Artifacts by marker enzyme adsorption on nanomaterials in cytotoxicity assays with tissue cultures

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Abstract. We used precision cut lung slices (PCLS) to study the cytotoxicity of cobalt ferrite nanomaterials with and without bovine serum albumin (BSA) stabilization. Using mitochondrial activity as an indicator of cytotoxicity (WST-1 assay) increasing concentrations of cobalt ferrite nanomaterial caused increasing levels of cytotoxicity in PCLS irrespective of BSA stabilization. However, there was no increase in released lactate dehydrogenase (LDH) levels caused by BSA stabilized nanomaterial indicating concentration depended cytotoxicity. Moreover, non-stabilized nanomaterial caused a decrease of background LDH levels in the PCLS culture supernatant confirmed by complementary methods. Direct characterization of the protein corona of extracted nanomaterial shows that the LDH decrease is due to adsorption of LDH onto the surface of the non-stabilized nanomaterial, correlated with strong agglomeration. Preincubation with serum protein blocks the adsorption of LDH and stabilizes the nanomaterial at low agglomeration. We have thus demonstrated the cytotoxicity of nanomaterials in PCLS does not correlate with disrupted membrane integrity followed by LDH release. Furthermore, we found that intracellular enzymes such as the marker enzyme LDH are able to bind onto surfaces of nanomaterial and thereby adulterate the detection of toxic effects. A replacement of BSA by LDH or a secondary LDH-on-BSA-corona were not observed, confirming earlier indications that the protein corona exchange rate are slow or vanishing on inorganic nanomaterial. Thus, the method(s) to assess nanomaterial-mediated effects have to be carefully chosen based on the cellular effect and possible nano-specific artifacts.

1. Introduction

Toxicological testing of nanomaterials demands test systems which take into account the unique physicochemical characteristics of nanomaterials compared to the bulk material. For human safety, respiratory exposure to dust atmospheres is considered to be the route of highest concern for nanomaterials.[1] In vivo inhalation studies on rats or mice have established that there is not a general hazard due to the nanoscale structure, but that the hazard potential depends on specific material properties [2,3]. Hazard assessment is hence required for each nanomaterial, at least until cross-reading and groupings become validated in future REACH revisions. Considering the large number of materials that will fall under the upcoming regulatory definitions, the reduction of animal tests is a pressing issue. Precision cut lung slices (PCLS) may offer a test system to assess the lung toxicity of nanomaterials without in vivo inhalation studies. This method offers the opportunity to prepare around...
100 lung slices from a single *ex vivo* rat lung. Thus, 50 different conditions can be tested and this qualifies it as an alternative method to *in vivo* studies. Biophysical interactions have not been reported so far for the PCLS assay, but a sign of warning is raised by the dose-response shift of inorganic nanomaterials due to adsorption effects [4] and the generally recognized importance of characterization for in vitro assays.[5,6]. Significant artifacts have been reported for hydrophobic nanomaterials (carbon nanotubes) adsorbing MTT [7] or essential nutrients [8]. The biophysical mechanisms of particulate nanomaterials interacting with proteins was pioneered by the Dawson group with a focus on serum interactions of polymeric model particles [9-12], and first evidence emerges that similar effects occur with inorganic nanomaterials in ecological media [13; 14], in blood serum [14-18] or in alveolar environments [19,20]. Incubation of metal oxides with dysopsonins (BSA, IgA) decreased resorption [21] and prolonged circulation in the bloodstream[22], and incubation at unphysiologically high nanomaterial concentrations >10 mg/ml can even induce buffer depletion,[23] unless the adsorption was blocked by pre-incubation in serum.[24]

Here we used PCLS to study the cytotoxicity of cobalt ferrite (CoFe) nanomaterial. Bovine serum albumin (BSA) was added to prevent agglomeration of the nanomaterial during the test, but lead to the discovery that artifacts (false-negative results) occur in the determination of cytotoxicity by means of released levels of LDH depending on preincubation of the nanomaterial to be tested. The endpoint of LDH release can therefore be underestimated by adsorption of LDH onto non-stabilized CoFe nanomaterial. The in situ characterisation of the nanomaterial in PCLS revealed that the protein corona of either LDH or BSA correlates directly with the state of agglomeration and does not exchange noticeably in successive incubations. The cytotoxicity of nanomaterials in PCLS does not correlate with disrupted membrane integrity followed by LDH release unless the nanomaterials are carefully pre- incubated. These results are essential to enable the safety evaluation by alternative methods.

