Published in final edited form as:

Toxicology. 2011 September 5; 287(0): 99–104. doi:10.1016/j.tox.2011.06.011.

Validation of an LDH Assay for Assessing Nanoparticle Toxicity

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Abstract

Studies showed that certain cytotoxicity assays were not suitable for assessing nanoparticle (NP) toxicity. We evaluated a lactate dehydrogenase (LDH) assay for assessing copper (Cu-40, 40 nm), silver (Ag-35, 35 nm; Ag-40, 40 nm), and titanium dioxide (TiO₂-25, 25 nm) NPs by examining their potential to inactivate LDH and interference with β -nicotinamide adenine dinucleotide (NADH), a substrate for the assay. We also performed a dissolution assay for some of the NPs. We found that the copper NPs, because of their high dissolution rate, could interfere with the LDH assay by inactivating LDH. Ag-35 could also inactivate LDH probably because of the carbon matrix used to cage the particles during synthesis. TiO₂-25 NPs were found to adsorb LDH molecules. In conclusion, NP interference with the LDH assay depends on the type of NPs and the suitability of the assay for assessing NP toxicity should be examined case by case.

Keywords

nanoparticle; titanium dioxide; LDH assay; cytotoxicity; dissolution assay; NADH

1. Introduction

With a widening spectrum of applications of nanotechnology, concerns were raised over the environmental and human health impacts of nanoparticles (NPs, defined as particles of about 1–100 nm), the building blocks of nanotechnology. Safety evaluation of NPs, however, can be quite challenging given the great variety of physicochemical properties that could confound the biological/toxicological profiles of NPs. Some unique properties of NPs such as size-dependent surface reactivity could give rise to uncertainties in toxicological evaluation of NPs. One uncertainty, among others, lies in the toxicological profiling of NPs using standard assays that have been well established for assessing toxicity of chemicals. There is no guarantee that these assays would work equally well for NPs, warranting research on validating these assays for assessing NP activities. There have been several studies evaluating some of the commonly used cytotoxicity assays for NP toxicity research.

Some of these studies found that carbon nanotubes could interfere with certain assays such as the MTT assay (Wörle-Knirsch et al., 2006; Casey et al., 2007; Belyanskaya et al., 2007).

In this paper, we evaluated the suitability of a lactate dehydrogenase (LDH) assay for evaluating nanoparticle toxicity. In the assay, the level of extracellular LDH released from damaged cells is measured as an indicator of cytotoxicity. The assay relies on measuring the activity of LDH in catalyzing the reaction: $NADH + Pyruvate \xrightarrow{LDH} NAD^+ + Lactate$, where NADH is reduced to β -nicotinamide adenine dinucleotide. Since NADH has a peak absorbance at 340 nm, the rate of decrease in NADH level can be measured and used to determine LDH activity if the reaction starts with known levels of NADH and pyruvate and an unknown level of LDH. The absolute values of the slopes were taken as surrogates for LDH activity of the samples (in a unit of $\mu\text{M}/\text{min}/\text{ml}$).

The reliability of this assay, when used for measuring nanoparticle toxicity, could be influenced by several factors. NPs could inactivate LDH molecules nonspecifically in a solution via generation of free radicals on their surface. Metallic NPs in particular could cause metal-catalyzed oxidation (MCO) of LDH. Studies have shown that ROS produced in MCO reactions can cause site-specific damage to proteins (Madurawe et al., 1997; Breccia et al., 2002), though metal ions, not NPs, were usually studied in the existing literature. A NP could be a target on which LDH molecules are adsorbed, inactivated, and come off for new LDH molecules to be adsorbed, forming a dynamic process (dynamic adsorption). If the rate of this dynamic inactivation of LDH by NPs (in an NP-treated sample) is higher than the rate of natural LDH degradation without the presence of NPs (control sample), the measured LDH levels of the NP-treated samples would decrease more quickly over time than the control if both are measured multiple times over a time period. Alternatively, LDH molecules could be adsorbed firmly onto the surface of these particles without coming off (static adsorption), thus making the LDH molecules inactive or unavailable for measurement (unavailable because these adsorbed LDH molecules would be spun down together with the NPs before measuring the samples for LDH activity). The amount of remaining active LDH molecules would decrease initially and then become stable after the static adsorption is finished. In the case of a static adsorption process, the LDH levels over time of NP-treated samples do not have steeper slopes after the adsorption reaches equilibrium; but they do have lower LDH readings at all time points (except time zero) than the control due to the initial loss of LDH. Actually, some enzymes have been shown to be inactivated upon adsorption onto the surface of single-walled carbon nanotubes (SWCNTs) (Karajanagi et al., 2004).

