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Particle-induced artifacts in the MTT and LDH viability assays

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Abstract

In vitro testing is a common first step in assessing combustion generated and engineered nanoparticle related health hazards. Commercially available viability assays are frequently used to compare the toxicity of different particle types and to generate dose response data. Nanoparticles, well known for having large surface areas and chemically active surfaces, may interfere with viability assays, producing a false assessment of toxicity and making it difficult to compare toxicity data. The objective of this study is to measure the extent of particle interference in two common viability assays, the MTT reduction and the lactate dehydrogenase (LDH) release assays. Diesel particles, activated carbon, flame soot, oxidized flame soot, and titanium dioxide particles are assessed for interactions with the MTT and LDH assay under cell-free conditions. Diesel particles, at concentrations as low as 0.05 µg/ml, reduce MTT. Other particle types reduce MTT only at a concentration of 50 µg/ml and higher. The activated carbon, soot, and oxidized soot particles bind LDH to varying extents, reducing the concentration measured in the LDH assay. The interfering effects of the particles explain in part the different toxicities measured in human bronchial epithelial cells (16HBE14o). We conclude that valid particle toxicity assessments can only be assured after first performing controls to verify that the particles under investigation do not interfere with a specific assay at the expected concentrations.

Keywords

nanoparticles; particulate matter; cytotoxicity; in vitro models

Introduction

Airborne particles pose a serious hazard to human health; examples include cigarette smoke, ambient air particles¹, combustion generated particles², and welding fume³. With the increasing production of engineered nanoparticles in commercial products there is a potential for a wide variety of particles with diverse physical and chemical characteristics to be released to the air in the workplace or environment. With the multitude of different core and surface compositions, shapes, and sizes, in vitro assessment is expected to be an important step in screening for potential hazards from particles⁴. High throughput screening and standardized in vitro assays with multiple cell lines are the most promising approaches to screening the great magnitude of particle types^{5–6}. A core component to screening programs will be simple in vitro toxicity assays, because they are easy to perform and can provide repeatable results. However, these assays can be vulnerable to interactions with the compound being tested, resulting in false positives or false negatives. Interactions can be an

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even greater problem when determining particle induced toxicity, as particles often adhere to cell surfaces and are difficult to rinse off. Possible interactions from particles are (1) particle optical properties that interfere with light absorption or fluorescence used for detection, (2) chemical reactions between the particles and assay compounds, and (3) adsorption of assay molecules to the particle surface⁷.

Many different viability assays rely on absorbance or fluorescence measurements for quantification. Examples include the frequently used tetrazolium salt viability assays, MTT, WST-1, and XTT that are reduced by viable cells to form the colored formazan molecule, or the dichlorofluorescein assay which uses a fluorescence measurement. Most carbonaceous and some metal oxide particles absorb light over the spectral regions used in these systems. When present these particles change the resulting absorption spectrum, confounding the results of the assay⁸. In general, the absorbance due to particles is dependent on the particle composition and increases with increasing mass concentration⁵.

Reactions between particles and assay compounds may also interfere with viability assessments. Single-walled carbon nanotubes and carbon black reduce the tetrazolium compound in the MTT and XTT to the colored formazan without any cells present^{9–10}. Likewise, copper and silver nanoparticles were shown to cause an inactivation of the LDH enzyme, interfering with LDH assay to assess membrane integrity¹¹. Both of these effects could obscure a toxic response or even show an increase of viability in exposed cells.

Another possible interference is the binding of assay molecules to the particle surface. Evidence is building that many biological molecules have a strong affinity for particles. Several investigators have found that single walled carbon nanotubes adsorb dye molecules from the neutral red, alamar blue, MTT, and WST-1 assays, preventing accurate measurement^{9, 12–13}. Monteiro-Riviere and Inman¹⁴ initially suspected and later confirmed¹⁰ that dyes in the neutral red and MTT assays were adsorbed to activated carbon. A variety of biological molecules, such as vitamins, amino acids, serum proteins, and cytokines have all been found to adsorb to the surface of a variety of particles^{15–17}. Particle characteristics are expected to play an important role in the adsorption process^{16–17}.

