Chemical Research in Toxicology

Perspective

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Use of Cause-and-Effect Analysis to Design a High-Quality Nanocytotoxicology Assay

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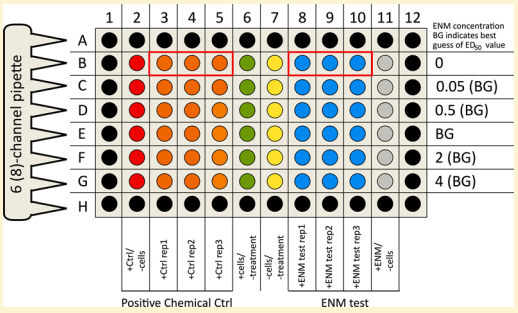
Supporting Information

ABSTRACT: An important consideration in developing standards and regulations that govern the production and use of commercial nanoscale materials is the development of robust and reliable measurements to monitor the potential adverse biological effects of such products. These measurements typically require cell-based and other biological assays that provide an assessment of the risks associated with the nanomaterial of interest. In this perspective, we describe the use of cause-and-effect (C&E) analysis to design robust, high quality cell-based assays to test nanoparticle-related cytotoxicity. C&E analysis of an assay system identifies the sources of variability that influence the test result. These sources can then be used to design control experiments that aid in establishing the validity of a test result. We demonstrate the application of C&E analysis to the commonly used 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) cell-viability assay. This is the first time to our knowledge that C&E analysis has been used to characterize a cell-based toxicity assay. We propose the use of a 96-well plate layout which incorporates a range of control experiments to assess multiple factors such as nanomaterial interference, pipetting accuracy, cell seeding density, and instrument performance, and demonstrate the performance of the assay using the plate layout in a case study. While the plate layout was formulated specifically for the MTS assay, it is applicable to other cytotoxicity, ecotoxicity (i.e., bacteria toxicity), and nanotoxicity assays after assay-specific modifications.



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INTRODUCTION

Engineered nanomaterials (ENMs) are manufactured nanoparticles often with unique physicochemical properties when compared to bulk materials. These unique properties such as high surface reactivity and quantum confinement will allow ENM to play a role in a variety of commercial applications such as textiles, environmental remediation, and medicine.[1−5] However, these same properties may also result in ENMs having unintended and potentially harmful effects on ecological receptors or humans during the manufacturing, use, and disposal of nanomaterial-enabled products.[6−8] It is important to

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be able to accurately assess the effects of nanomaterials on biological systems to inform risk−benefit models that guide how to regulate these specialized materials.

A tiered testing approach similar to that used for chemical compound testing[9,10] has been proposed for assessing the potential hazard associated with ENM.[11,12] The approach starts with cell-based toxicity assays as rapid screening tools and suggests further testing based on the screening results, the exposure mode, and the physicochemical characteristics of the ENM.[13] However, this approach requires the availability of cheap, reliable, and well-controlled cell-based assays to assess ENM−biological system interactions. Unfortunately, different laboratories often obtain substantially differing results when testing cellular interactions with ENMs.[14] For example, the reported effects of nanoparticulate TiO_2, carbon nanotubes (CNT), silica, and ZnO nanoparticles on cellular systems appear contradictory.[15−18] Uncontrolled conditions such as laboratory lighting or interference with toxicity assay readouts have been shown to affect assay results for ENM (i.e., TiO_2 and CNT).[19−23] Moreover, a literature survey of nanomaterial toxicity papers using biochemical techniques published since 2010 revealed that approximately 95% of these papers did not account for ENM interferences.[24] Incomparability of data for the effects of ENM on cells can result from poorly defined information on dosage, as well as differences in assay procedures, poor information on assay system performance, and weak or absent assay quality control experiments.[14] Physico-chemical properties of ENM, such as composition, size, shape, crystal structure, coating, and dissolution and dispersion techniques, also influence the measured ENM−cell interactions and can cause differences in assay results. The presence of these factors, which are not often encountered in soluble chemical based assays, calls for assay design and standards to ensure the comparability of the ENM cytotoxicity assay test results among different laboratories.

One approach that has been used to identify sources of variability in analytical tests is cause-and-effect analysis (hereafter referred to as C&E analysis). C&E analysis is based on the application of measurement science to fields such as quality manufacturing and propagation of measurement error in analytical chemistry.[25] C&E analysis identifies steps within a process where modification and quality monitoring may improve the quality of the manufacturing process or a measurement test result. C&E diagrams graphically summarize the potential causes of variations in a given test method, which can help develop a strategy for gaining control over the sources of variability. Critical to using these process analysis techniques is the initial identification of sources of variability or the "causes" of the variability and their "effect" on the result of a process. Application of C&E analysis to nanocytotoxicity assays will help identify specific control experiments that could be designed and integrated into the assay to monitor variability associated with the assay test system. While some studies have thoroughly investigated ENM interferences in certain nanotoxicology assays,[24,26,27] potential ENM interferences are only one source of uncertainty that is assessed by C&E analysis.

Here, we specifically utilize C&E analysis to design a robust 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) cell viability assay for ENM cytotoxicity testing. The MTS assay is widely used in cytotoxicity testing because it is one of the simplest assays with only a few major steps in the protocol and is useful for hypothesis testing. We present a prototype 96-well plate layout that incorporates a number of control experiments that assess the quality of the MTS assay system for a nanocytotoxicity measurement and demonstrate the application of this plate layout in a case study. The sources of variability revealed in the C&E diagram and the resulting 96-well plate layout may be generalized to other cell-based and biological assays for evaluating the environmental or human health effects of ENMs or other compounds. However, the nuances of each method need to be carefully considered with regard to how the reagents interact with the biomolecules of interest or may interact with different ENMs to identify the sources of variability for that assay.

GENERAL FEATURES OF THE MTS ASSAY

The MTS assay is a "live−dead" assay where the signal is related to the number of metabolically active cells in a sample (see Figure 1 for an example summary protocol). The

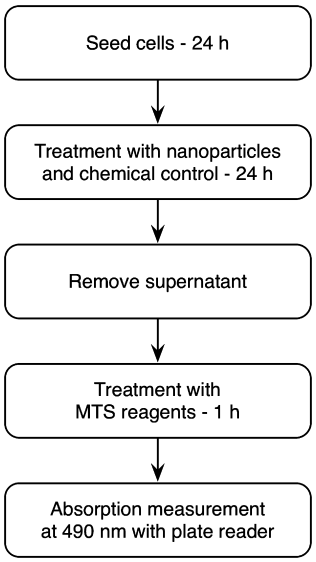

Figure 1. Flowchart with the main process steps of the MTS assay.

absorbance at 490 nm is measured in the sample as the MTS assay reagents are converted to a purple formazan product by intracellular reductase enzymes within living cells. The effect of an ENM dose on the viability of cells can be estimated from the MTS readout as a function of time. Confounding effects arising from factors such as other absorbing species at 490 nm, enzyme activity change, and cell line culture artifacts give rise to variability and bias in the measurement.[1] While the summary protocol shown in Figure 1 indicates a 24 h exposure time with ENM, it is important to assess cytotoxicity after multiple time periods to understand the time dependence of the toxicological effects.

