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Buoyant Nanoparticles: Implications for Nano-biointeractions in Cellular Studies

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Abstract

In the safety and efficacy assessment of novel nanomaterials, the role of nanoparticle (NP) kinetics in in vitro studies is often ignored although it has significant implications in dosimetry, hazard ranking and nanomedicine efficacy. Here, we demonstrate that certain nanoparticles are buoyant due to low effective densities of their formed agglomerates in culture media, which alters particle transport and deposition, dose-response relationships, and underestimates toxicity and bioactivity. To investigate this phenomenon, we determined the size distribution, effective density, and assessed fate and transport for a test buoyant NP (polypropylene, PP). To enable accurate dose-response assessment, we developed an inverted 96-well cell culture platform in which adherent cells were incubated above the buoyant particle suspension. The effect of buoyancy was assessed by comparing dose-toxicity responses in human macrophages after 24 h incubation in conventional and inverted culture systems. In the conventional culture system, no adverse effects were observed at any NP concentration tested (up to 250 $\mu\text{g ml}^{-1}$), whereas dose-dependent decreases in viability and increases in reactive oxygen species were observed in the inverted system. This work sheds light on an unknown issue that plays a significant role in vitro hazard screening and proposes a standardized methodology for buoyant NP assessments.

Keywords

nanotoxicology; nanomedicine; engineered nanomaterials; dosimetry

1. Introduction

Nanotoxicology and nanomedicine share the common goal of understanding the relationships between the properties of nanoparticles and their interactions with biological systems in order to promote the safety and betterment of society.[1-4] While the nanotoxicology field has focused on illuminating relationships between physicochemical and morphological properties and toxicological outcomes to identify potential health hazards and enable design of safer nanomaterials, [5-9] nanomedicine has sought to understand and

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exploit the unique properties of nano-sized materials to devise advanced diagnostic and therapeutic platforms that could improve patient outcomes.[10-12] [13, 14] Both nanotoxicology and nanomedicine utilize physiologically-relevant in vitro testing to assess hazard ranking, mechanisms of biointeractions and bioactivity.[15]

It is well known that nanoparticles (NPs) can form agglomerates when dispersed in culture media. [16] Agglomerates are protein corona-wrapped particle bundles containing trapped cell culture fluid and proteins (Figure 1a).[17] The agglomerate diameter and effective density (density of the agglomerate unit) primarily define sedimentation and diffusion in culture systems and thus determine dose metrics such as the NP mass, surface and particle number delivered to cells over the time of exposure.[18] Typical nanomaterials have densities substantially greater than that of cell culture media, but because their agglomerates contain relatively large proportions of media, their effective densities in suspension can be much lower than that of the raw material, and are often only slightly greater than that of the media.[17] Nevertheless, as long as the raw material density is greater than media density, the agglomerate effective density will always be greater as well, and the agglomerates will settle over time. However, if the raw material density is lower than the media density, as in the case of some conjugated polymers, the agglomerates will also have a lower density, and will be buoyant when dispersed in media. In this case their suspended forms would not settle, but would in fact rise or float away from cells over time making the dose response relationship impossible to determine (Figure 1b).

NPs such as nanobubbles and liposomes, used in nanomedicine,[19, 20] and other applications[21, 22] are typical examples of NPs which may have effective densities lower than that of the culture medium and may become buoyant.

This phenomenon of buoyant NPs has not been recognized or reported in the literature in part due to the fact that effective density is rarely measured in in vitro cellular studies, and because of a general lack in harmonization and standardization of analytical techniques for colloidal dispersion and characterization of nanomaterials. Because particle or agglomerate size and effective density determine particle kinetics in suspension, accurate characterization of both properties is essential for accurate dosimetry and assessment of biological activity in vitro.[23]