These previous studies have shown that many types of particles can interfere with viability assays; however, it is not yet clear what role particle characteristics play. The objective of this study is to determine if particles with very different characteristics (composition, surface area, size, hydrophobic/hydrophilic nature) interfere with two commonly used viability assays, the LDH and MTT assays, preventing rank toxicity assessments. Several carbonaceous particle types were studied, all with a similar core composition but with differing surface treatments and trace compounds. Additionally, titanium dioxide particles were used to determine if interference effects were limited to carbonaceous particles.

A description of the possible mechanisms by which particles may interfere with the MTT and LDH assays and the hypothesized effect on the viability measurement are described in Table 1. Both assays use an absorbance measurement of a colored compound to quantify cellular viability that can be affected by the presence of light absorbing particles. The MTT assay measures the metabolic activity of cells by measuring the amount of formazan generated by active mitochondria that cleave the MTT tetrazolium ring. Formazan generated from the MTT assay is insoluble and is solubilized with dimethyl sulfoxide before an absorbance measurement. We hypothesize that particles can react with the tetrazolium compound, generating the formazan molecule in the absence of healthy cells, and that the formazan molecule may adsorb to particles and be removed in rinsing or centrifugation steps. The LDH assay assesses the membrane integrity of cells by measuring the concentration of the cytosolic LDH enzyme in the extracellular medium. Active LDH

enzyme in the supernatant from centrifuged exposure medium is measured by its ability to generate NADH which is used to generate a colored compound. We hypothesize that particles in the exposure medium can adsorb the LDH enzyme or deactivate the LDH enzyme, with both mechanisms preventing released LDH from being measured.

Experimental Procedures

Particle Characteristics

Commercially available carbonaceous particles used in this study are National Institute of Standards and Technology standard reference material 1650, derived from a heavy-duty diesel engine, and Norit Ultra C activated carbon. To investigate the effects of surface composition, flame soot from a methane-air diffusion flame described by Stipe et al.¹⁸ was collected on a filter. In an alternative arrangement, the flame soot was oxidized by ozone (28 ppm) in a flow reactor with a residence time of 14 min, described in Holder et al.¹⁹. Ozone reacts with the soot surface, creating oxygen-containing functional groups such as carboxyl and alcohols that transform particles from hydrophobic to hydrophilic²⁰. Aeroxide P25 titanium dioxide was used as an example of a metal oxide particle.

The characteristics for commercially available particles as reported by the manufacturer are described in Table 2. Corresponding measurements were made for the flame generated soot and oxidized soot particles as described in detail in Holder et al.¹⁹ and briefly presented here. Soot composition was determined to be almost entirely elemental carbon using thermo-optical analysis²¹. BET surface area was measured with a Micromeritics gas adsorption analyzer (Tristar 3000). Particle size was determined from images obtained on an electron microscope (FEI, Tecnai 12) and analysis of the fractal aggregate structure soot particles according to the method outlined in Koçlu et al.²². The hydrophobic/hydrophilic nature of the soot and oxidized soot was observed by the differing degree of wettability of the particles. Furthermore, FTIR measurements showed an increase of oxygen containing function groups on the oxidized soot surface, which increases the hydrophilic nature of these particles¹⁹.

Particle Suspensions

Particle suspensions used for cell exposures and assay interference assessments were made in Laboratory of Human Carcinogenesis (LHC) basal medium (Gibco) with 0.004% dipalmitoyl phosphatidylcholine (DPPC) from Sigma. The phospholipid DPPC is a constituent of the mucus layer and is used to aid in the dispersion of particles in the cell culture medium²³. All exposures were performed without serum because of previous evidence of serum proteins impacting cytokine binding by particle type²⁴. As an alternative to serum, LHC medium was used, which was designed for culturing bronchial epithelial cells under serum free conditions²⁵. Stock suspensions of 200 µg/ml were made by weighing particles into sterile tubes and adding LHC – 0.004% DPPC. The particles were dispersed by stirring with a vortex stirrer for 30 seconds and sonicating for five minutes in a bath sonicator. Solutions were kept at 8 °C no longer than 1 week. Before use with an assay or cell exposure, the suspensions were warmed to 37 °C and stirred on the vortex stirrer for 30 seconds.