When the MTS assay is used with a dose−response experimental design, the test result is typically an ED_{50} value, the effective dose that causes a 50% effect. The following data analysis steps are used to generate the ED_{50} value: (1) absorbance values at 490 nm are collected from wells of treated cells, nontreated cells (maximum absorbance value), and wells containing no cells (background absorbance value). (2) Background absorbance values are subtracted from all the absorbance values from each well. (3) The background-corrected absorbance values of treated wells are then normalized to the background corrected absorbance values of

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Table 1. General Categories and a Short Description of the Sources of Variability in the MTS Assay

source of variability to be addressed (branch number corresponds to Figure 2)	short description
cell maintenance (branch 1)	includes variability in the maintenance of a cell line such as the following:
	cell passage number
	cell freeze passage
	passaging procedure
	cell vendor
	serum vendor and lot number
	different DNA/genotype
pipetting (branch 2)	Addresses differences in pipetting reproducibility from one well to the other due to the pipetting process. Includes differences in
	cell seeding density
	reagent volume (either of the disturbant of interest or finally the MTS assay reagent)
instrument performance (branch 3)	Addresses issues concerning nonlinearity or general functional problems with the instrument needed for assay readout.
toxic chemical positive control (branch 4)	This branch represents the sources of variability in a toxic response to a positive control reference material. Many of these sources are common for the chemical control and ENM testing system. This branch serves as an assay test system performance control.
assay protocol (branch 5)	includes conditions and protocol specifications, which can influence the mechanistic part of the assay readout such as the following:
	age, storage temperature, and freeze/thaw-cycle numbers of the assay reagent
	change in background absorbance
	optical degradation of reagents
engineered nanomaterial handling and characterization (branch 6)	includes all aspects of ENM:
	dispersion method and quality
	physicochemical properties (e.g., surface charge and chemistry, surface area and reactivity, size, shape, etc.)
	agglomeration behavior in cell-culture medium
	interference reactions with the assay itself (e.g., quenching events)

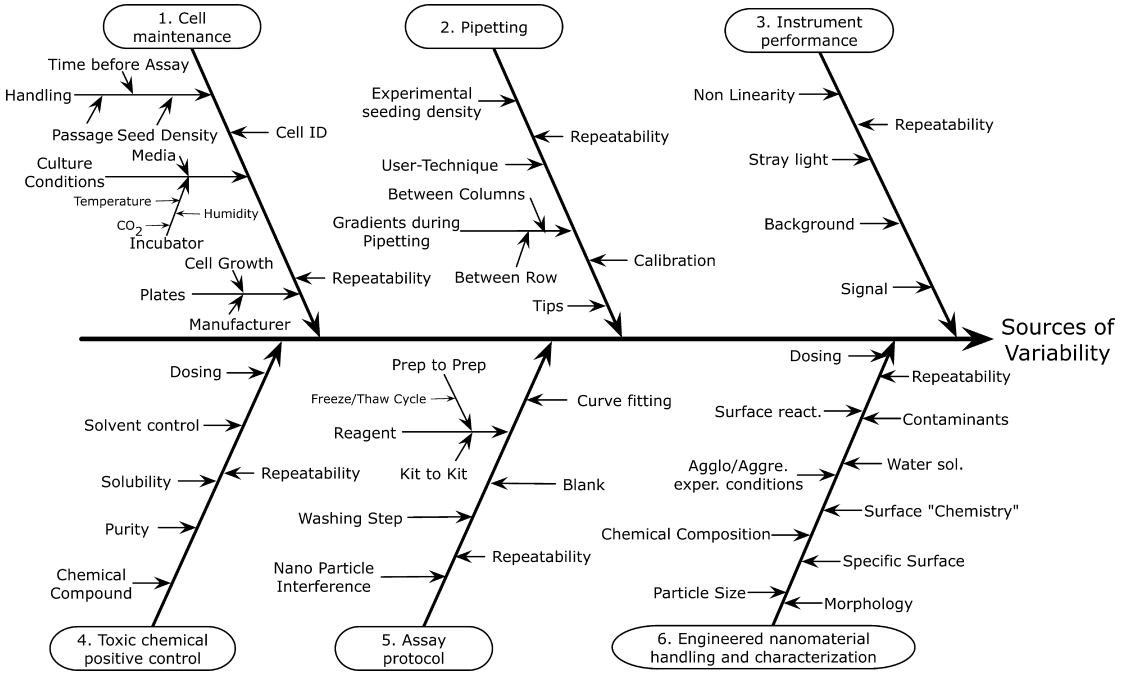

Figure 2. Cause and effect diagram.

the nontreated cell samples. After these calculations, a normalized absorption value near 1 is typically interpreted as no effect of the treatment condition on the cells, whereas a measurement of 0 represents a complete toxic event where no viable cells remain in the cell culture well. (4) The normalized absorbance values are then fitted to a sigmoid dose−response curve, and the ED_{50} value for the curve is calculated.[28] Three or more replicate dose−response curves are used to evaluate the variability of the ED_{50} value.