Particle size distributions are typically determined by either dynamic light scattering (DLS), disk centrifugation, or more recently, by tunable resistance particle sizing (TRPS). Because TRPS directly measures sizes of individual particles it may provide greater accuracy and resolution than DLS.[24] Effective density can be measured using analytical ultracentrifugation (AUC) techniques.[25, 26] However this method is time consuming and requires access to expensive instrumentation. The Volumetric Centrifugation Method (VCM) was recently developed as an inexpensive high-throughput alternative for effective density measurement suitable for cellular studies.[18] Despite the availability and proven utility of these tools, effective density is rarely measured or considered in nanotoxicity or nanomedicine cellular studies.[23]

Likewise, dosimetric consideration or the delivered dose rate is an important parameter that is usually overlooked in nano-related in vitro cellular studies. Recently, integrated dosimetric approaches have been developed and validated to address this issue.[15, 27] Such approaches are usually two step approaches and include: 1) proper preparation and characterization of nanoparticle suspensions and include measurement of size distributions and effective density of formed agglomerates; 2) use of numerical fate and transport models to estimate delivered to cell mass, particle number or surface area over time.[28] Typical numerical models include the In vitro Sedimentation, Diffusion and Dosimetry model (ISDD)[29] and its adapted version, VCM-ISDD which takes into consideration the effective density measured by VCM.[28] More recently, the Distorted Grid (DG) model was developed to provide both concentration and deposition metrics, both at the bottom of the culture and as a function of vertical position, and to accommodate variable nanoparticle-cell adsorption kinetics.[30]

In this study, we investigated the particle kinetics and their effects on toxicological outcomes for a buoyant nanoparticle (NP) derived from nanoparticles released during incineration of nano-enabled thermoplastics. Because the density of polypropylene (0.9 g cm^{-3}) is less than typical media density ($\sim 1.0 \text{ g cm}^{-3}$) we hypothesized that the PP NP would be buoyant in suspension. This was verified by measuring the effective density of the NP in suspension using the VCM method, which was adapted to enable measurement of buoyant particle effective densities. Colloidal size distribution was determined using both DLS and TRPS. Fate and transport modeling to determine the concentration profile across the well and delivered to cell dose rate in vitro was performed using the DG model. To assess the effect of buoyancy on cellular bioactivity, we compared cytotoxicity dose-responses in human alveolar macrophage-like monocyte derived macrophages (AM-HMDMs) in a conventional in vitro culture system with that in an inverted cell culture system, which we developed for use with buoyant NPs.

2. Results and Discussion

2.1 Test NP generation and characterization

Test NPs were generated using the Integrated Exposure Generation system (INEXS), which provided a controlled environment to assess the release of particulate matter during thermodegradation of nano-enabled thermoplastics.[31] The particle size distribution and mode diameter of the generated test NP evolved from low to high values as a function of time and particle concentration reaching a maximum at a thermal decomposition temperature $\sim 400^\circ\text{C}$, then subsequently decreased over time. The maximum particle concentration was $2.0 \times 10^6 \text{ particles cm}^{-3}$ and the mean aerodynamic diameter of the released aerosol was 65.7 nm (Supplemental Figure 1), which is consistent with size distributions reported in the literature.[31, 32] Organic carbon and elemental carbon (OC/EC) analysis revealed that the PP NP consisted of 100% organic carbon. Additional details of the exposure generation platform, chemical composition and NP size distributions can be found in the recent publications by Sotiriou et al.[31, 32]

The test NP used in this study is of high relevance in nanosafety assessment. Upon disposal, numerous nano-enabled thermoplastics enter the waste stream and are either recycled or

incinerated. It is generally known that the thermal decomposition of plastics generates toxic gases and nano-sized particulate matter.[32] The biological activity of this nano-sized particulate matter is largely unknown and may potentially pose significant environmental and public health risks.

2.2 Validation of test NP buoyancy in a culture media environment

Proper colloidal dispersion and characterization is an essential part of cellular nanotoxicology studies (See methods below for details). The formed agglomerate size distributions and effective density determine the fate and transport of the suspended materials, which in turn determine the dose (e.g., mass or concentration) delivered to cells. Figure 2 illustrates the mean volume-weighted size distribution of test NP suspensions as determined by both DLS and TRPS. Table 1 provides a summary of the colloidal characterization results.