Cell Culture

A bronchial epithelial cell line 16HBE14o (gift from Dieter Gruenert, Pacific Northwest Medical Center) was used to determine the toxicity of each particle type. Bronchial epithelial cells are a suitable model for studying the effects of inhaled nanoparticles, and this particular cell line has previously been used to assess the effects of a variety of particle types including diesel particles and ambient air particles^{26–27}. Cells were grown submerged on

collagen coated surfaces in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) in an incubator maintained at 37 °C and 5% CO₂. For experiments, cells were seeded at a density of 2×10⁵ cells/well (0.5×10⁵ cells/cm²) onto collagen coated 12-well plates.

Cell Exposure to Particles

Exposures were done after cells were approximately 75% confluent, which was two days after being seeded into the 12-well plates. Before the exposure, the medium was removed and each well was rinsed with 0.5 ml of PBS (pH = 7.4). Wells were dosed with 0.76 ml of LHC medium, LHC – 0.004% DPPC, 25 mM H₂O₂ (positive control), or particle suspension at a concentration of 200 µg/ml. Each particle suspension was tested in triplicate wells, LHC – 0.004% DPPC and H₂O₂ were tested in nine wells, and LHC was tested in six wells. The cells were exposed to particle suspensions for four hours in an incubator maintained at 37 °C and 5% CO₂. In previous measurements with this cell line, four hours was found to be a sufficient exposure time for measurable reductions of toxicity for the soot and oxidized soot particles. After exposure, the medium was collected for the LDH assay, the wells were rinsed twice with 0.5 ml PBS, and the MTT assay was performed.

MTT Assay – Particle Interference

MTT purchased from Invitrogen was used for measurements of cell viability and particle interference. A stock solution of 12 mM MTT was made in PBS and kept at –8 °C for no more than 4 weeks. The tendency of each particle type to reduce MTT to formazan was measured in a cell-free system. Suspensions of each particle type were made at concentrations of 0.1, 1.0, 10, and 100 µg/ml. The suspension was mixed with 1.2 mM MTT in PBS at a ratio of 1:1 and incubated at 37 °C for three hours. DMSO was added at a ratio of 2:1 to the MTT-particle mixture and was incubated for another 10 minutes at 37 °C. The mixture was centrifuged at 115 × *g* for 10 minutes and the supernatant absorbance was measured at 540 nm on a UV/vis spectrometer (Ocean Optics, HR4000). The background absorbance was removed from the peak value using equation 1:

$$MTT_{540\text{ nm}}\text{Corrected} = MTT_{540\text{ nm}} - MTT_{850\text{ nm}} \times \text{Abs Ratio}_{540\text{ nm}/850\text{ nm}} \quad \text{Eq. 1}$$

where MTT_{540 nm} Corrected is the background subtracted value, MTT_{540 nm} is the assay absorbance at the peak, MTT_{850 nm} is the assay absorbance at 850 nm, AbsRatio_{540 nm/850 nm} is the ratio of the particle absorbance at the peak value to that at the background value and is determined from the spectra of the particle suspensions.

The potential for particles to interfere with the MTT assay by binding the MTT molecule, preventing its reduction to formazan, or binding the formazan produced was also investigated. MTT was converted to formazan using ascorbic acid, which reduces MTT in a cell-free environment²⁸. After the three hour incubation, 0.16 ml of the MTT – particle mixture (described above) was mixed with 0.066 ml ascorbic acid (0.05 mM) and incubated another hour at 37 °C. DMSO was added to the MTT – particle – ascorbic acid mixture at a ratio of 2:1 and incubated another 10 minutes at 37 °C. The mixture was centrifuged at 115 × *g* for 10 minutes and the supernatant absorbance was measured at 540 nm, the background absorbance at 850 nm was subtracted from the peak.