CAUSE-AND-EFFECT ANALYSIS OF THE MTS ASSAY

The example summary protocol for an MTS cell viability assay for ENM shown in Figure 1 was used to generate a C&E

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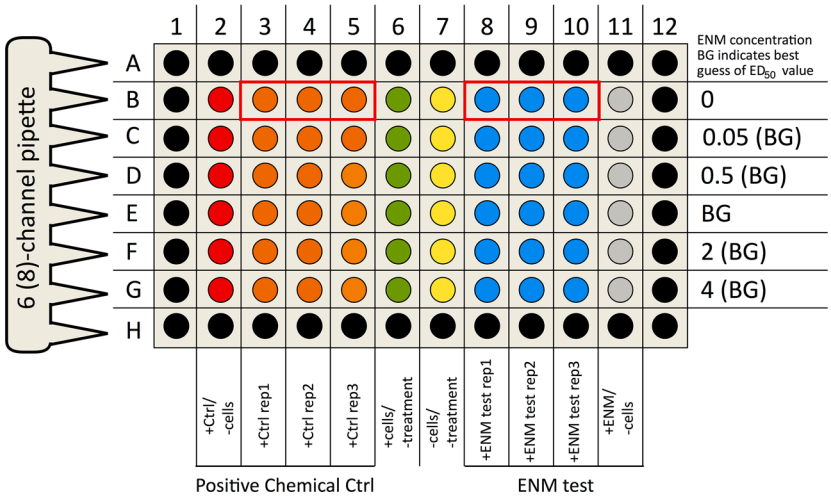

Feature Number	*Branch Number from Table 1 and Figure 2*	*Wells Depicted in Figure 3*	*Brief Control Description*
1	1: cell procurement 2: pipetting	Green wells B6-G6	These wells assess **within** multichannel pipetting variance of cell seeding number. Non-treated cells are seeded with a single multichannel pipette ejection step. This feature can detect technical problems with the pipette and the absolute absorbance measurement provides insight on nominal cell growth.
2		Wells inside red squares B3-B5 & B8-B10	These wells assess **between** multichannel pipetting variance in cell seeding density. Vehicle treated cells (compare B3-B5 for chemical control, B8-B10 for ENMs) are seeded in different ejection steps. This feature can indicate handling problems of the operator during cell seeding procedure and possible effects of the vehicle by comparing to "no treatment" wells (B6-G6)
3	3 & 5: instrument performance and assay protocol	Yellow wells B7-G7	These wells contain only MTS reagent (last step of assay procedure). Allows for determination and evaluation of the background absorbance correction value. Together with outermost wells (control No. 4) assesses possible internal measurement gradients due to instrument malfunction or culture plate variability. Large variations may further indicate issues with the MTS reagent.
4	3, 5 & 1: instrument performance, assay protocol & cell procurement and handling	Black wells A1-12; H1-12 & B1-G1; B12-G12	Additional considerations that exceed those described in No. 3 "no cells no treatment." These wells contain medium from the time of cell seeding to the addition of the MTS reagent. This helps to circumvent so called edge effects that might occur during longer incubation times of cells seeded in small volumes in the outermost wells (i.e., evaporation). Addition of the MTS reagent to these wells aids in establishing instrument performance.
5	4: Toxic chemical positive control	Orange wells B3-G5	Triplicate reference chemical control. ED_{50} measurement is used to assess the overall assay system performance.
6		Red wells B2-G2	Background correction for the toxic chemical positive control. Also serves as an interference control between the toxic chemical compound and the MTS reagent
7	6: engineered nanomaterial handling and characterization	Grey Wells B11-G11	Background correction for the ENM dosing. Also serves as an ENM interference control. It allows detecting if increasing concentrations of the ENM change the final MTS measurement result.
8	actual test samples	Blue wells B8-G10	Measures the influence of ENMs on cell viability in triplicates. These are replicate unknown ENM test samples.

Figure 3. Position of control and test experiments deduced from the cause-and-effect analysis and implemented into a 96-well plate layout. The word "treatment" in this figure refers to both the positive chemical control and ENM test material. All wells contain the MTS reagent.

diagram. We identified major categories for sources of variability in the test result based on the steps on the summary protocol (Table 1). Major categories for sources of variability (i.e., pipetting)[29] can impact many steps in the protocol and are

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considered the main branches of the C&E diagram (Figure 2). Additional details about the contributory factors that may introduce variability in the assay result are then added to each of the main branches (Figure 2). These factors can be found in the manufacturer's protocol, other high-quality protocols, expert opinion, and best-practice guidance documents.[2] The final goal is to add as many reasonable factors that may influence the assay result while minimizing the number of factors that have a negligible effect on the measurement readout (i.e., plate reader manufacturer). After identification of important factors, control experiments were designed to assess the variability in these factors. The results of these control experiments serve to establish quality parameters that can be tracked with control charts to ensure confidence in the test measurement system.

DESIGN OF THE 96-WELL PLATE MTS ASSAY

The control experiments identified during the C&E analysis of the MTS assay were incorporated in a novel 96-well plate layout (see Figure 3). In the 96-well plate layout, only 18 wells (Feature 8, Figure 3) correspond to the actual test samples, which are used to investigate dose-dependent effects of ENM on cell viability. The remaining 78 wells on the 96-well plate serve as seven system-control experiments for qualifying the reliability, reproducibility, and comparability of the test measurement. The ENM dose concentrations are multiples of the best guess concentration (BG) for the ED_{50} value obtained from preliminary experiments or literature values. When choosing the concentrations, it is advisible to include one concentration which elicits no effect, one concentration which elicits a complete effect, and several concentrations which elicit effects on the transition part of the ED_{50} curve.[30] Judicious choices for the test concentrations will help minimize the uncertainty due to fitting the ED_{50} value. Measurements from these control experiments should be charted over time to characterize the natural variability of the test system. Specifications based on the control charts can then be used to define the acceptable operating range of the MTS test system. For each assay, the results from the control experiments must be within the predefined specifications for a valid ENM test result.

CAUSE-AND-EFFECT DIAGRAM AND CONTROL EXPERIMENTS FOR THE MTS ASSAY

Branch 1: Variability Due to Cell Maintenance. Although cell culture is routinely performed in laboratories, many factors that can give rise to variability in ENM cytotoxicity results are not frequently reported. Cell culturing factors that may influence the ENM test results include thaw passage number, passage number at the time of experiment, experimental and passaging cell seeding density, and cell passaging procedures in general including cell detachment techniques and variability in uncontrolled substances such as fetal bovine serum (FBS) (see Table 1 and Figure 2).[3] In addition, the identity of the original cells may be questionable if the DNA integrity has not been confirmed.

The following steps can be taken to address these potential sources of variability. At a minimum, it is critical to document the cell culture handling and maintenance procedures in great detail. This ensures that the steps can be repeated at a later time or in a different laboratory. This documentation can also be used to identify other sources of variability in cell-based nanotoxicity assays. For example, if there is a systematic change in control experiments and the test result, documentation can be used to assess if a change in FBS serum lots or manufacturer could be the source of the change in the control experiment results.