An additional concern is possible oxidation of NADH by certain NPs. Some studies have suggested that certain NPs, like gold or platinum NPs, could catalyze NADH oxidation (Huang et al., 2005; Hikosaka et al., 2008). However, NP size dependency of the NADH oxidation effect was not examined in these studies. Regarding the specific concerns above, we want to know whether these interferences, if any, can significantly influence the results of the LDH assay.

2. Methods

2.1. Reagents and NPs

Potassium phosphate monobasic (KH_2PO_4 , P0662), potassium phosphate dibasic (K_2HPO_4 , P3786), cupric sulfate (CuSO_4 , C7631), reduced β -nicotinamide adenine dinucleotide (NADH, N8129) and sodium pyruvate (P2256) were purchased from Sigma. Sodium sulfate (Na_2SO_4 , SX0763-1) was from EM Science. Phenol red free RPMI 1640 cell culture medium (11835), HBSS (14175), gentamicin (15750), and trypsin (25300) were from GIBCO® Invitrogen. Fetal bovine serum was from Hyclone (SH30070.03). Membrane discs for the dissolution assay were from Spectrum Laboratories, Inc (Spectra/Por® Regenerated Cellulose, 33 mm, Product No.: 132488) with a cutoff molecular weight of 3.5 kDa (~2 nm equivalent pore size).

NPs used for this study include a commercially available TiO_2 -25 (TiO_2 Degussa, 25 nm), a metallic copper NP (Cu-40, 40 nm, from Nanotechnologies), two silver NPs (Ag-35, 35 nm, from Nanotechnologies and Ag-40, 40 nm, from Applied NanoScience). Ag-35 NPs have carbon matrix while Ag-40 NPs do not. Carbon matrix used in Ag-35 NPs was supposed to prevent aggregation of the NPs during the preparation stage. TiO_2 -25 has been frequently studied and it is generally benign. On the other hand, Cu NPs have been shown to be very active in our laboratory (Rushton et al., 2010) as well as by others (Chen et al., 2006). Silver NPs have intermediate activity in terms of generating reactive oxygen species (ROS) (Rushton et al., 2010).

2.2. Physicochemical characterization of the NPs

Hydrodynamic size measurement was performed using a Malvern ZetaSizer (ZetaSizer Nano ZS, Malvern Instruments Inc., Malvern, PA, USA) for the NPs dispersed in water. Malvern ZetaSizer measures the size of particles in a solution based on the principle of dynamic light scattering (DLS). BET isotherm (Autosorb-1, Quantachrome) was used to obtain the specific surface area of the NPs in the dry state (Brunauer et al., 1938). The BET method works by measuring the quantity of a gas (usually nitrogen) adsorbed onto the surface of the material of interest at low temperature (77 K for nitrogen). However, some errors are fine since the purpose of this characterization in aqueous solution to judge agglomeration/aggregation state which does not have to be quantitatively accurate. The accuracy of these measurements is not directly related to other results in this work.

2.3. Cell culture

Used in this study was a rat lung epithelial Type-I cell line R3/1, which was originally isolated from fetal male Han-Wistar rat lung tissue. The cell line demonstrates a predominantly Type I alveolar epithelial cell phenotype and it has surface markers such as T1 α , Caveolin-1 and 2, and Connexin-43 (Kosłowski et al., 2004).

Cells were maintained in cell culture flasks (75 cm²) with RPMI 1640 cell culture medium containing 10% fetal bovine serum (FBS) in an incubator (37 °C, 5% CO₂, and 98% humidity) and were passaged every 3 or 4 days. To passage cells, the cell culture flasks were

washed with HBSS three times and trypsin was added to detach cells from the bottom of the flasks.