MTT Assay – Cellular Viability

For cellular viability measurement, the stock MTT solution was thawed and diluted 1:10 with MEM (without phenol red or FBS). After the exposure, the cells were incubated with the diluted MTT solution (0.8 ml/well) at 37 °C and 5% CO₂ for three hours. A portion of the MTT solution was removed leaving 0.25 ml per well. Dimethyl sulfoxide (DMSO) was

added (0.5ml/well) to solubilize the formazan crystals. The plates were gently agitated and incubated at 37 °C for another 10 minutes. The assay liquid was centrifuged at $115 \times g$ for 10 minutes to remove the particles. The absorbance of the supernatant was measured at 540 nm, the background absorbance was corrected for as described by equation 1.

LDH Assay – Particle Interference

We hypothesized that particles may interfere with the LDH assay by either adsorbing the LDH protein or alternatively inactivating the LDH protein, with both mechanisms causing the same result of decreased absorbance in the LDH assay. Particle interference was tested by incubating particle suspensions with LDH protein of known concentration and measuring LDH concentration using an LDH kit (Sigma). An LDH standard (Sigma) suspended in ammonium sulfate was diluted to a concentration of 2 U/ml in PBS. To obtain a measurable LDH concentration, 20.5 μ l of the 2 U/ml LDH standard was added to 0.25 ml of the particle suspension at varying concentrations (10, 25, 50, and 100 μ g/ml). The mixture was incubated at 37 °C for 15 minutes. The effect of incubation time was assessed by incubating the 50 μ g/ml particle suspension with the LDH standard for 5, 15, 30, and 60 minutes. After incubation, the mixture was centrifuged at $115 \times g$ for 10 minutes and the supernatant was assayed for LDH concentration following the manufacturer's instructions. Briefly, the supernatant was mixed with the LDH assay mixture at a ratio of 1:2 and incubated at room temperature in the dark for 30 minutes. The reaction was stopped by the addition of 1 N HCl (1/10 of mixture volume), and the absorbance at 490 nm (corrected for background absorbance in the same way as was done for the MTT assay described in equation 1) was interpreted as the apparent LDH concentration since inactivated LDH may be present but not measured.

LDH Assay – Cellular Viability

Measurements of LDH release were performed following the manufacturer's instructions. After cells were exposed to particles, the exposure medium was collected and centrifuged at $115 \times g$ for 10 minutes and assayed as described above. Viability in relation to the control (the unexposed group) is calculated from equation 2:

$$Viability (\%Control) = \frac{LDH_{lysed} - LDH_{Exposed}}{LDH_{lysed} - LDH_{control}} (100\%) \quad \text{Eq. 2}$$

where LDH_{lysed} is the LDH released from wells treated with the LDH lysing solution (total cellular LDH content), $LDH_{exposed}$ is the LDH released from wells exposed to particle suspensions, and $LDH_{control}$ is the LDH released from cells in the control group.

Results

Interference from Absorbing Particles

Viability assays frequently employ an absorbance measurement to determine toxicity. The presence of absorbing particles in the assay sample increases the absorbance and leads to misleading viability results. The spectra of the particles used in this study are shown in Figure 1a. Even a low concentration of 3 μ g/ml of soot in the assay sample resulted in a 120% absorbance increase at the formazan peak wavelength, with the absorbance increasing linearly with particle concentration (data not shown). Subtracting the background absorbance at 850 nm from the formazan peak at 540 nm removes only about 90% of the absorbance at the formazan peak for the soot suspension, since the particle absorption is wavelength dependent. Measuring the entire particle spectrum to determine the proper correction factor could improve the measurement at the targeted wavelength. In the cell exposure and cell-free experiments, all assay samples were centrifuged at $115 \times g$ for 10

minutes to remove the majority of the particles (Figure 1b). Only the oxidized soot and the diesel particles appeared to remain in the suspension to some extent after centrifugation. Because not all of the particles were removed for all the samples all assay absorbance measurements were corrected to remove the particle contribution to the peak absorbance as described in equation 1.