Assuring the DNA integrity in cells used in cell-based assays is critical given recent high profile reporting on the prevalence of contaminated or misidentified cell lines.[31] This can be performed using DNA integrity tests, which are commercially available for human cell lines and have been recently developed for mouse and vervet monkey,[32,33] before initiating experiments. These tests function by confirming the retention of short-nucleotide tandem repeats (STR) within the genomic DNA and are relatively rapid and highly confirmatory.[34] Changes in the STR results can indicate cell line contamination, changes in chromosome structure, or chromosome deletions, all of which can lead to variability in the ENM test result.

Cell line characteristics can also be specified and monitored such as the calculated proliferation rate, isoenzyme analysis for species verification (ATCC), and two-dimensional projected morphology.[35] Each of these parameters is sensitive to culture conditions, cell contamination, extracellular matrix, and cell handling conditions. Ideally, all of these cell-assay-specific factors should be documented, but practically, the benefit of these tests should be weighed against expert opinion before they are specifically described in the testing protocol for improving reliability in the ENM cell assay.

The 96-well plate layout includes a nontreatment cell control experiment (Feature 1, Figure 3). Measurement results (i.e., absolute absorbance) from this control can be compared between different experimental assay plates to generate specifications for nominal cellular behavior data and provide information about the variability of the cell culture conditions. This control provides insight on cellular growth and variability in seeding density.

Branch 2: Variability Due to Pipetting and Cell Seeding. The protocol for the MTS assay[4] begins with seeding a given number of cells per well, known as the experimental seeding density, in a multiwell plate (Figure 1). Pipettes are also used for rinsing and MTS reagent addition during the protocol. It is critical that nontreatment and treatment wells initially have similar numbers of cells to reduce variability during fitting of the dose–response curve.

Control experiments to assess within-pipet channel variability, between-pipetting step variability, and evidence of pipet maintenance/technique can substantially improve assay reliability. An advantage of using a multichannel pipet is that the regularity of the cell seeding density across the pipettes is likely governed by the homogeneity of the suspended cells before they are picked up with the individual pipettes, and the calibration of each pipet channel volume. For well-maintained multichannel pipettes, it is likely that within-pipet-channel variability in cell seeding density is significantly lower than the variability between separate pipetting steps due to cell settling and resuspension requirements. Figure 3 shows an example of a 6-channel pipet and a recommended orientation for cell seeding. By seeding each column with a single pipet ejection, variability in seeding density between the nontreatment and treatment wells for a single dose–response replicate is likely minimized. This can reduce variability in the determination of the ED_{50} values for the assay.

Two control experiments (Features 1 and 2 in Figure 3) introduced into the 96-well plate layout assess *pipetting-specific*

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issues based on cell seeding column-wise into the 96-well plate using a multichannel pipet (6-channels at a time). Feature 1 can be used to measure the within-multichannel-pipet variation. Feature 2 serves as a control for variability between multichannel pipetting steps. The mean absolute absorbance values and the variability of these wells after MTS reagent addition should be charted. This allows identifying trends that can indicate a malfunction of the multichannel pipet or a change in the pipetting technique. Data from these control experiments allow evaluation of the quality in the pipetting steps.

Branch 3: Variability Due to Instrument Performance. For the MTS assay, the absorbance value of each well containing the MTS reagent is measured in a standard multiwell plate-UV absorbance plate reader. There are a number of factors that can lead to increased variability in assay results: nonlinearity of absorbance measurements, stray light, background differences across the 96-well plate possibly due to internal mechanical issues with a plate reader, and lower signal-to-noise ratios for low level responses.

While modern absorbance plate readers can evaluate instrument performance as part of their startup cycle, several control experiments (Features 3 and 4 in Figure 3) are also included in the 96-well plate design to evaluate background absorbance and general instrument functionality. The wells for Feature 3 do not contain cells or other substances (i.e., chemical control or ENM). They contain culture media during the course of the experiment and receive the same amount of MTS reagent before the final measurement. For the MTS reagent in the absence of cells, the expected absorbance at 490 nm should be low unless unexpected changes in reagent quality have occurred. The absorption measurement at each of these wells should be consistent and can be used to calculate background absorbance corrections. If large variations or an unexpected change in absorbance is observed, this may indicate either an instrument malfunction or quality problems with either the cell culture plates or the MTS assay reagent itself. While these controls are intended to detect such problems, they do not necessarily provide sufficient information to identify the source of the problem. If the variability exceeds a specification-based threshold, further experiments would be required to identify the experimental factor that is not performing as expected. Furthermore, these controls do not directly evaluate the linearity of the instrument response or the possibility of inaccurate measurements in particular wells. Experiments to assess these particular controls can also be performed using plates with absorbance standards.

Branch 4: Toxic Chemical Positive Control. A well-defined chemical control toxicity experiment (e.g., using reference material such as a toxic metal salt) can establish proper function of the complete test system by assessing if a dose-dependent cytotoxic response of the cell line is within predefined specifications.[31] There are several reasons that a dose–response curve can add more confidence to a cell assay system than a simple one well or one concentration positive and negative control experiment. Unlike a positive control measurement which tests a single powerful effect, a dose–response curve allows data collection on the transition from nontreatment to full treatment of a control molecule. The measurements at each concentration in a well can be used to analyze the between-technical-replicate reproducibility. A dose–response control experiment also provides information about the variability in the ED_{50} values for a certain number of replicate curves, information which can provide insight into the variability observed for the ED_{50} value from the ENM exposure.

An appropriate chemical control is highly stable, antibacterial, can be accurately and reproducibly prepared, has a cytotoxic mechanism that is general to many different types of cells, and has a concentration that can be readily quantified in solution.[36,37] A metal salt, such as $CdCl_2$, fits these criteria. At high concentrations, $CdCl_2$ is antibacterial, stable at millimolar stock concentrations in water for long periods of time, has a concentration that can be measured using several widely available analytical techniques (e.g., ICP-OES), and is toxic to many types of cells.

We thus selected $CdCl_2$ as a *toxic chemical positive control* (Features 5 and 6 in Figure 3) in the 96-well plate design. Increasing concentrations of this metal salt are applied in columns 2 to 5 (Features 5 and 6). The cell-free wells in Feature 6 enable detection of the extent to which the chemical itself influences the MTS absorbance readout in the absence of cells. This information is used for background correction of the cell-based measurements, which are performed in triplicate in columns 3 to 5 (Feature 5, Figure 3) from which a dose–response curve and an ED_{50} value of the chemical toxin are calculated. The specified wells of row B in the 96-well plate (Feature 2, Figure 3) contain cells that have been treated with the solvent vehicle of the chemical control or the ENM, respectively. Thus, comparing the results of the solvent treated wells to the nontreated wells of Feature 1 provides information about any possible effect of the respective solvent on cell viability. Researchers are encouraged to chart the ED_{50} results of the reference chemical over time to identify trends that indicate the MTS assay is not responding as expected. Control charting of these system control measurements can be used to evaluate plate-to-plate and lab-to-lab variability of the assay response and also to propose validity specifications for the chemical control results. If the assay does not perform within specifications for the chemical control, results from the ENM effects should be considered invalid.