2.4. LDH assay

To prepare samples for the LDH assay, cells of passage numbers 11-20 were used. One milliliter of cells at a density of 5×10^5 cells/ml (RPMI containing 10% FBS) were seeded in each well of 12-well plates and grown for 48 hr before NP exposure. The cells were washed with HBSS three times and dosed with different concentrations of NPs in RPMI medium containing 1% FBS. After 24 hr exposure, the 12-well plates were shaken briefly to homogenize the released LDH in the cell culture medium and the medium was transferred to 1.5 ml microcentrifuge tubes and were centrifuged at 12,000 xg and 4 °C for 15 min to remove any cell debris and NPs. One hundred microliters (100 μ l) of each sample was added to the substrate solution and the absorbance at 340 nm was measured using a spectrophotometer (UV-Vis HP-8453). The LDH activity of the samples was obtained by measuring the decreasing rate of NADH absorbance over time (slopes) and therefore all the slopes thus obtained were negative.

2.5. LDH inactivation study

The general idea of studying the inactivating effects of NPs is to observe the LDH activity over time in a sample with a fixed amount of LDH (no additional source of LDH such as cells). To achieve this goal, purified LDH molecules can be used (but not used in this study). Alternatively, lysed cells can be directly used as LDH samples without further steps to purify LDH since the real concern of LDH stability is not purified LDH but LDH in cell lysate in the presence of NPs.

We lysed cells by first freezing cells (5×10^6 cells in a small amount of medium containing 1% FBS) at -80 °C over night. The cells were then thawed and vigorously shaken at 4 °C for 60 min. The cell lysate was then centrifuged at 14,000 g and 4 °C for 30 min and the supernatant was collected. The aliquots were ready for use upon dilution with 5 ml of RPMI medium containing 1% FBS with or without NPs. Note that samples in all the groups contain the same starting level of LDH. One hundred sixty microliters (160 μ l) of diluted samples with or without NPs was placed into each well of 24-well cell culture plates and incubated (37 °C, 5% CO₂, and 98% humidity) for 1, 4, 8, or 24 hours. The remaining samples were measured immediately for LDH levels and recorded as LDH levels at “time zero”.

2.6. Dissolution assay

To investigate the role of released metal ion, we performed a dissolution assay for Ag-40 and Cu-40 NPs. A certain amount of NPs to be tested was dispersed in simulated lung fluids, one with a pH of 7.4 with CO₂ bubbling and the other has a pH of 4.5 (Potter and Mattson, 1991). Particles to be tested are placed in the upper chamber of a cell that is separated by an ultrafiltration membrane. The equivalent pore size of the membrane is about 2 nm so that the original NPs with an average size of 40 nm cannot get across until they dissolve or become much smaller. The simulated fluid flows through the lower part of the cell at a constant rate (50 μ l/min) and the hourly fractions were collected for elemental analysis using atomic

absorption spectroscopy (Perkin-Elmer model AAnalyst 600 with longitudinal Zeeman background correction, Shelton, CT, USA) measured at 328.1 nm for silver or direct current plasma atomic emission spectrometry (DCP-AES; Beckman SpectraSpan V, Beckman Inst., Somerset, NJ) at 324.8 nm for copper. More information about the dissolution assay was provided in other published works (Potter and Mattson, 1991; Eastes and Hadley, 1994; Maxim et al., 2006).

2.7. NADH oxidation assay

The LDH assay was performed on NPs dispersed in the substrate solution (containing the substrates NADH and pyruvate) without LDH. If the NPs tested behave like LDH molecules and have significant effect on NADH oxidation, an increase in “LDH” reading should appear because the assay works by measuring the oxidation rate of NADH, no matter what causes NADH oxidation. NPs of interest were dispersed in the LDH assay substrate solution (NADH and pyruvate) and the LDH levels of these samples were measured. Note that this process does not involve any source of LDH molecules so that any oxidation of NADH should occur either naturally or be caused by the NPs. Though the measurement process for LDH samples only takes about two minutes, we also measured the ratio of NADH:NAD⁺ over time for up to 21 hr. We measured the absorbance of the samples at both 340 nm (A_{340} , NADH alone) and 265 nm (A_{265} , NADH and NAD⁺) and used the ratio A_{340}/A_{265} as a surrogate for the relative amount of NADH. This ratio should decrease over time in the samples and the decreasing rate can serve as an indicator for the rate of NADH oxidation.