2. Materials and Methods

2.1. Preparation of precision cut lung slices (PCLS)

Animals (8-10 weeks old nulliparous and non-pregnant female Wistar Crl:WI (Han) rats, Charles River, Germany) were euthanized with an overdose of pentobarbital-Na. Lung tissue was prepared directly post mortem to conserve the viability of the tissue. Through the trachea the lung was carefully filled in situ with 10 mL / 200 g body weight prewarmed 1.5 % agarose – medium solution. The lung was removed and put on ice for 20 minutes, allowing the agarose to polymerize. Lung lobes were separated, placed on wax, and 8 mm tissue cylinders were subsequently prepared. The cylinders were placed into the Krumdieck tissue slicer (Alabama Research and Development, USA) filled with ice cold salt solution (EBSS) and slices with a thickness of approximately 200 µm were prepared. The PCLS were then washed three times with prewarmed DMEM/F-12 to remove the agarose.

2.2. Preparation of the cobalt ferrite nanomaterial suspension

Cobalt ferrite (CoFe) nanomaterial suspensions were prepared in serum-free DMEM/F12 medium as well as in DMEM/F12 medium with BSA (Sigma-Aldrich, Germany) to stabilize the surface of the nanomaterial and to avoid the aggregation of the particles. NFeCo132 was provided by Colorobbia Italia SPA (Vinci, Italy) in a concentration of 117 mmol/kg as suspension in water (PDI = 0.225; D (V mean) nm = 16,76; Fe/Co ratio = 1.91; ICP % w/w = 2.74 and grain size by XRD = 5 nm). For a 10 mM stock solution a total of 0.8547 g NFeCo132 and 9.1453 g were added to serum-free DMEM/F12 medium (Gibco Product No. 21041-025). For the preparation of a 10 mM stock solution with BSA, 0.8547 g of the NFeCo132 nanomaterial were suspended by shaking in 5 % BSA solution (0.5 g in ultrapure Milli-Q water) for 30 minutes. Then, 8.6453 g DMEM/F 12 medium was added to the mixture. PCLS were exposed to CoFe nanomaterial suspensions in DMEM/F12 medium (0, 0.01, 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 2.5, and 5.0 mM). In addition, a BSA solution (5 % in ultrapure Milli-Q water) was used as a negative control. For cytotoxicity assays, 1% Triton X-100 (in DMEM/F-12, Sigma-Aldrich, Germany) was used as positive control.
2.3. Cytotoxicity assays
After a 24 hours incubation of nanomaterial semi-quantitative determinations of the mitochondrial metabolic activity in PCLS using the water-soluble tetrazolium assay (WST-1, Roche, Germany) and determination of the cell membrane integrity by measuring the released levels of lactate dehydrogenase in the supernatant (LDH Cytotoxicity Detection Kit, Roche, Germany) were performed. Triton X-100 lysed PCLS were served as positive control (100% LDH release). Results were calculated as percentage of the total LDH content or maximum viability. All tests were performed in duplicate.

2.4. Incubation protocol
CoFe nanomaterial concentrations were incubated PCLS-free under different conditions for 24 hours: (1) in serum-free DMEM/F12 medium, (2) in serum-free DMEM/F12 medium containing human serum LDH (at a concentration of 0.05 U/mL, Prezinorm U, Roche, Germany) (3) non-BSA-stabilized NP suspension in serum-free DMEM/F12 containing 20% of PCLS-lysate, and (4) BSA-stabilized nanomaterial suspension in serum-free DMEM/F12 containing 20% of PCLS-lysate. As positive control (100% LDH release) PCLS were treated for 24 hours with 1% Triton X-100 and examined in the LDH assay. After a PCLS-free incubation of 24 hours the LDH content of the nanomaterial incubates was determined as described above.