Branch 5: Variability Due to Assay Protocol. The MTS assay and other cell viability assays use chemical reagents that change chromogenic properties when they interact with living cells. The use of chemical reagents in the assay can cause variability if the reagents are changed by extended exposure to ambient light, freeze–thaw cycles, or elevated temperatures, or if there is substantial lot-to-lot variability in the reagents. Thus, control experiments to monitor reagent quality will help ensure confidence in assay results. The manufacturing specifications (e.g., lot number), storage conditions, and the results of these control measurements should be documented.

A novel and critical consideration for using toxicity assays requiring chemical reagents is that ENM may interact with the reagents and cause false positive or false negative results.[19,21,22,26,38] For the MTS assay, it is important to determine that the nanoparticle being tested does not directly affect the MTS reagent optical properties in the absence of cells, change the background absorbance through ENM precipitation in the well, or adsorb the reagent.[19,22]

The 96-well plate design (Figure 3) includes an interference control experiment for the MTS assay mechanism. This control includes duplication of the ENM dose concentrations in wells that do not contain cells (Feature 7 in Figure 3) but are treated with the MTS reagent. If the ENM does not cause an effect with the MTS reagents, background level absorptions are expected. Changes to the absorbance in these control wells can

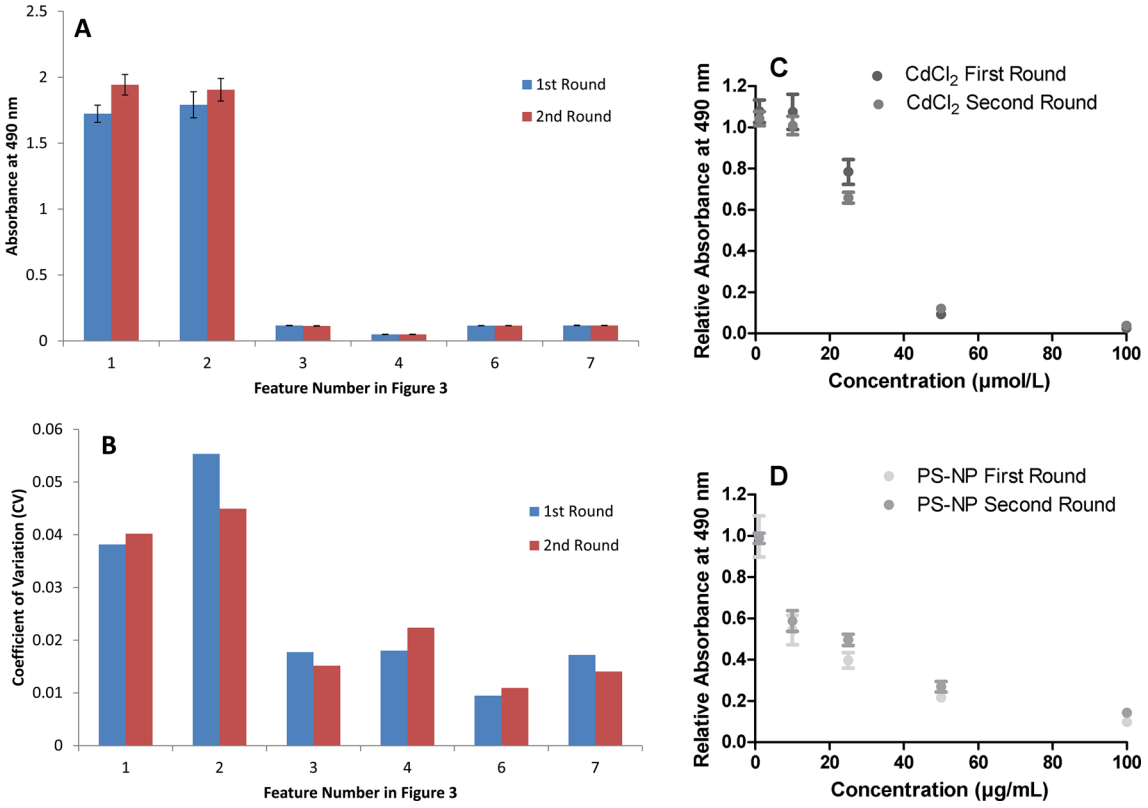

Figure 4. Results from the case study on PS-NPs: the measured absorbance values at 490 nm are shown for Features (see Figure 3) 1, 2, 3, 4, 6, and 7 for two replicate rounds (A), coefficients of variation for all of the Features (B), and the dose–response curves for $CdCl_2$ (C) and PS-NPs (D). Data in panels A, C, and D represent the mean values, and the error bars represent standard deviation values from the different Features on the plate. Error bars that are not visible are smaller than the data points.

be the result of precipitation of ENM agglomerates or interactions between the ENM and the assay reagents at the different ENM concentrations. Changes in the assay protocol or assay readout mechanism may be required to minimize this source of variability on the ENM test result.

Removing the supernatant after cell treatment or washing and before MTS reagent application may introduce another source of variability to the assay process. As the signaling pathways that lead to toxicity are (at least initially) unknown, it might be possible that cells start detaching from the culture plate before actually dying. Thus, cells that are only loosely attached to the cell culture plate but still viable may be removed with the supernatant and are thus missing in the final measurement. Rigorous rinsing will lead to the loss of more cells than gentle rinsing, and this would lead to lab-to-lab or experiment-to-experiment variations. Development of a highly reproducible rinsing protocol and clear articulation of the protocol in the assay procedure can reduce this variability. This variability can be evaluated by many of the pipetting controls described above.