2.8. Statistical analysis

Four replicates were used in all experiments except data in Figure 7 where duplicates were used after finding that variability in that experiment was very small. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Comparisons between two groups were based on t-test for two independent samples. One phase exponential decay was applied for curve fitting in Figure 4 with the equation $y = \text{Span} \cdot \exp(-k \cdot t) + \text{Plateau}$. In the formula, “Span” and “Plateau” were set as 100 and 0 respectively, k is the decay rate, and t is time.

3. Results

Some of the physicochemical properties of these NPs were summarized in Table 1 in another paper of our group (Rushton et al., 2010). The hydrodynamic sizes of all these NPs are much larger than their primary particle sizes, indicating that these NPs form agglomerates or aggregates in water. According to the measuring principle of the Zeta-sizer, the measurement of Ag-40 NPs involves more error since it shows more than one peak and the peak sizes are based on distribution by volume.

We observed LDH stability in different media. We found good stability of LDH in either HBSS (Hank's Balanced Salt Solution) or RPMI 1640 cell culture medium containing 1% FBS but not in water (Figure 1). Therefore, LDH inactivation potentials of these NPs were evaluated in RPMI with 1% FBS, finding that TiO₂-25 or Ag-40 NPs did not have

significant inactivating potential in cell culture medium with 1% FBS while Cu-40 and Ag-35 were able to inactivate LDH in a dose-dependent manner (Figure 2).

The fact that TiO₂-25 NPs do not inactivate LDH can help explain the dose-response curve of the released LDH upon exposure of R3/1 cells to TiO₂-25. In such a dose-response curve (Figure 3), the LDH response increases over increasing doses and then decreases beyond a certain dose limit (100 µg/ml).

A comparison between Cu-40 NPs and Cu²⁺ found similar profiles of LDH inactivation of the two at equal molar concentration of Cu NPs and Cu²⁺ (Figure 4). Curve fitting assuming one-phase exponential decay suggested that the half lives of LDH in 20 µg/ml of Cu-40 NPs (4.4% per hr) and Cu²⁺ (4.2% per hr) were very close. To validate that the LDH inactivating potential of CuSO₄ was caused by Cu²⁺ and not by SO₄²⁻, a comparison between CuSO₄ and Na₂SO₄ was compared at equimolar concentration of SO₄²⁻. Results showed that CuSO₄ but not Na₂SO₄ could inactivate LDH, confirming our assumption that the LDH inactivation potential of CuSO₄ was indeed due to the presence of Cu²⁺ but not SO₄²⁻ (Figure 5).

The similar profiles of LDH inactivation of Cu-40 and Cu²⁺ (Figure 4) seem to suggest that Cu-40 mainly inactivates LDH via the release of copper ions (Cu⁺ and/or Cu²⁺). To test this hypothesis, we measured the dissolution rate of Cu-40 in simulated lung fluids and found that Cu-40 has a high rate indeed (Figure 6). In clear contrast, Cu-40 NPs had a half life of about 9-10 hr in the fluid with a pH of 7.4 while less than 0.1% of Ag-40 NPs dissolved 30 hr later. However, we do not know whether the dissolved ions are Cu²⁺ or Cu⁺.

Finally, we determined whether NPs themselves can oxidize NADH and thereby interfere with the outcome of the LDH assay. We found that the reading did not change in the presence of NPs compared to controls in two minutes which is the required time for completing the reading (data not shown), eliminating the concern that a significant LDH-mimicking effect of NPs could exist to invalidate the assay. The samples were measured three times over a time scale of 21 hr and the presence of NPs still did not lead to statistically higher rate of NADH oxidation (Figure 7). In Figure 7, all samples including the controls showed decreased readings over time, indicating that NADH oxidation can occur without the presence of NPs or LDH.