The mean volume-weighted sizes determined by DLS and TRPS were 479.2 nm and 392.1 nm, respectively (Figure 2). Although the difference between these values is relatively small (~22%), because the sedimentation coefficient is proportional to the square of the diameter, the actual difference in sedimentation rate would be closer to 50%, which would have a significant effect on fate and transport of PP NP, and thus influence dose metrics over time. We also observed a truncation of the size distribution starting at 700 nm using TRPS in comparison to DLS (Figure 2). Figure 2 inset shows the morphology of a representative PP NP agglomerate from electron microscopy analysis of the colloid. Although electron microscopy allows only a qualitative assessment, the size of the formed agglomerate is in agreement with the DLS and TRPS results. Although TRPS has been shown to provide greater accuracy and higher resolution size data, [24] we chose to use the DLS size distribution data for fate and transport modeling because most labs are equipped with DLS equipment or use equivalent methods.

We utilized the volumetric centrifugation method (VCM) to determine the effective density of our test NP formed agglomerates (see method section below for details). Interestingly, in the test media (RPMI containing 3% FBS) the effective density of these agglomerates was found to be identical to that of the raw material (0.90 g cm^{-3}), suggesting a tightly packed agglomerate form containing relatively little media. Because this effective density is less than that of culture media (1.0008 g cm^{-3}), the PP NP formed agglomerates were classified as buoyant.

2.3 Nanoparticle buoyancy determines partico-kinetics in vitro and dose metrics

It is now widely understood that estimating fate and transport to account for particle kinetics is essential for accurate in vitro dosimetry and comparative safety assessment of NPs.[27] Yet, many studies are still conducted without such fate and transport modeling, and assume that either initial total mass or concentration represent adequate dose metrics for generating dose-response relationships. We now know that this is an erroneous approach even for settling, non-buoyant NPs, but it is particularly problematic, as we will show, in the case of buoyant particles, because the particles are “fleeing” from the cells from the outset, and there is essentially zero dose delivered to the cells at the bottom of the well. To address this

question, we employed the DG computational fate and transport model, which allows the determination of both concentration and deposition metrics, both at the bottom and top of the culture well and as a function of vertical position, in addition to accommodating polydispersity, dissolution and variable nanoparticle-cell adsorption kinetics (see methods for details on the DG model).

Figure 3 shows the time progression and mean concentration and dose metrics at the bottom and top of the well. After 24 h, the deposited mass at the bottom of the well in conventional culture system is negligible (Figure 3a) in comparison to the fraction deposited or accumulated mass at the top of the well (Figure 3b). Likewise, the concentration at the bottom of the well is 70 times lower (25.7 $\mu\text{g/ml}$) (Figure 3c) than the concentration at the top of the well (1821 $\mu\text{g/ml}$) (Figure 3d)". Thus, it is clear that the fraction deposited and NP concentration is greater at the top of the well than the bottom of the well for this class of NPs. Mean concentration and delivered dose metrics for each administered NP concentration are given in Table 2.

We should note that this paper focuses solely on determining the dose that cells experience as a function of exposure time, and does not address the role of intrinsic and extrinsic properties associated with nanoparticles and the biological environment on bioactivity. The role of intrinsic properties of nanoparticles such as size [33], shape, crystallinity, charge [34] on biointeractions is well established in the literature [35, 36]. Similarly, the importance of the protein coronas on bio-activity is well recognized [37, 38]. The fundamental rule of toxicology is that the "dose makes the poison". Therefore, the more accurate the dose estimate is the more accurate and predictive the dose response relationships would be and would help in understanding better the bioactivity and extract correlations with the aforementioned factors.