Curve fitting and parametrization of the dose–response curve for both the positive chemical control and the ENM viability test also give rise to variability. Different algorithms can lead to different results. A clear indication of how curve fitting was accomplished should be reported with the value of the test result. The accuracy of the curve fitting can be improved by using a dosing strategy that includes a no-treatment response and a maximum dose–response and other dosing concentrations that cover as much of the transition between the minimum and maximum responses as possible.[30] Fitting errors in the estimation of the ED_{50} value with a logistic curve can be significant if there are few or no dose points that aid in defining the logistic curve transition.[39]

Branch 6: Variability Due to Nanoparticle Handling and Characterization. Stock ENM suspensions need to be evaluated to ensure that they have the expected physicochemical characteristics such as morphology, zeta-potential, size, size distribution, surface activity and composition.[6] In addition, ENMs often contain additional substances such as impurities (metal catalysts or endotoxins) and other molecules that improve the stability of the ENM dispersion (surface coatings, detergents, etc.). Thus, the toxicity of several compounds is typically being tested at once, and careful experiment design is needed to distinguish between effects caused by these additional chemicals and those from the ENM itself.[5] Moreover, preparation of ENM dispersions, such as the dispersion of fullerenes using tetrahydrofuran, can cause artifacts in nanotoxicology assays.[40,41]

Preparation of the final working concentration of ENM dispersed in biological media is an additional source of variability in the ENM branch of the cause-and-effect diagram. The unique physicochemical properties of ENM can cause them to behave as colloids and not as fully dissolved chemical compounds. Effects such as agglomeration, precipitation, and particle dissolution can occur within the ENM dispersion, and this changes the nature of the dosing treatment over the time course of the toxicity experiment. The extent of these effects can be greatly influenced by the method used to disperse the nanoparticles (i.e., sonication, stirring, vortexing, etc.) and the type of dispersion media.[42−44] Characterization of the

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dispersion must be performed throughout the time of the toxicity experiment to ensure that these characteristics and exposure dose are reproducible and accurate.[45,46] Sonication power, stirring speed, time after the addition of ENM, time after dispersion before cell treatment, ionic strength of the biological media, and the presence of serum protein should be controlled and reported to ensure reproducible particle dispersions. While several studies have suggested methods to disperse ENM,[43,45,46] it is likely that each ENM will require testing for dispersion procedure development. It is important to note that a high-quality MTS assay does not require a well-dispersed primary ENM. Even if a final ENM dispersion exhibits agglomeration or dissolution, the reproducibility of the dispersion technique can be established and the specific procedure included in the assay protocol. Both the treatment volumes and the dosing concentration should be clearly described in the assay protocol so that the total number of particles or total ENM mass in the treatment well can be estimated. This enables one to calculate toxicity values using ENM mass or number concentration metrics.

CASE STUDY

A case study was conducted using positively charged polystyrene nanoparticles (PS-NPs) to demonstrate the applicability of the plate layout; nanoparticles are a class of ENMs with all three dimensions less than 100 nm. The method used to conduct the case study and the raw data from the case study (Tables S1 and S2) are provided in the Supporting Information. This method follows the general steps described in Figure 1 but contains substantially more detail. The results from the case study are shown in Figure 4. One observation from the absorbance measurements is that there was good repeatability of the procedure between the two rounds (Figure 4a). The measurements of within- and between-cell pipetting variabilities (Features 1 and 2) had substantially larger absorbance values compared to those of the controls without cells (Features 3, 4, 6, and 7) but also had the highest coefficient of variation (CV) values (Figure 4b). This indicates that pipetting cells results in larger well-to-well variability than pipetting solution volumes. The measurements to test the instrument performance (Features 3 and 4) were reproducible across the two rounds and had low CV values, thus suggesting that the instrument was operating with good day-to-day reproducibility and without gradients across the plate. The background correction measurements for the positive chemical control (Feature 6) and the PS-NPs (Feature 7) were both similar to the measurements with only the MTS reagent (Feature 3). This indicates that neither the PS-NPs nor the positive chemical control impacted the MTS reagent readings. If the wells with the nanomaterials and reagent had larger absorbance values compared to the wells with only the reagent, this would indicate nanomaterial interference in the absorbance measurement. Both $CdCl_2$ and the PS-NPs caused a dose-dependent toxic effect (Figure 4c and d), and the ED_{50} values are provided in Table 2. It was apparent that the two different statistical modeling approaches yielded different estimates of the ED_{50} values and the 95% confidence intervals. The mean and standard deviation values from Features 1 to 6 can be used to produce test specifications to ensure assay performance after they have been measured for an extended period of time.

Table 2. ED_{50} Values for the $CdCl_2$ and PS-NPs during the Two Rounds Using Two Different Fitting Programs[a]

	ED_{50} value GraphPad Prism modeling	ED_{50} value Markov chain Monte Carlo modeling
$CdCl_2$ round 1 (μmol/L)	25.4 (25.2, 25.6)	30.5 (24.1, 36.6)
$CdCl_2$ round 2 (μmol/L)	25.2 (25.1, 25.3)	27.1 (22.4, 33.7)
PS-NP round 1 (μg/mL)	9.83 (9.43, 10.2)	12.0 (5.9, 19.4)
PS-NP round 2 (μg/mL)	9.85 (9.37, 10.3)	15.2 (9.4, 21.2)

[a]Values represent the mean response, and values in parentheses are the 95% confidence intervals. The ED_{50} values were calculated using two different statistical approaches.

CONCLUSIONS

The presence of contradictory test results from cell-based assays in nanotoxicology journals has been described in several reviews.[6,14] To systematically define the significant sources of variability in a nanocytotoxicity assay, we applied C&E analysis to assess the MTS assay for use with ENM and performed a case study. It is important to note that C&E analysis does not provide quantitative information on the nominal variability in these cause factors and the size of the effect these factors have on the test result. C&E diagrams are a highly ordered approach for cataloging sources of variability. Using these C&E diagrams, we designed a novel 96-well plate layout for the MTS assay, which consists of 7 system control measurements in addition to the ENM test result (see Figure 3). By monitoring or charting of the results from these control experiments for the instrument, assay reagent, cell seeding density, and positive chemical control performance, we generate a graphical tool (i.e., chart) that enables performance assessment of the assay measurement system. Continued monitoring of the assay performance serves multiple functions: (1) highlighting unexpected trends in the control data, (2) supporting rapid identification of outlier results indicative of changes in the assay system, (3) enabling comparison of assay performance within and between laboratories and for each measurement performed by a scientist, and (4) providing confidence checks on the test ENM results. A test ENM result should only be considered valid when all of the control parameters lie within specifications defined by the charting process. If the control measurements do not meet specification, issues such as pipet calibration, chemical and MTS reagent quality, cell quality, and instrument quality should be tested and corrected. This approach also facilitates sensitivity assessment of assays in which the magnitude of variation caused by different factors is tested.