Particle Interference – MTT Assay

The effect of particles on the reduction of MTT to formazan was determined by incubating MTT with suspensions of particles for three hours (Figure 2a). Activated carbon did not react with MTT at any concentration. Soot, oxidized soot, and titanium dioxide reacted with MTT to produce formazan only at the highest particle concentration of 50 $\mu\text{g/ml}$, with oxidized soot producing the largest concentration of formazan. In contrast, diesel particles produced formazan at all concentrations tested. At the highest diesel particle concentration, the formazan peak absorbance of 0.36 is comparable to the absorbance measured in the cell exposure, which ranged from 0.09 to 0.69.

Binding of MTT or formazan to particles was determined by incubating particles with MTT, reducing the MTT to formazan with ascorbic acid, and measuring the formazan concentration after centrifugation (Figure 2b). The formazan generated is normalized to that formed by ascorbic acid without particles present to determine the percentage of the original absorbance at 540 nm. The diesel particles react with MTT generating formazan, adding to the formazan generated from the ascorbic acid. The other particle types cause only slight reductions in the measured formazan. At the 50 $\mu\text{g/ml}$ concentration, the formazan measured is 83% and 77% of the particle free absorbance for the soot and oxidized soot respectively.

Particle Interference – LDH Assay

The percentage of the added LDH protein either adsorbed or inactivated by different particles in 15 minutes is shown in Figure 3a. The diesel particles were the only particle type that did not change the apparent LDH concentration at any of the measured particle concentrations. The other carbonaceous particles reduced the measurable LDH protein by a substantial amount, with the untreated soot reducing the free LDH concentration by 70% even at the lowest concentration tested (9 $\mu\text{g/ml}$). The oxidized soot, activated carbon, and TiO_2 particles also reduced the apparent LDH concentration.

Increasing the time in which diesel particles were incubated with LDH had little effect after the first few minutes (Figure 3b). Apparent LDH concentrations were not impacted by diesel particles at the concentrations tested here. The reduction of the apparent LDH concentration caused by the soot particles was rapid, with the LDH concentration decreased by 85% within five minutes. The oxidized soot particles also rapidly reduced the apparent LDH concentration, with a 70% reduction of free LDH after 15 minutes incubation.

Cell Exposure

Cells were exposed to particle suspensions to determine how particle interference with viability assays may confound viability results (Table 3). H_2O_2 (25 mM) was used as a positive control to ensure that both viability assays detected a toxic response. H_2O_2 caused a large increase in the LDH release and a large decrease in MTT absorbance, demonstrating the efficacy of the assays (Table 3).

All particles, except for the diesel particles, reduced cell viability compared to the control group (LHC with 0.004% DPPC) as measured by the MTT assay. Activated carbon caused the greatest reduction in MTT cellular viability. The diesel particles increased MTT

absorbance compared to control. LDH release from exposed cells was increased by all particle types except the oxidized soot. Flame soot caused the greatest LDH release from exposed cells. Cells treated with oxidized soot had an LDH concentration that was 3% of the control group due to LDH adsorption to these particles. A comparison of the viability (represented as the percentage of the control level) measured with the LDH and MTT assays is shown in Figure 4. There is no clear correlation between the two assays for any of the particle suspensions tested.

Discussion

We assessed the degree to which several different particle types interfered in the LDH and MTT viability assays under cell-free conditions. Particles were found to interfere with absorbance measurements, react with assay compounds, and to adsorb proteins. Two approaches were taken to remove the absorbance due to particles in the MTT assay: background subtraction and sample centrifugation. Unlike the MTT assay, the LDH assay absorbance measurement was not strongly affected by particles since the assay already includes a centrifugation step. At the modest concentrations used in this study, subtraction of a background absorbance at a different wavelength from the MTT absorbance peak was easily done on a plate reader. The spectrum of each particle type needed to be determined prior to subtraction to correctly account of the absorption at the MTT absorbance peak.