4. Discussion

We evaluated the usefulness of an LDH assay for assessing NP cytotoxicity. We first examined the stability of LDH in different media and showed that LDH was quite stable in either HBSS or RPMI 1640 cell culture medium containing 1% FBS but not water (Figure 1). Many macromolecules are evolutionarily adapted to the physiological osmolarity for maintaining normal structure and function and therefore a significant shift from the optimal osmolarity could change their structures and functions (Clark and Zounes, 1977; Jenkins and Tanner, 1977; Gróf et al., 1996).

When evaluating the stability of LDH in RPMI containing 1% FBS, Cu-40 and Ag-35 were found to cause decreased LDH activity over time (Figure 2). Since TiO₂-25 does not

inactivate LDH, a reasonable explanation for the decreasing part of the curve in Figure 3 is that a significant portion of the LDH activity could not be measured due to adsorption of LDH molecules onto the TiO₂-25 particle surface. The similar LDH inactivation pattern shared by Cu-40 NPs and Cu²⁺ (in form of CuSO₄) (Figure 4) at an equimolar concentration seems to suggest that LDH inactivation by Cu NPs was predominantly through a dissolution process that gradually releases copper ions. Though we did not study the exact form of these ions, Cu⁺ ions could be the directly released form and they were probably quickly oxidized into Cu²⁺, the more stable form of copper ions. There are some differences between the curves of the copper NP and Cu₂ at time zero and 1 hr time point and these could have been caused by a time delay for Cu NPs to release copper ions. Note that one-phase exponential decay model used for fitting the curves in Figure 4 may not be the best model for fitting Cu NP data because the model assumes a stable level of copper ions and this assumption does not hold for the Cu-40 samples in the LDH inactivation assay due to a continuous release of copper ions in the dissolution process of the particles that leads to an increase in the level of copper ions. In addition, the fitting for 5 µg/ml Cu²⁺ group was not as good either, which could have been caused by the unstable Cu²⁺ level (relative to 10 and 20 µg/ml Cu²⁺ groups) over time because of possible conjugation of Cu²⁺ by macromolecules in cell lysate.

Cu-40 NPs had a very remarkable rate of dissolution in a simulated lung fluid at a pH of 7.4 (Figure 6). The quick dissolution of copper NPs could be caused, in part, by oxidation that has already occurred on the surface of the particles via brief exposures to room air when taking out samples from the storage bottle for use, though the bottle is always flushed and refilled with argon for storage. Chen et al. (2005) suggested that surface oxidation of copper NPs (~14 nm average size) was a self-limiting process at 298 K (25 °C) and that the outer oxidation layer (Cu₂O) did not progress further after reaching a maximum thickness of about 2.3 nm (Chen et al., 2005). Nevertheless, it is advisable to aliquot a new type of NP for proper storage when its chemical stability is still in question.

The finding that copper NPs were able to inactivate LDH suggests that data from the LDH assay have to be interpreted with caution. This result also underscores the importance of combining several cytotoxicity assays in the safety evaluation of a chemical or NP. While the LDH assay was evaluated by focusing on several mechanistic aspects within the assay itself, it is a different issue whether toxicity results from an *in vitro* assay are a valid indication of the *in vivo* situation. The ultimate criterion for validating an *in vitro* assay in terms of *in vivo* relevance should be to demonstrate the correlation between *in vitro* and *in vivo* results.

A high dissolution rate of Cu-40 NPs may underlie the remarkable LDH inactivation potential of the NPs in cell culture medium. The *in vivo* relevance of the dissolution assay is a concern when using it as a tool for toxicity evaluation. To the best of our knowledge, correlation studies regarding *in vitro* vs. *in vivo* dissolution with NPs have not been carried out.