Overall, the development of a C&E diagram for an assay is a useful strategy for understanding the factors that can affect assay performance resulting in noncomparable test results. Although the diagram shown in Figure 2 is based on the MTS assay, it is likely that similar cause-and-effect diagrams are applicable to many cytotoxicity assays. The data generated in the case study indicate how assay specifications can be developed using the 96-well plate design. The use of such a protocol in an interlaboratory comparison can provide further insight into the parts of the protocol that require more detailed procedures to reduce large variabilities observed in the control measurements between the laboratories. This plate design is currently being used to develop a high quality protocol that

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allows comparability of nanocytotoxicity data among international laboratories.

■ ASSOCIATED CONTENT

Supporting Information

The protocol for the case study and the raw data obtained from the case study. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

CNT, carbon nanotube; DNA, deoxyribonucleic acid; ED_{50}, median effective dose; ENM, engineered nanomaterial; FBS, fetal bovine serum; ICP-OES, inductively coupled plasma−optical emission spectrometry; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; NP, nanoparticles; OD, optical density; PS, polystyrene; STR, short tandem repeat

■ REFERENCES

(1) De Volder, M. F. L., Tawfick, S. H., Baughman, R. H., and Hart, A. J. (2013) Carbon nanotubes: present and future commercial applications. *Science 339*, 535−539.

(2) Wagner, V., Dullaart, A., Bock, A.-K., and Zweck, A. (2006) The emerging nanomedicine landscape. *Nat. Biotechnol. 24*, 1211−1217.

(3) Huang, Q., Shi, X., Pinto, R. A., Petersen, E. J., and Weber, W. J. (2008) Tunable synthesis and immobilization of zero-valent iron nanoparticles for environmental applications. *Environ. Sci. Technol. 42*, 8884−8889.

(4) Petersen, E. J., Pinto, R. A., Shi, X., and Huang, Q. (2012) Impact of size and sorption on degradation of trichloroethylene and polychlorinated biphenyls by nano-scale zerovalent iron. *J. Hazard. Mater. 243*, 73−79.

(5) Grainger, D. W., and Castner, D. G. (2008) Nanobiomaterials and nanoanalysis: opportunities for improving the science to benefit biomedical technologies. *Adv. Mater. 20*, 867−877.

(6) Krug, H. F., and Wick, P. (2011) Nanotoxicology: an interdisciplinary challenge. *Angew. Chem., Int. Ed. 50*, 1260−1278.

(7) Som, C., Wick, P., Krug, H., and Nowack, B. (2011) Environmental and health effects of nanomaterials in nanotextiles and façade coatings. *Environ. Int. 37*, 1131−1142.

(8) Petersen, E. J., Zhang, L., Mattison, N. T., O'Carroll, D. M., Whelton, A. J., Uddin, N., Nguyen, T., Huang, Q., Henry, T. B., Holbrook, R. D., and Chen, K. L. (2011) Potential release pathways, environmental fate, and ecological risks of carbon nanotubes. *Environ. Sci. Technol. 45*, 9837−9856.

(9) Bhattacharya, S., Zhang, Q., Carmichael, P. L., Boekelheide, K., and Andersen, M. E. (2011) Toxicity testing in the 21 century: defining new risk assessment approaches based on perturbation of intracellular toxicity pathways. *PLoS One 6*, e20887.

(10) Hartung, T. (2009) Toxicology for the twenty-first century. *Nature 460*, 208−212.

(11) Rushton, E. K., Jiang, J., Leonard, S. S., Eberly, S., Castranova, V., Biswas, P., Elder, A., Han, X., Gelein, R., Finkelstein, J., and Oberdörster, G. (2010) Concept of assessing nanoparticle hazards considering nanoparticle dosemetric and chemical/biological response metrics. *J. Toxicol. Environ. Health A 73*, 445−461.

(12) Som, C., Nowack, B., Krug, H. F., and Wick, P. (2013) Toward the development of decision supporting tools that can be used for safe production and use of nanomaterials. *Acc. Chem. Res. 46*, 863−872.

(13) Zhang, H., Ji, Z., Xia, T., Meng, H., Low-Kam, C., Liu, R., Pokhrel, S., Lin, S., Wang, X., Liao, Y.-P., Wang, M., Li, L., Rallo, R., Damoiseaux, R., Telesca, D., Mädler, L., Cohen, Y., Zink, J. I., and Nel, A. E. (2012) Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. *ACS Nano 6*, 4349−4368.

(14) Schrurs, F., and Lison, D. (2012) Focusing the research efforts. *Nat. Nanotechnol. 7*, 546−548.

(15) Kaiser, J.-P., Roesslein, M., Buerki-Thurnherr, T., and Wick, P. (2011) Carbon nanotubes - curse or blessing. *Curr. Med. Chem. 18*, 2115−2128.

(16) Landsiedel, R., Kapp, M. D., Schulz, M., Wiench, K., and Oesch, F. (2009) Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations–many questions, some answers. *Mutat. Res. 681*, 241−258.

(17) Park, H., and Grassian, V. H. (2010) Commercially manufactured engineered nanomaterials for environmental and health studies: important insights provided by independent characterization. *Environ. Toxicol. Chem. 29*, 715−721.

(18) Buerki-Thurnherr, T., Xiao, L., Diener, L., Arslan, O., Hirsch, C., Maeder-Althaus, X., Grieder, K., Wampfler, B., Mathur, S., Wick, P., and Krug, H. F. (2013) In vitromechanistic study towards a better understanding of ZnO nanoparticle toxicity. *Nanotoxicology 7*, 402−416.

(19) Worle-Knirsch, J. M., Pulskamp, K., and Krug, H. F. (2006) Oops they did it again! Carbon nanotubes hoax scientists in viability assays. *Nano Lett. 6*, 1261−1268.

(20) Gerloff, K., Albrecht, C., Boots, A. W., Förster, I., and Schins, R. P. F. (2009) Cytotoxicity and oxidative DNA damage by nanoparticles in human intestinal Caco-2 cells. *Nanotoxicology 3*, 355−364.

(21) Monteiro-Riviere, N. A., Inman, A. O., Zhang, L. W. Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. *Toxicol. Appl. Pharmacol. 234*, 222−235.

(22) Petersen, E. J., Henry, T. B., Zhao, J., Maccuspie, R. I., Kirschling, T. L., Dobrovolskaia, M. A., Hackley, V., Xing, B., and White, J. C. (2014) Identification and avoidance of potential artifacts and misinterpretations in nanomaterial ecotoxicity measurements. *Environ. Sci. Technol. 48*, 4226−4246.