Centrifugation was also effective at removing the majority of the particles for the MTT assay. However, centrifugation is only useful if the particles do not adsorb assay dyes thus preventing their measurement. For the particles tested here, the MTT formazan dye solubilized in DMSO did not adsorb to the particle surface and remained in the supernatant after centrifugation. Conversely, single-walled carbon nanotubes have been shown to adsorb a variety of assay dyes. Wörle-Knirsch et al.¹³ found that formazan generated from the MTT assay was adsorbing to the surface of single-walled carbon nanotubes, reducing the peak absorbance and causing a false toxic response. Since this initial discovery, several other groups have confirmed that carbon nanotubes bind viability assay dyes such as formazan from the MTT assay (MTT-formazan)^{9–10}, formazan from the WST assay (WST-formazan), alamar blue, and neutral red¹², causing false results for these assays. Wörle-Knirsch et al.¹³ proposed that the insolubility of the MTT-formazan product caused it to bind to the nanotubes, as they did not observe the water soluble WST-formazan adsorbing to the nanotubes. However, Casey et al.¹² observed that WST-formazan did adsorb to nanotubes, but to a lesser extent than the MTT-formazan. In our results, MTT incubated with particles and subsequently reduced by ascorbic acid resulted in only a minimal reduction of measurable formazan, indicating that the particles did not adsorb the formazan dye to any great extent. Previous studies only demonstrated an effect with single-walled carbon nanotubes and not the particles tested here (soot, carbon black, and titanium dioxide). Additionally, previous studies with carbon nanotubes used acidified isopropanol, sodium dodecyl sulfate, or acetone rather than DMSO to solubilize formazan¹³. It is possible that DMSO may be more effective at reversing formazan binding to nanoparticles, thus reducing this form of particle interference with the MTT assay.

All of the particles tested, except for activated carbon, exhibited evidence of chemical reactions with MTT assay compounds. The diesel particles were the most reactive, reducing the MTT tetrazolium compound under cell-free conditions, generating formazan concentrations that were similar to those observed from viable cells in the control group. Single-walled carbon nanotubes^{9–10} and carbon black¹⁰ have also been found to convert MTT to formazan under cell-free conditions. As we have demonstrated, this reduction occurs for many particle types such as diesel particles, titanium dioxide, soot, and oxidized soot. Particles reacted with MTT in a concentration dependent manner, with only the diesel

particles generating formazan dye at low concentrations. Similarly, Kroll et al.⁵ observed no interference with a variety of different nanoparticles compositions when the nanoparticle concentration was limited to below 10 $\mu\text{g}/\text{cm}^2$ (32 $\mu\text{g}/\text{ml}$). Because particles can stick to cells even after rinsing, it may be difficult to predict the remaining mass of particles in the assay sample and to what extent the particles may interfere. Additionally, the effectiveness of the rinsing is dependent on the particle surface characteristics. For example, oxidized soot particles tended to stick to the cell surface more than untreated soot particles. Thus, the MTT assay is only valid for toxicity assessment of particles at concentrations that do not reduce MTT. Our results and that of Kroll et al.⁵ place this limit at approximately 30 $\mu\text{g}/\text{ml}$, except for the NIST diesel particles, in which the MTT assay should not be used for any concentration.

The LDH assay was found to be vulnerable to interference from particle adsorption or protein inactivation. The exact mechanism could not be determined by our experiments since both cause a decrease of the measurable LDH protein resulting in a false indication of a nontoxic response. The measurable LDH concentration was reduced with increasing incubation time until a seemingly steady state condition was achieved, which was different for each particle type. The amount of LDH reduction was also impacted by particle type, with the diesel particles binding the least, followed by titanium dioxide, activated carbon, soot, and oxidized soot. The LDH reduction by the oxidized soot particles was substantial even at a particle concentration as low as 10 $\mu\text{g}/\text{ml}$. These measurements predict that about 50% of the LDH released from cells exposed to activated carbon, soot, or oxidized soot bind to the particle and is not measured in the LDH assay. This is in contrast to measurements made by Kroll et al.⁵ in which none of the 23 nanoparticle types tested, including carbon black and titanium dioxide were found to bind LDH at any concentration. However, Han et al.¹¹ found indications of LDH binding to titanium dioxide nanoparticles in a cell exposure, but did not confirm with cell-free measurements of LDH adsorption. The differences between our results and other groups may be due to the presence of other proteins or biological compounds that impact LDH adsorption to particles. Our measurements were performed with pure LDH protein in LHC media with DPPC, whereas other studies have used different media or have had the additional presence of intracellular proteins and cell debris generated during cell exposure.