Whereas the effective density and hydrodynamic diameter dictate nanoparticle fate and transport, it is important to note that the protein corona can influence observed cellular responses. Lynch and coworkers established the concept of protein corona and described the dynamic interaction of proteins and the nanoparticle surface [39]. The authors surmised that proteins with longer residence times on the particle surface will have a greater influence on biological outcomes, and that identifying these proteins is crucial to understanding nano-biointeractions. In line with these observations, another study moreover clearly demonstrated that the protein corona formed from serum or lung lining fluid decreased the cytotoxic and hemolytic activity of functionalized polymeric nanoparticles [40], suggesting that these protein corona can provide protection in nano-biointeractions. Other studies have also noted that the composition of the protein corona can affect recognition and subsequent uptake of particles by target cells [35]. Recent advances in measuring particle-cell interactions in real time have further underscored the significance of the protein corona in nano-safety assessments [4, 9]

2.4 Buoyant NPs diminish dose response relationship using conventional culturing approach

As demonstrated above, the effect of buoyancy on the delivery of NPs to the cells at the bottom of the well (conventional approach) is problematic because the particles will move

away from the cells. Even at very high initial concentrations the dose delivered to cells will remain very low. It is therefore clear that conventional in vitro cell systems are not suitable for evaluating the biological activity of buoyant NPs. In order to circumvent this problem, we developed a 96-well inverted cell culture system in which adherent cells are oriented above the NP suspension, so that suspended buoyant particles or agglomerates move toward, rather than away from the test cells, and the concentration and mass delivered to cells increase over time, just as concentration and delivered mass of non-buoyant NPs increase over time in a standard culture system (Figure 4). It should be noted that the proposed methodology applies only to studies of adherent cells, which is the usual case in nanotoxicology studies. In the case of cells incubated in suspension, estimating cellular dosimetry using numerical fate and transport modeling is complicated by the differing settling rates of particles and cells. To the best of our knowledge, there is no methodology in the published domain to estimate the dose in this case. Since cells are very large and thus will settle very quickly, it is likely that the situation can be approximated by assuming the cells rest almost immediately at the well bottom, or at the well top in an inverted system, and dosimetry can be approximated accordingly.

We performed dose-response assessment using both the conventional and inverted approach for comparison purposes. In the conventional cell culture system, AM-HMDMs displayed no changes in metabolic activity (Figure 5a) or membrane integrity (Figure 5b) with increasing administered concentrations of the buoyant test NP. In contrast, in the inverted system, viability decreased and cytotoxicity increased with increasing administered concentrations, and viability was significantly reduced relative to that in conventional culture system at administered doses of 100 and 250 $\mu\text{g ml}^{-1}$ (delivered dose 1821 and 4553 $\mu\text{g ml}^{-1}$, respectively).

In addition, whereas a very slight initial dose-dependent increase in oxidative stress (ROS generation) was observed in the upright culture system, a much stronger dose-dependent increase in oxidative stress was seen in the inverted system. This appeared as concentrated foci of ROS surrounding cell nuclei and mitochondria (Figure 5c and d). These observations are consistent with current evidence in the literature regarding polymer particle mediated oxidative stress, where oxidative stress is a well-known mode of action and early harbinger of cytotoxicity for NPs. [41] The small dose-dependent increase in oxidative stress in the standard system can be accounted for by the small but non-zero average concentration of test NP at the bottom of the well, which is greater when the initial concentration is greater. Indeed, it may be true that dose-response data could be obtained in a standard system if initial concentrations are made sufficiently high, but this would be an impractical approach, and would likely be complicated by changes in colloidal suspension properties, which can become unstable at high concentrations. By contrast, the inverted culture approach allows a more controlled assessment of biological responses over a typical range of initial concentrations.