The fact that TiO₂-25 in a wide dose range of 10 – 200 µg/ml did not lead to LDH inactivation (Figure 2) and that the LDH response to TiO₂-25 exposure in R3/1 cells would

decrease beyond a certain dose limit (100 µg/ml) (Figure 3) implied that adsorption of LDH molecules onto NP surface was a reasonable explanation for the decreasing part of the dose-response curve. The concentration-dependent increase-decrease pattern of LDH measurement following cellular exposure to the NPs is determined by two counter-acting processes: LDH release from cells and LDH adsorption onto NP surface. Though the high toxicity at high NP concentration range can lead to high LDH release, the release is limited by the limited number of cells available. When all cells are dead and no further LDH can be released, further increase of NP concentration will decrease the released LDH by providing more surface area for LDH adsorption.

Adsorption and protein inactivation are two common types of NP-protein interaction. Some studies on NP-protein interactions (Cedervall et al., 2007; Linse et al., 2007) suggested that NP-protein interactions can be mechanistically diversified and the degree of interactions can be different depending on the types of NPs and the types of proteins. Many of the interactions are likely to be relatively nonspecific, suggesting that one should be very careful in validating any protein-based or other macromolecule-based assays for nanotoxicological research. An assay is not invalidated simply upon finding interference from the tested NPs; instead, the interference should be quantified so that one can reach a reliable conclusion about its usefulness.

The difference between the LDH inactivation potentials of Ag-40 and Ag-35 (Figure 2) could be caused by the presence of carbon matrix that is used for preventing aggregation of Ag-35 NPs during synthesizing process while Ag-40 NPs do not contain carbon matrix. This finding, together with some studies on NP-protein interactions (Cedervall et al., 2007; Linse et al., 2007), suggest that carbon-based or carbon-containing nanomaterials such as carbon nanotubes might be able to inactivate protein molecules in a relatively nonspecific manner. Obviously, more studies need to be performed to confirm this suggestion. This again support the point that validating a protein-based assay should be conducted on a case-by-case basis because the presence and degree of interference could depend on the surface properties of NPs tested and the type of protein measured in the assay.

Since some studies have found that certain NPs, like gold and platinum, could catalyze the oxidation of NADH (Huang et al., 2005; Hikosaka et al., 2008), it was necessary to examine possible direct interference with the LDH assay from the NPs via oxidation of NADH. The temporal pattern of NADH oxidation of the NP groups did not differ significantly from the control (Figure 7), eliminating the concern that NP could interfere with the assay by directly affecting the LDH substrate (i.e., NADH). For validating the LDH assay, we did not examine the effect of NPs on NADH alone, as has been done in the cited studies above (Huang et al., 2005; Hikosaka et al., 2008); instead, we focused on NADH oxidation by NPs in the presence of pyruvate, because it is the mixture of NADH and pyruvate that is used as the substrate solution for performing the LDH assay. Even if NPs could have some effect on NADH oxidation on a time scale of hours, this effect could still be negligible after a few minutes, which leaves enough time to complete a normal measurement session of the assay.

In summary, among the several NPs examined, Cu-40 and Ag-35 NPs were able to inactivate LDH. Therefore, data from LDH assay have to be interpreted with caution

whenever the assay is used for assessing cytotoxicity of an ROS-generating material. This result also confirmed the importance of combining several cytotoxicity assays when evaluating a chemical or NP.

Acknowledgments

The dissolution apparatus was kindly provided by Dr. Russell M. Potter at Owens Corning Science and Technology Center, Granville, OH. This work was supported by AFOSR MURI Grant FA9550-04-1-0430, NSF SGER grant BES-0427262, UR-EPA PM Center Grant RD83241501 and the University of Rochester Toxicology Training Grant T32ES07026.

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Figure 1. LDH stability in deionized water, HBSS, or RPMI 1640 containing 1% FBS

Samples were prepared by dissolving LDH-containing cell lysate (from R3/1 cells) in different media including deionized water, Hank's Balanced Salt Solution (HBSS), and RPMI cell culture medium containing 1% FBS. The samples were then placed in a cell culture incubator for various times. LDH activity of the samples was measured using LDH assay. LDH levels were found to be stable in either HBSS or RPMI but not in water. Data were normalized with respect to HBSS (** p<0.01).