What factors are important in these protein-particle interactions are currently unknown. However, this is an important interaction to understand as the binding of biologic molecules to particles is thought to not only to interfere with viability assays such as the LDH assay^{8, 29–30}, but also to govern particle interactions with biological systems³¹ and possibly deplete essential nutrients in the medium^{15, 32}. We were unable to identify any trends in our measurements. The hydrophobic/hydrophilic nature of the particle did not appear to play a role; the hydrophobic diesel particles did not reduce measurable LDH concentrations, but the hydrophobic soot particles caused the largest reduction of measurable LDH. Additionally, surface area was not a factor; activated carbon with the largest surface did not reduce LDH as much as oxidized soot with a much smaller surface area. A similar collection of diverse results was reported for particle adsorption of IL-8. Seagrave et al.¹⁷ found that carbon black (Elftex-12) did not bind IL-8, but Kocbach et al.²⁴ observed another carbon black (Printex 90) did bind IL-8. Kocbach et al.²⁴ found that quartz particles did not bind cytokines and Veranth et al.³³ observed that metal oxide particles did bind several cytokines. Until the fundamental principles governing the particle-protein interaction can be determined, particle interaction with the LDH protein needs to be determined on a case-by-case basis, severely limiting the utility of the LDH assay.

Evidence of the particle interference with the MTT and LDH viability assays is apparent in the results from the human bronchial epithelial cell exposure. For the particles tested here,

toxicity measured by the LDH and MTT assays did not correlate for most particle types. Only the H₂O₂ positive control and the soot exposed cells showed similar decreases of viability in both assays. Furthermore, even the ranking of the toxicity of each particle type did not agree between the two assays. Both the activated carbon and the titanium dioxide exposed cells showed a large decrease in viability measured by the MTT assay, but only a minimal decrease of viability measured by the LDH assay. Considering that both of these particle types adsorbed or inactivated the LDH protein, it is possible that the lack of a strong toxic response in the LDH assay is an artifact. Cells exposed to diesel and oxidized soot particles displayed what is likely to be an artifact, in that they had an apparent viability increase over the control group. These diesel particles have previously been shown to increase inflammatory mediators²⁷, oxidative stress²⁶, and toxicity²⁷ (measured by LDH release) in this cell line. Additionally, the diesel particles reacted with MTT to generate formazan in cell-free conditions, thus adding to the formazan generated from viable cells; therefore we believe that the apparent increased metabolic rate is an artifact. In cell free conditions oxidized soot reduced the measurable LDH concentration, possibly obscuring a toxic effect. Although flame soot was also shown to reduce LDH in cell-free conditions, cells exposed to flame soot had a reduced viability measured by the LDH assay. This effect may be attributable to other proteins with a greater affinity adsorbing to the particle and displacing the LDH protein³¹.

Other approaches to identify hazardous particles that are free from particle interference need to be investigated further. One way of doing this is by performing the assay under conditions that mitigate assay interference from particles. Examples of mitigation would be to limit particle concentrations to below levels that interfere, such as was done by Kroll et al.⁵, or by adding compounds such as FBS which can decrease the adsorption of cytokines to particles²⁴. Alternatively, if the interference is well characterized for the exposure conditions, a correction factor may be applied²⁴. Herzog et al.³⁴ have suggested avoiding many of the pitfalls of standard toxicity assays by using a clonogenic assay, but this would necessitate an exposure duration of 7 to 10 days. As yet there remains a gap for a robust toxicity assay that can be used for high throughput screening of particle toxicity. Ayres et al.³⁵ have discussed a variety of cell-free systems to assess oxidative stress, such as the measurement of antioxidant depletion, which may provide information on the comparative toxicity of particles. More recently, impedance based measurements of cellular toxicity, which do not use labels or optical measurements have shown great promise at detecting nanoparticle induced toxicity without interference³⁶. Until methods that avoid nanoparticle interference have been fully developed, toxic assessment will necessitate careful consideration of particle interference.