2.5. Analytical ultracentrifugation with interference detection (AUC)
A mighty tool for the characterization of nano-colloids (0.5 – 10,000 nm diameter) is the Analytical Ultracentrifuge (AUC)[25-27], especially the universal interference optics Beckman XLI™ with widespread use in the proteome business[28-30], and, only to a lesser extent, also the disc centrifuges (Brookhaven Instruments XDC, CPS Instruments DC24000) with their rather limited detection optics and lower speeds. Schlieren, turbidity, interference, UV-VIS absorption and X-Ray absorption detection are published.[26,31] The optical AUC method detects the time- and radius-dependent concentration of the solutes simultaneously with the sedimentation at 600 to 60,000 rpm. Thereby, we quantify the amount and the diameter of each component independently.[25] At present, AUC is the only method that detects all components from the agglomerates, to the dispersed nanoparticles and the sub-10-nm proteins.[3]

Here we use interference optics (Beckman model `XLI proteome lab`) at 10,000 rpm and 44,000 rpm. The raw data is fitted by the free-ware software SedFit, resulting in a distribution of sedimentation coefficients $s$.[32] In order to convert the observed sedimentation coefficients $s$ to diameters $d$, we rely on the simplifications of the Stokes-Einstein relation:

$$d = \frac{18\eta s}{\Delta\rho}$$  \hspace{1cm} (1)

where $\eta$ is the viscosity of the solvent and $\Delta\rho$ is the density difference between solute and solvent. The concentration shares $c$ are read directly from the interference fringe shift $j$ with

$$j = c\frac{(dn/dc)l}{\lambda}$$  \hspace{1cm} (2)

where $dn/dc$ is the refractive index increment, $l = 12$ mm the length of the optical cell, and $\lambda = 680$ nm the wavelength of the laser. Here we evaluated the particulate signal with a density difference $\Delta\rho=4.6$ g/cm$^3$. The protein signal was evaluated with $\Delta\rho=0.36$ g/cm$^3$ and $dn/dc=0.18$ cm$^3$/g.

Samples were prepared by mixing 0.1975 g stock particle suspension with 4.7628 g DMEM. For conditioning by albumin, 0.1974 g stock particle suspension were mixed with 0.2737 BSA solution, stirred for 30 minutes, then added to 4.5659 g DMEM and stirred again. Either of these suspensions were stirred for 30 minutes, then mixed 1:1 with the PCLS lysate and stirred again. During this preparation, intermediate and final samples were measured by AUC.
2.6. Time of flight secondary ion mass spectrometry (TOF-SIMS)

Time of flight secondary ion mass spectrometry (TOF-SIMS) is a powerful surface analysis technique that allows chemical characterization of the first atomic layers of any vacuum stable solid surface. Being a fragmenting mass spectrometry technique, it provides a wealth of chemical information and allows identifying compounds with very high sensitivity. This makes TOF-SIMS ideally suited to characterize and study biomolecules on surfaces, such as proteins.[33] For SIMS measurements, the particulate fraction was separated by sedimentation at 10,000 rpm for 4 minutes. The supernatant was discarded, and the volume was refilled with DMEM, stirred shortly and pelleted again for washing of non-adsorbed proteins. The clean particle sediment was prepared for SIMS analysis by their placing on clean silicon wafers. On the thus prepared sample positions, no silicon wafer secondary ion mass signal could be detected any more, confirming that the sample layer thickness well exceeded the SIMS information depth of typically 1-3 nm.

Static TOF-SIMS spectra were recorded using a TOF-SIMS V spectrometer (Iontof GmbH, Germany). A pulsed mass-filtered primary ion beam of 25 keV singly charged bismuth (Bi+) was used. This primary ion beam, resulting in a spot size of typically 5 µm on the sample surface, was raster scanned over an area of 250 x 250 µm to record spectra of positive and negative secondary ions. The primary ion dose density was always kept well below 10-12 ions/cm² and thus in the static SIMS regime. To prevent charging of the sample surface, a low-electron energy flood gun was used.

3. Results

3.1. CoFe nanomaterial induced cytotoxicity

Cell viability (WST-1 assay) is presented as relative mitochondrial activity normalized to the vehicle control (100% mitochondrial activity). The mean mitochondrial activity was decreased from 0.4 mM concentration to almost the value of the positive control (1% Triton X-100) when compared to the vehicle or BSA control. Therefore the WST-1 assay indicates at increasing concentrations of nanomaterial (without and with BSA stabilization) increasing levels of cytotoxicity in PCLS. However, by means of the LDH release assay, presented as percentage of the total LDH content (calculated from Triton X-100 lysed samples, 100% LDH release), was not increased when compared to the vehicle control. This was independent of incubating PCLS with or without BSA-stabilized nanomaterial. Moreover, upon incubation with non-stabilized nanomaterial decrease of background LDH levels in the PCLS culture supernatant were measured (Figure 1).

