based upon porcine phospholipids, with a remainder of surfactant proteins (SP) SP-B and SP-C. The influence of the dispersing agents on in vitro particikinetics was assessed by comparing the NMs' hydrodynamic diameters, the proportion of non-agglomerated NMs, the amount of (non-)adsorbed albumin or phospholipid, and the calculated effective dosages.

2. Material and methods

Table 1 presents the substance names and primary particle sizes of the 16 NMs. All NMs were delivered as powders, except for Ag NM-300K, a polymer-stabilized dispersion. For further details of the supplier's physico-chemical characterization, cf. Sauer et al. (2014).

In preparing the NM dispersions, as a rule, 3 mg NMs were added to 1 mL DMEM/F-12 (Invitrogen, Germany) containing 1% (v/v) Penicillin/Streptomycin (10,000 IU/10,000 μg/mL; Biochrom AG, Germany). Only Ag NM-300K, TiO2 NM-103, ZnO NM-111, and TiO2 NM-102 were suspended at concentrations of 1, 4, 10, and 15 mg/mL, respectively (cf. Supplementary Information for the underlying considerations).

To homogenize the dispersions, they were shaken manually and sonicated twice for 2.5 min at 200 Watt with a probe sonicator (Sonopolus Ultraschall-Homogenisator, Bandelin Electronic, Germany; power: 20–30%; cycle: 100%), cooled on ice. Afterwards they were vortexed for 10 s.

For the BSA-supplemented dispersions, one part of a 5% (w/v) BSA solution (Sigma–Aldrich, Germany) was added to nine parts of dispersed NMs. All NMs were delivered as powders, except for Ag NM-300K, TiO2 NM-103, ZnO NM-111, and TiO2 NM-102 that were concentrated, and the dispersion was stirred on a magnetic stirrer at 700 rpm at room temperature for 24 h. Thereby, at least 10 mg BSA was provided per mL of dispersed particulate surface. Excess albumin is recognized as stabilizing dispersed NMs (Nepal and Geckeler, 2007; Schaefer et al., 2012).

For the Curosurf® dispersions, 1 mg test substance was prepared in 2 mL of a 1:37 dilution of Curosurf® (Nycomed Pharma, Germany, for Chiesi Farmaceutici, Italy) in water. Based upon a personal recommendation by H.J. Gall (University of Münster, Germany), this dilution was chosen to obtain 2 mg/mL Curosurf® (i.e. phospholipids) in the final preparation. The suspension was ultrasonicated and stirred as detailed for the BSA-based suspensions.

The nonmaterial dispersions were characterized by analytical ultracentrifugation (AUC) using the Beckman Ultracentrifuge XL™ with integrated interference optics (Beckman Coulter, USA). By fitting the ultracentrifuge with an optical detection system, the fractionation and sedimentation process of dispersed particles can be observed in real time (Planken and Coelfen, 2010; Wohlleben, 2012). Due to their black coloration, the MWCNT suspensions.