Figure 2. The effect of NPs on the stability of LDH in RPMI 1640 containing 1% FBS
 The NPs were dispersed in RPMI medium containing 1% FBS and equal concentration of LDH (derived from R3/1 cells) and incubated in the incubator for various times. LDH was measured at these time points. Ag-35 and Cu-40 NPs inactivate LDH while TiO₂-25 and Ag-40 don't (** p<0.01). Data were normalized with respect to the control.

Figure 3. Dose-response data of *in vitro* LDH release from R3/1 cells following exposure to TiO₂-25 NPs

A dose-response curve of TiO₂-25 NPs (24-hr LDH, cultured R3/1 cells) shows a decreasing trend following an initial increase as the dose increases. This is probably due to LDH adsorption onto NP surface (** p<0.01 compared to maximum LDH response at a dose of 100 μg/ml). Note that doses are log-transformed (base 10).

Figure 4. Cu²⁺ plays a dominant role in LDH inactivation by Cu-40 NPs

Time trend of LDH inactivation by Cu-40 NPs was compared to that of Cu²⁺ (in form of CuSO₄) in a cell-free condition. Cu-40 and CuSO₄ were dispersed in RPMI containing 1% FBS and equal concentration of LDH and incubated for various times. LDH was measured and comparison was made between the LDH time trends of the particle and the salt. LDH inactivation profiles of the copper NP and Cu²⁺ are similar exponential decay curve fitting results. Data were normalized as percentages of controls.

Figure 5. Cu^{2+} , not SO_4^{2-} , plays a dominant role in the LDH inactivation of CuSO_4

To distinguish the roles of Cu^{2+} and SO_4^{2-} in LDH inactivation by CuSO_4 , CuSO_4 and Na_2SO_4 were examined for their effects on the stability of LDH. The salts were dispersed in RPMI containing 1% FBS and equal concentration of LDH and incubated for various times. The decreasing LDH level in the presence of CuSO_4 but not Na_2SO_4 suggests that Cu^{2+} plays a dominant role in the LDH inactivation potential of CuSO_4 and SO_4^{2-} doesn't seem to have a significant effect on LDH activity (** $p < 0.01$).

Figure 6. Dissolution trends of Ag-40 and Cu-40 NPs in simulated lung fluids

Dissolution assay was performed as described in the text. Cu-40 NPs have a very rapid rate of dissolution (almost 100% in 30 hr) while Ag-40 NPs have a very slow dissolution though both have statistical significance when comparing some of the data points on the right side with the control ($p < 0.0001$). Why weren't the Ag-35 particles tested? Answer: Because Ag-35 NPs are not as pure as Ag-40 NPs and they are also slightly smaller on average.

Figure 7. TiO₂-25 and Ag-40 NPs do not affect the stability of NADH in LDH assay buffer without LDH

NPs of interest were dispersed in the substrate solution (NADH and pyruvate) and absorbance of the samples was measured at 340 nm (A_{340} for NADH alone) and 265 nm (A_{265} for NADH and NAD⁺). The decreasing rate of A_{340}/A_{265} over time was used as an indicator of the oxidation rate of NADH. Similar patterns of the NP groups with that of the control suggest that these NPs do not have a significant effect on NADH oxidation. Data were first expressed as the ratio A_{340}/A_{265} and then standardized as percentage of value at time zero. (** $p < 0.01$ compared to time zero).

Table 1
Some of the physicochemical properties of the NPs used in the study (Most of the data was from Rushton et al., 2010)

NP	Source	Primary size reported by manufacturer (nm)	Hydrodynamic size (nm) ^(a)	Specific surface area (m ² /gm) ^(b)
TiO ₂ -25	Degussa Chemicals	25	576	57
Cu-40	Nanotechnologies	40	850	31
Ag-35 (carbon matrix)	Nanotechnologies	35	483	21
Ag-40	Applied	40	45 (50%)	10
	NanoScience		185 (35%)	
			5390 (15%)	

Notes:

^(a) Hydrodynamic size of the NPs was measured using Malvern ZetaSizer (ZetaSizer Nano ZS) and peak size calculation was based on particle volume

^(b) Specific surface area of the NPs was measured using the BET method (Brunauer et al., 1938) except Ag-40 for which a calculation based on size distribution data from TEM images was used.