Our results demonstrate that ranking the toxicity of particles by in vitro exposure is no trivial matter, as commonly used viability assays, such as the LDH and MTT assay, are ineffective in ranking the particle-induced toxicity even at low particle concentrations. Despite suggestions that multiple assays should be used to account for interference^{7, 37}, we show that even with a small subset of particles of similar type, each assay used can still be subject to interference from particles with diverse characteristics. Our comparison of the soot and oxidized soot show that changes in surface chemistry alone will result in very different interferences. We suspect other parameters that may impact surface chemistry, such as different media or additives like FBS, may also impact particle interference. Therefore, we recommend that a thorough characterization of particle interference be carried out for each particle toxicity assessment to account for the impacts of different particles, media, additives, and assay compounds.

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Abbreviations

LDH	lactate dehydrogenase
DPPC	dipalmitoyl phosphatidylcholine
MEM	minimum essential medium
FBS	fetal bovine serum
DMSO	dimethyl sulfoxide

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Figure 1.

(a) Particle spectra at a concentration of 50 $\mu\text{g/ml}$. (b) Spectra of the supernatant of particle suspensions centrifuged for 10 minutes at $115 \times g$.

Figure 2.

Percentage of formazan generated from particles under cell-free conditions. (a) Particle suspensions (0, 0.05, 0.5, 5, 50 μg/ml) were incubated with 0.6 mM of MTT for 3 hours at 37 °C. (b) Ascorbic acid was added to the above samples to reduce remaining soluble MTT to formazan. All samples were centrifuged for 10 minutes at $115 \times g$ to remove the particles and corrected for background absorption.

Figure 3.

Percentage of the 0.15 U/ml LDH measured in the supernatant after incubation with particle suspensions. (a) Mixtures with particle concentrations of 0, 9, 23, 46, 92 µg/ml were incubated for 15 minutes. (b) Mixtures with a particle concentration of 46 µg/ml were incubated for 5, 15, 30, and 60 minutes. All samples were centrifuged for 10 minutes at $115 \times g$ prior to performing the LDH assay and absorbance values were corrected for the background.

Figure 4.

Comparison of the viability (percent of the control group) measured by the LDH assay and the MTT assay. $MTT_{viability} = MTT_{exposed} / MTT_{control} \times 100$. LDH viability is calculated from equation 1. The control group are cells exposed to LHC-DPPC ($MTT = 0.52 \pm 0.05$, $LDH = 0.25 \pm 0.04$). Error bars represent the SE. * Statistically significant at $p < 0.05$ for the MTT assay, † statistically significant at $p < 0.05$ for the LDH assay.

Table 1

Hypothesized particle induced artifacts and the impact on the measured viability (increase, decrease, or no change) for the MTT and LDH assays.

Artifact	MTT		LDH	
Light Absorption	Particle absorbance adds to the MTT absorbance	↑	Particles are not present in the assay medium	—
Adsorption	Particle adsorbs formazan compound, reducing the assay absorbance	↓	Particles adsorb LDH preventing LDH from being measured	↓
Reactivity/Inactivation	Particles generate formazan, adding to the assay absorbance	↑	Particles inactivate the LDH enzyme preventing LDH from being measured	↓

Table 2

Particle composition, surface area, and size

Particle Type	Composition	Surface Area (m ² /g)	Mean Dry Aggregate Diameter (nm)
Diesel Particles	Carbonaceous ^a 20% extractable mass in dichloromethane	108 ^a	180 ^a
Activated Carbon	Steam activated carbon ^a 0.2% Chloride 0.2% Sulfate 0.005% metals	1200 ^a	23,000 ^a
Flame Soot	Elemental Carbon ^b	124 ^c	278 ^d
Oxidized Flame Soot	Elemental Carbon ^b	123 ^c	291 ^d
Titanium Dioxide	99.5% TiO ₂ ^a 0.3% Al ₂ O ₃ 0.2% SiO ₂ 0.01% Fe ₂ O ₃	95 ^a	21 ^a

^aReported by manufacturer^bThermo-optical analysis 21^cBET surface area measured with a micromeritics (Tristar 3000 gas adsorption analyzer)^dMeasured with a transmission electron microscope (FEI Tecnai 12)