Table 1

<table>
<thead>
<tr>
<th>OECD reference number</th>
<th>Test material</th>
<th>Primary crystal/particle size (nm)b</th>
<th>NM dispersion in BSA-containing mediuma</th>
<th>NM dispersion in Curosurf® phospholipida</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NM diameter with BSA (nm)</td>
<td>Dispersed NM (mg/mL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM-100</td>
<td>Anatase TiO2</td>
<td>42–90 (TEM)</td>
<td>262 (28, 2.4)</td>
<td>196 (18, 3.1)</td>
</tr>
<tr>
<td>NM-101</td>
<td>Anatase TiO2</td>
<td>6 (XRD)</td>
<td>428 (29, 2.5)</td>
<td>351 (11, 2.5)</td>
</tr>
<tr>
<td>NM-102</td>
<td>Anatase TiO2</td>
<td>20 (XRD)</td>
<td>495 (22, 3.2)</td>
<td>256 (13, 2.2)</td>
</tr>
<tr>
<td>NM-103</td>
<td>Rutile TiO2</td>
<td>20 (XRD)</td>
<td>118 (22, 2.6)</td>
<td>106 (23, 2.7)</td>
</tr>
<tr>
<td>NM-104</td>
<td>Rutile-anatase TiO2</td>
<td>22 (XRD)</td>
<td>105 (2, 2.6)</td>
<td>194 (33, 2.4)</td>
</tr>
<tr>
<td>NM-110</td>
<td>Unc coated ZnO</td>
<td>41.5 (XRD)</td>
<td>79 (66, 2.8)</td>
<td>196 (31, 3.0)</td>
</tr>
<tr>
<td>NM-111</td>
<td>Coated ZnO</td>
<td>33.8 (XRD)</td>
<td>176 (75, 2.2)</td>
<td>168 (25, 2.6)</td>
</tr>
<tr>
<td>NM-200</td>
<td>Amorphous SiO2</td>
<td>20 (TEM)</td>
<td>310 (33, 2.6)</td>
<td>222 (9, 2.6)</td>
</tr>
<tr>
<td>NM-203</td>
<td>Amorphous SiO2</td>
<td>20 (TEM)</td>
<td>65 (47, 2.8)</td>
<td>380 (25, 2.3)</td>
</tr>
<tr>
<td>NM-211</td>
<td>CeO2</td>
<td>103 (XRD)</td>
<td>58 (125, 2.9)</td>
<td>66 (128, 1.9)</td>
</tr>
<tr>
<td>NM-212</td>
<td>CeO2</td>
<td>33 (XRD)</td>
<td>146 (43, 3.0)</td>
<td>155 (8, 2.4)</td>
</tr>
<tr>
<td>NM-300K</td>
<td>Ag1</td>
<td>15 (TEM)</td>
<td>107 (84, 3.2)</td>
<td>176 (64, 2.3)</td>
</tr>
<tr>
<td>NM-400</td>
<td>MWCNT</td>
<td>Ø 9.5 nm, 3.3 μm length (TEM)</td>
<td>11 (100, 3.6)</td>
<td>13 (65, 0.6)</td>
</tr>
<tr>
<td>NM-401</td>
<td>MWCNT</td>
<td>Ø 10–30 nm, 5–15 μm length (TEM)</td>
<td>30 (90, 2.7)</td>
<td>416 (28, 1.8)</td>
</tr>
<tr>
<td>NM-402</td>
<td>MWCNT</td>
<td>Ø 5–15 nm, 0.1–0.1 μm length (TEM)</td>
<td>219 (94, 3.5)</td>
<td>187 (100, 2.8)</td>
</tr>
</tbody>
</table>

Notes:

- **a** In preparing the NM dispersions, as a rule 3 mg NMs were added to 1 mL DMEM/F-12, except for Ag NM-300K, TiO2 NM-103, ZnO NM-111, and TiO2 NM-102 that were added at concentrations of 1, 4, 10, and 15 mg/mL, respectively (cf. Supplementary Information for further details on the selection of these concentrations.) For the BSA-supplemented dispersions, one part (0.1 mL) of a 5% (w/v) BSA solution (Sigma–Aldrich, Germany) was added to nine parts of the freshly prepared NM dispersions (e.g. 5 mg BSA/2.7 mg NMs dispersed in 3 mL medium culture). Thereby, it was ensured that at least 10 mg/mL of dispersed particulate surface was added to the NM suspensions. This concentration ensures excess albumin content and prevents critical depletion of protein, so that protein adsorption on the NM surface reaches an equilibrium (Schaefer et al., 2012). For the Curosurf® dispersions, 1 mg test substance was prepared in 2 mL of a 1:37 dilution of Curosurf® in water.
- **b** Primary particle/crystal size as provided by the European Commission's Joint Research Centre, the supplier of all NMs; determined either with transmission electron microscopy (TEM) or X-ray diffraction (XRD); for further details on the primary particle characterization cf. Sauer et al. (2014).
- **c** The amount of BSA or phospholipids bound to the surface of the NMs can be calculated indirectly from the concentration of non-adsorbed BSA or phospholipids measured by AUC (Schaefer et al., 2012). Of note, however, these colloidal concentrations are not chemically specific and therefore do not necessarily fully correlate with the effective content of individual components in the samples.
- **d** Coated with 1–4% triethoxycaprylyl silan.
- **e** Bimodal particle size distribution.
- **f** Monodisperse with capping agent (monolayer) stabilizing agents: 4% each of polyoxyethylene glycerol tristearate and polyoxylethylene (20) sorbitan mono-laurate (Tween 20), silver content: 10.16% w/w.
dispersions were assessed using a UV–Vis detector (Wohlleben, 2012) coupled to the AUC.

During AUC, the actual particle concentration is read directly from the optical fringe shift, further providing information on the molecular weight of free proteins, particle size distribution, shape, concentration, and protein-particle interactions (Schaefer et al., 2012; Wohlleben, 2012).

Fractions of the suspensions having particle diameters below 10 μm were assessed as ‘dispersed NM fractions’ and thus distinguished from large NM agglomerates that settle within minutes and from the dispersing proteins or phospholipids. The weight-percentages of the dispersed nanoparticles and their diameters were recorded. The amount of BSA or phospholipids bound to the nanoparticle surface can be calculated indirectly from the concentration of non-adsorbed BSA or phospholipids (Schaefer et al., 2012). However, these AUC-based concentrations are not chemically specific and therefore do not necessarily coincide with the effective content of individual components in the samples.