(23) Petersen, E. J., Reipa, V., Watson, S. S., Stanley, D. L., Rabb, S. A., and Nelson, B. C. (2014) DNA damaging potential of photoactivated P25 titanium dioxide nanoparticles. *Chem. Res. Toxicol. 27*, 1877−1884.

(24) Ong, K. J., MacCormack, T. J., Clark, R. J., Ede, J. D., Ortega, V. A., Felix, L. C., Dang, M. K. M., Ma, G., Fenniri, H., Veinot, J. G. C., and Goss, G. G. (2014) Widespread nanoparticle-assay interference: implications for nanotoxicity testing. *PLoS One 9*, e90650.

(25) Ellison, S. L. R., and Barwick, V. J. (1998) Estimating measurement uncertainty: reconciliation using a cause and effect approach. *Accredit. Qual. Assur. 3*, 101−105.

(26) Horst, A. M., Vukanti, R., Priester, J. H., and Holden, P. A. (2013) An assessment of fluorescence- and absorbance-based assays to study metal-oxide nanoparticle ROS production and effects on bacterial membranes. *Small 9*, 1753−1764.

(27) MacCormack, T. J., Clark, R. J., Dang, M. K. M., Ma, G., Kelly, J. A., Veinot, J. G. C., and Goss, G. G. (2012) Inhibition of enzyme activity by nanomaterials: Potential mechanisms and implications for nanotoxicity testing. *Nanotoxicology 6*, 514−525.

(28) Huang, S., and Titus, S. J. (1993) An index of site productivity for uneven-aged or mixed-species stands. *Can. J. For. Res. 23*, 558−562.

(29) Marx, V. (2014) Pouring over liquid handling. *Nat. Methods 11*, 33−38.

(30) Robinson, D. S., Chapman, K., Hudson, S., Sparrow, S., Spencer-Briggs, D., Danks, A., Hill, R., Everett, D., Mulier, B., Old, S., and Bruce, C. (2009) *Guidance on Dose Level Selection for Regulatory General Toxicology Studies for Pharmaceuticals*, Laboratory Animal Science Association and National Centre for the Replacement, Refinement and Reduction of Animals in Research. http://www.lasa.co.uk/PDF/LASA-NC3RsDoseLevelSelection.pdf (accessed Aug 8, 2014).
(31) Chatterjee, R. (2007) Replies to cases of mistaken identity. *Science 315*, 928−931.
(32) Almeida, J. L., Hill, C. R., and Cole, K. D. (2011) Authentication of African green monkey cell lines using human short tandem repeat markers. *BMC Biotechnol. 11*, 102.
(33) Almeida, J. L., Hill, C. R., and Cole, K. D. (2014) Mouse cell line authentication. *Cytotechnology 66*, 133−147.
(34) Dirks, W., and Drexler, H. (2011) Online Verification of Human Cell Line Identity by STR DNA Typing, in *Methods in Molecular Biology* (Cree, I. A., Ed.), pp 45−55, Humana Press, Totowa, NJ.
(35) Mancia, A., Elliott, J. T., Halter, M., Bhadriraju, K., Tona, A., Spurlin, T. A., Middlebrooks, B. L., Baatz, J. E., Warr, G. W., and Plant, A. L. (2012) Quantitative methods to characterize morphological properties of cell lines. *Biotechnol. Prog. 28*, 1069−1078.
(36) Roesslein, M., Hirsch, C., Kaiser, J.-P., Krug, H. F., and Wick, P. (2013) Comparability of in vitro tests for bioactive nanoparticles: a common assay to detect reactive oxygen species as an example. *Int. J. Mol. Sci. 14*, 24320−24337.
(37) Salit, M. L., Turk, G. C., Lindstrom, A. P., Butler, T. A., Beck, C. M., and Norman, B. (2001) Single-element solution comparisons with a high-performance inductively coupled plasma optical emission spectrometric method. *Anal. Chem. 73*, 4821−4829.
(38) Liao, K.-H., Lin, Y.-S., Macosko, C. W., and Haynes, C. L. (2011) Cytotoxicity of graphene oxide and graphene in human erythrocytes and skin fibroblasts. *ACS Appl. Materi. Interfaces 3*, 2607−2615.
(39) Brown, C. C. (1978) The Statistical Analysis of Dose-Effect Relationships, *Principles of Ecotoxicology*, CRC Press, Boca Raton, FL.
(40) Henry, T. B., Menn, F.-M., Fleming, J. T., Wilgus, J., Compton, R. N., and Sayler, G. S. (2007) Attributing effects of aqueous C60 nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression. *Environ. Health Perspect. 115*, 1059−1065.
(41) Spohn, P., Hirsch, C., Hasler, F., Bruinink, A., Krug, H. F., and Wick, P. (2009) C60 fullerene: a powerful antioxidant or a damaging agent? The importance of an in-depth material characterization prior to toxicity assays. *Environ. Pollut. 157*, 1134−1139.
(42) Limbach, L. K., Li, Y., Grass, R. N., Brunner, T. J., Hintermann, M. A., Muller, M., Gunther, D., and Stark, W. J. (2005) Oxide nanoparticle uptake in human lung fibroblasts: effects of particle size, agglomeration, and diffusion at low concentrations. *Environ. Sci. Technol. 39*, 9370−9376.
(43) Zook, J. M., Maccuspie, R. I., Locascio, L. E., Halter, M. D., and Elliott, J. T. (2011) Stable nanoparticle aggregates/agglomerates of different sizes and the effect of their size on hemolytic cytotoxicity. *Nanotoxicology 5*, 517−530.
(44) Zook, J. M., Rastogi, V., Maccuspie, R. I., Keene, A. M., and Fagan, J. (2011) Measuring agglomerate size distribution and dependence of localized surface plasmon resonance absorbance on gold nanoparticle agglomerate size using analytical ultracentrifugation. *ACS Nano 5*, 8070−8079.
(45) Taurozzi, J. S., Hackley, V. A., and Wiesner, M. R. (2011) Ultrasonic dispersion of nanoparticles for environmental, health and safety assessment − issues and recommendations. *Nanotoxicology 5*, 711−729.
(46) Taurozzi, J. S., Hackley, V. A., and Wiesner, M. R. (2013) A standardised approach for the dispersion of titanium dioxide nanoparticles in biological media. *Nanotoxicology 7*, 389−401.