Released LDH-levels (red bars, “LDH R.”) and mitochondrial metabolic activity (blue bars, “Mit. Act.”) were assessed after 24 hours exposure of PCLS to CoFe nanomaterial without (solid bars, “w/o BSA”) and with BSA stabilization (diagonal stripes, “w/ BSA”). Controls were PCLS incubated with BSA (horizontal stripes) and Triton X-100 with and without BSA (100% LDH release, open bars and horizontal stripes).

3.2. Biophysical interactions of CoFe nanomaterial in the PCLS assay

3.2.1. Adsorption: Indirect assessment by quantification of non-adsorbed LDH. We have hypothesized the discordance of the two cytotoxicity assays to be an artifact: the LDH released from the disintegrated cell membrane at particle concentrations was adsorbed to the non-stabilized nanomaterial in the supernatant. Figure 2 shows that the LDH is adsorbed onto the surface of the non-stabilized nanomaterial whereas BSA stabilization blocked the absorption of LDH (Figure 2).
Figure 1. Assessment of LDH release and mitochondrial activity after 24 hour exposure of PCLS to different concentrations of CoFe nanomaterial.

LDH-levels were assessed after 24 hours exposure (solid bars, “w/o BSA”) and with BSA stabilization (diagonal stripes, “w/ BSA”). Controls were PCLS incubated with Triton X-100 (100% LDH release, open blue bar) and 20% PCLS lysate in medium without (purple bar, solid) and with BSA (purple bar, stripes). Assessed were CoFe nanomaterial in serum-free DMEM/F12 medium (green bars), in serum-free DMEM/F12 medium containing 0.05 U/mL human serum LDH (yellow bars), non-BSA-stabilized nanomaterial suspension in serum-free DMEM/F12 containing 20% of PCLS-lysate (blue bars, solid), and BSA-stabilized nanomaterial suspension in serum-free DMEM/F12 containing 20% of PCLS-lysate (blue bars, stripes).

As a cross-check with a complementary technique, we determined the concentrations of free proteins by analytical ultracentrifugation with interference detection. The concentration of proteins in PCLS rises from 2.7 mg/mL to 3.3 mg/mL (measured by the refractive index signal below 10 nm diameter in the AUC, data not shown) when the nanomaterial was stabilized in BSA before incubation.

Figure 2: Assessment of LDH adsorption to CoFe nanomaterial by the LDH Detection Kit.
with PCLS. The BSA signal is clearly identified by the refractive index signal at 66 kDa and 132 kDa, attributed to monomer and dimer, respectively. The concentration of non-adsorbed BSA (monomer and dimer added) did not increase when the nanomaterial was transferred from DMEM into PCLS lysate, indicating that BSA is not replaced by other ligands. These results with hydrodynamic separation of proteins by mass and unselective detection confirmed the results obtained with the enzymatic LDH measurement.

3.2.2. Adsorption: direct assessment of the protein corona on CoFe nanomaterial. In the context of this study, TOF-SIMS is employed to characterize the nanomaterial adsorbates and elucidate the influence of prior particle conditioning with BSA. As reference, the untreated nanomaterial was investigated by TOF-SIMS revealing the elementary ion signals of iron and cobalt in the positive partial mass spectrum shown in Figure 3a (marked with blue, diagonally striped circles). In addition, bulk samples of DMEM, BSA and the PCLS lysate were analyzed for reference (not shown).

Nanomaterial treated only with PCLS lysate (non-BSA stabilized) showed a distinct change in their surface mass spectra (Figure 3b), which indicates the formation of an adsorbate layer. Upon comparison with the reference spectra, it can clearly be shown by distinct marker secondary ion peaks (98, 137 and 142 amu, labeled with orange circles, vertically striped) that this adsorbate stems form the PCLS lysate. On the other hand, such signals are not detected from the surface of the sample conditioned with BSA prior to exposure to PCLS lysate. In this case (Figure 3c) only clear BSA fragments secondary ions are detected, e.g. at 70, 84, 86, 110 and 120 amu (plain green circles).

![Figure 3](image-url)

**Figure 3.** Secondary ion mass spectra recorded from (a) untreated reference particles, (b) non-stabilized particles exposed to PCLS lysate and (c) BSA stabilized particles exposed to PCLS lysate.