Taking into account NM sedimentation and diffusion properties, particle agglomeration and density, the effective in vitro dosage upon 12- or 24-h incubation was calculated using AUC-measured parameters. Additionally, for well-dispersed NMs (i.e. <50% effective dose after 24-h incubation), the effective dose was calculated using the ‘in Vitro Sedimentation, Diffusion, and Dosimetry’ model (Hinderliter et al., 2010; cf. SI for further information on these calculations).

3. Results

Overall, the NMs were better dispersed in the BSA-supplemented medium. Compared to the Curosurf® dispersions, BSA-addition combined with ultrasonication proved most effective in deglomerating MWCNTs, but also reduced agglomeration for most metal oxide NMs. In BSA-containing medium, the largest agglomerates were observed for TiO2 NM-102 and NM-103. However, all materials were at least partially agglomerated in either dispersing agent (Table 1).

BSA adsorption was lowest for the polymer-stabilized Ag NM-300K. The thicker MWCNT NM-401, which also had a lower specific surface area than NM-400 or NM-402, adsorbed less BSA than these two MWCNTs. The highest affinity of BSA was observed for three TiO2 NMs and both ZnO NMs. Hydrophobic TiO2 NM-103 adsorbed more BSA than hydrophilic TiO2 NM-104.

As a rule, Curosurf® dispersed lower amounts of NMs than BSA. Although anatase TiO2 NM-100, NM-101 and NM-102 and both ZnO NMs had smaller diameters in Curosurf® than in the BSA-supplemented dispersions, their overall level of agglomeration was higher.

For SiO2 NM-203, sedimenting masses >100% were recorded in both dispersing agents, while the Curosurf® content dropped considerably, suggesting either formation of a lipid corona or SiO2 NM-203 bridging several multilamellar Curosurf® vesicles. MWCNT NM-401 did not adsorb phospholipids, whereas Curosurf® interacted with MWCNT NM-400 and NM-402 and induced pronounced agglomeration.

As compared to full dispersion in BSA-containing medium, only 65% of Ag NM-300K was dispersed in Curosurf®, and free lipid vesicles were reduced to 0.6 mg/mL, with significantly increased vesicle masses (data not shown). This observation was unique for the suspension-delivered Ag NM-300K. Presumably, the stabilizing agents in the suspensions precipitated the lipid vesicles remote from the NMs. Such effects were not observed for any of the powder-based NMs, where the lipids had overall low affinities.

Adding either BSA or Curosurf® to the NM preparations, applying otherwise identical dispersion protocols, considerably affected the in vitro effective dosages derived from the AUC-sedimentation rates (Table 2 and Supplementary Information). But also the sampling procedure and the incubation time (12-h or 24-h) affected in vitro particokinetics: Especially for particle suspension in Curosurf®, which often resulted in low dispersed fractions, the effective dosage to adherent cells when administering a freshly generated or constantly stirred suspension (%dose of total weight) differed considerably from the administration of an unstirred suspension, whereby only the dispersed fraction is sampled (%dose of dispersed fraction).

Comparing BSA and Curosurf® suspensions, particokinetics differ least for 24-h incubation and administration of the ‘total weight fraction’, while particokinetics are divergent for 12-h incubation and for pipetting from the dispersed fraction only. The most sensitive dependence of delivered dose on suspension media, sampling method and incubation time was observed for TiO2 NM-100 and NM-102, CeO2 NM-212, Ag NM-300K and the MWCNTs. The differences between the AUC- and ISDD-based calculations underline the need to take into account the individual NM’s sedimentation and diffusion properties when determining effective dosages.

4. Discussion

In vitro test systems should be relevant for the in vivo situation they mimic. Reaching the alveoli, inhaled, uncoated nanoparticles come into contact with pulmonary surfactants forming a ‘corona’ on the particle surface, which affects particokinetics (Landsiedel et al., 2012). Dispersing agents rendering NM preparations more stable and NMs less likely to sediment are applied in in vitro assays to reflect NM deposition in the lower respiratory tract.