3.2.3. State of agglomeration in situ of the PCLS assay. The degree of nanomaterial agglomeration in physiological media highly depends on the composition of the used solvent. In DMEM the nanomaterial agglomerates with a diameter distribution between 200 and 1200 nm. When the nanomaterial is first stabilized in BSA and then added to DMEM, the degree of agglomeration decreases to a mean diameter below 200 nm (Figure 4). Transfer of BSA-stabilized nanomaterial from DMEM into PCLS lysate maintains the low degree of agglomeration. In contrast, when nanomaterial without BSA stabilization is transferred from DMEM directly into PCLS lysate, the degree of agglomeration is higher.
4. Discussion

In comparison to the relatively constant values of the LDH release of the nanomaterial stabilized with BSA, the non-stabilized nanomaterial seemed to induce, with increasing concentrations, a reduced LDH release. This was hypothesized to be an artifact and the LDH released from the cells at particle concentrations disrupting the membrane integrity adsorbed to the non-stabilized nanomaterial in the supernatant. Therefore the detection of LDH as indicator of the membrane integrity was shown to be limited to assess the cytotoxic effects of nanomaterials.

The results of this study demonstrate that released LDH from the culture supernatant of the lysed PCLS bound to the surface of non-stabilized CoFe nanomaterial, whereas the stabilization of the nanomaterial with BSA prevented the adsorption of the marker enzyme to the particle surface. Serum-free DMEM/F12 medium had no effect on the released LDH levels, such that an artifact by the interaction of the nanomaterial with the components of the assay was excluded. The constant LDH level in the approach with defined LDH concentration in human serum can be explained by the fact that other proteins of the human serum adsorb to the particle surface and therefore serve as particle stabilization. The adsorption of proteins and organic molecules at the surface of nanomaterial has been described in the literature and is used in medicine for the improvement of the efficiency of drug effects. Against this background, the reduced released LDH levels determined in the cytotoxicity assay can be explained by the adsorption of released LDH at particle surfaces. We have substantiated this hypothesis by direct analysis on the nanoparticle surfaces, where the TOF-SIMS data confirms that components from PCLS lysate, while clearly adsorbing on neat nanomaterial, are not able to displace previously adsorbed BSA or form an adsorbate layer on top of the BSA conditioning film. The surface of dispersed colloids determines their mutual interactions. From this point of view, it is obvious that adsorbed proteins can act as protection colloids, if the specific protein has flexible solvated loops that stabilize the nanomaterial by steric mechanisms. Especially albumins in water or DMEM have dispersed and stabilized a wide variety of nanomaterials: CNTs, metal nanoparticles, metal carbide nanoparticles, and metal oxide nanoparticles, whereas bronchoalvelolar lavage induces the opposite effect of increasing agglomeration.
Since an exchange between BSA and LDH in the lysate was not observed on the 1 h-time scale of this experiment, this supports earlier indications that the protein corona exchange rate is slow or vanishing on inorganic nanomaterial.[16; 44], in contrast to nanomaterials with polymeric surface functionalization.[9; 45] It may well be that morphological changes of the proteins upon adsorption impose a strong enthalpic barrier against dissociation, leading to an effective non-equilibrium adsorption mechanism.

5. Conclusion
The results of this study demonstrate that LDH from the culture supernatant of the lysed PCLS bound to the surface of non-stabilized CoFe nanomaterial, whereas the stabilization of the nanomaterial with BSA prevented the adsorption of the marker enzyme to the particle surface. We have shown that, besides BSA, intracellular enzymes such as the marker enzyme LDH can bind at the surfaces of nanomaterial and thus falsify the detection of cytotoxic effects. We characterized the resulting hard protein corona by several complementary techniques – indirect by the loss of free LDH, direct by the nanomaterial's surface – and found a non-exchanging corona that correlates exactly also with the state of agglomeration, which is determined by the adsorbates on the colloidal surfaces. The enzymatic detection of LDH as indicator of the membrane integrity was shown to be limited to assess the cytotoxic effects of nanomaterial, but a proper preincubation of the tested nanomaterial could restore the applicability of the LDH assay. For this reason, the method for the examination of cytotoxicity of nanomaterials including the dispersion / preincubation protocol has to be carefully chosen. Even though it has been argued that preincubation 'masks' the nanomaterial surface and hence supposedly lowers physiological effects, the hypothetical disadvantage is overcompensated by the advantage of avoiding false positive results by adsorption of marker enzymes.

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