It remains to be determined which degree of dispersion best simulates the situation in the lung. In the present study, Curosurf®, containing porcine lung surfactant phospholipids, was less effective than BSA in dispersing the tested NMs. Similarly, addition of 10 μg/mL Curosurf® to 100% fetal calf serum hardly reduced TiO2 agglomeration (Vippola et al., 2009), just as TiO2 NMs were poorly deagglomerated in pulmonary surfactant surrogates (Maynard, 2002). However, in epithelial cells or alveolar macrophages, addition of 0.25% Curosurf® diminished amorphous silica uptake, presumably by reducing the dose delivered by sedimentation, although also cellular uptake may have changed (Vranic et al., 2013). Pre-coating of MWCNTs with Curosurf® increased the levels of reactive oxygen species and decreased intracellular glutathione depletion and release of tumor necrosis factor-α in human macrophages (Gasser et al., 2012). Schlieh et al. (2013) cautioned that Curosurf® addition to NM suspensions does not mimic the pulmonary situation since it hardly contains hydrophilic SP-A or -D, which affect NM cellular uptake and toxicity.

Bihari et al. (2008) obtained best deagglomeration when rutile TiO2 NMs were first sonicated with 4.2 × 105 kJ/m3 in distilled water, then human serum albumin, and finally PBS was added. In the present study, NM dispersions in DMEM/F-12 were sonicated before and after BSA addition, and the specific sonication energy corresponded to 3.6 × 104 kJ/m3 (determined as described by Taurozzi et al., 2011).

Hydrophobic nanoparticles are generally poorly dispersed in biological fluids and culture media (Schaefer et al., 2012; Zhu et al., 2013). Also in the present study, hydrophobic materials had a stronger tendency to agglomerate and a higher BSA affinity than the hydrophilic variants of the same inorganic core thereby minimizing the total interfacial energy of the suspension.

The biological proteins and lipids surrounding a nanoparticle determine its biological fate, since it is this corona that cells encounter and interact with. When nanoparticle-protein complexes pass from one biological fluid to another, the corona is assumed to change due to competitive adsorption of different biomolecules (Monopoli et al., 2012).
Table 2
Estimation of effective dose of the applied 1000 µg/mL test substance concentration of 16 OECD reference nanomaterials reaching in vitro test systems after 12- and 24-h test substance exposure.

<table>
<thead>
<tr>
<th>OECD reference number</th>
<th>Test material</th>
<th>NM dispersion in BSA-containing medium (modified and extended from: Sauer et al., 2014)</th>
<th>NM dispersion in Curosurf® (based on Table 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AUC</td>
<td>ISDD&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12-h Exp.</td>
<td>24-h Exp.</td>
</tr>
<tr>
<td>NM-100</td>
<td>TiO₂</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>NM-101</td>
<td>TiO₂</td>
<td>46</td>
<td>85</td>
</tr>
<tr>
<td>NM-102</td>
<td>TiO₂</td>
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<td>96</td>
</tr>
<tr>
<td>NM-103</td>
<td>TiO₂</td>
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<td>91</td>
</tr>
<tr>
<td>NM-104</td>
<td>TiO₂</td>
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<td>75</td>
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<tr>
<td>NM-105</td>
<td>TiO₂</td>
<td>24</td>
<td>63</td>
</tr>
<tr>
<td>NM-110</td>
<td>ZnO</td>
<td>90</td>
<td>99</td>
</tr>
<tr>
<td>NM-111</td>
<td>ZnO</td>
<td>84</td>
<td>99</td>
</tr>
<tr>
<td>NM-200</td>
<td>SiO₂</td>
<td>15</td>
<td>60</td>
</tr>
<tr>
<td>NM-203</td>
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<td>94</td>
</tr>
<tr>
<td>NM-212</td>
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<td>73</td>
</tr>
<tr>
<td>NM-300K</td>
<td>Ag</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>NM-400</td>
<td>MWCNT</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>NM-401 MWCNT</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>NM-402 MWCNT</td>
<td>1</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculations based upon the ‘dispersed fraction’ (df) assume that the test samples are pipetted from the supernatant of the 1000 mg/mL test substance preparations and therefore do not contain large agglomerates that settle within minutes.

5. Conclusion

The biomolecules that NMs come into contact in test substance preparations affect the effective dose. Further, different NMs react differently in different dispersing agents. Dispersion protocols need to consider, on a case-by-case basis, which property, size, or corona of the dispersed NMs is relevant to the corresponding in vivo situation. In designing nanotoxicological in vitro systems, study protocols should address whether culture additives aim at the best-possible dispersion of the NMs or at simulating more realistically in vivo tissue uptake and distribution. Calculations of the effective dose delivered to the in vitro system are indispensable for relevant in vitro data interpretation.

Conflict of Interest

Some of the authors are employees of BASF SE, a company producing and marketing nanomaterials. The authors alone are responsible for the content and writing of the paper.

References


Transparency Document

The Transparency document associated with this article can be found in the online version.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tiv.2014.10.011.


Schlechte, C., Kroeyond, W.C., Lehr, C.M., 2013. Pulmonary surfactant is indispensable in order to simulate the in vivo situation. Part. Fibre Toxicol. 10, 6.