To validate the principle that the direction in which a particle settles, depending on its buoyancy, determines the utility of conventional vs. inverted culture systems, we evaluated a non-buoyant nanoparticle, CuO NP, in both conventional and inverted systems. The CuO NPs (Sigma Aldrich) (Sigma, St. Louis, MO) possessed a specific surface area of 16.2 m^2/g

and the equivalent BET diameter of primary particles was 58.7 nm.[7] Colloidal characteristics of CuO NP, including volume weighted mean hydrodynamic diameter and effective density, is shown in Supplemental Table 1. The effective density was found to be 1.578 g cm^{-3} (non buoyant nanoparticle). Concentrations and deposited fractions of CuO NP at the bottom and top of the well (corresponding to delivered doses in the conventional and inverted systems, respectively) as a function of time, as determined by fate and transport modeling, are shown in Supplemental Figure 2. Mean concentrations and deposited fractions at the top and bottom of the well are summarized in Supplemental Table 2. As expected, CuO NP concentration at the bottom of the well (delivered dose in conventional system) increased over time with a mean value ($925.9 \mu\text{g ml}^{-1}$) far exceeding the administered concentration ($50 \mu\text{g ml}^{-1}$), whereas the concentration at the top of the well (delivered dose in the inverted system) decreased over time with a mean value ($1.4 \mu\text{g ml}^{-1}$) far less than the administered concentration. Consequently, as predicted, only minimal reduction in metabolic activity was observed in the inverted system, whereas a striking dose-dependent toxicity was observed in the conventional culture system (Supplemental Figure 3). The slight toxicity observed in the inverted system may have resulted from copper ions produced by dissolution of the CuO NP, from the short initial exposure that would occur before the particles sedimented away from the cells, or from particles that adhered to cell surface during the same initial exposure period. These results correlate to previous reports in the literature concerning the cytotoxic nature of CuO NPs.[42-45]

3. Conclusions

In summary, current in vitro toxicological and bioactivity screening methods used in the development of nanomedicine and safety assessment of NPs may fail to detect or underestimate bioactivity for buoyant particles, with major implications in these emerging fields. Due to their low material and effective density in culture media, this class of NPs is buoyant and may never settle and interact with cell surfaces at bottom of the well, leading to inaccurate dose response assessments of toxicity or efficacy. We demonstrated that buoyant NPs in suspension have no cytotoxic effects on cells in a standard culture system, but produce a dose-dependent increase in cytotoxicity and ROS generation in an inverted system. From the results of fate and transport computational modeling for the two systems, it is clear that these differences in dose response are a result of upward movement and concentration of the material at the top of the well. The methodology presented underscores the importance of accurate characterization of colloidal properties to include measurement of effective density and particle kinetics in toxicological and bioactivity assessment, and suggests a need for specific adaptations to current methods for assessment of buoyant nanomaterials.

4. Experimental Section

Nanoparticle Generation, Extraction, and Colloidal Preparation

The Integrated Exposure Generation System (INEXS) was employed to generate polypropylene aerosols or PP NP, where the nano-enabled thermoplastic material was thermo-degraded in a quartz furnace at 800°C as described by Sotiriou et al.[31] The

resulting released aerosols were collected on Teflon filters using the Harvard Compact Cascade Impactor (CCI) system,[46] then extracted utilizing a previously developed method by the authors using sonication and solvent washing. [47] The recovered PP NP were dispersed using a dispersion protocol described by Cohen et al. [15] and Pal et al.[48], where the critical sonication energy (466 J/ml) was delivered to reduce agglomerate size and create stable colloidal suspensions. Dispersed stock solutions in DI H₂O were diluted to desired concentrations (10, 100, 250 µg/ml) in RPMI-1640 containing 3% FBS, HEPES, and 10,000 units of penicillin/streptomycin (Lonza) and vortexed for 30 seconds. Copper oxide NPs were obtained from Sigma Aldrich (Sigma, St.Louis, MO). Colloidal characterization details can be found in Supplemental Table 1.

Dynamic Light Scattering (DLS)

Time series measurements were conducted for PP NP suspended in water and in RPMI-1640 media containing 3% FBS using a dynamic light scattering instrument (Malvern Zetasizer Nano-ZS).

Scanning Electron Microscopy (SEM)

Scanning electron micrographs of PP NP in water were obtained for morphological characterization of the formed agglomerates. Briefly, PP NP suspensions were sonicated above the DSE_{cr}, then vortexed before imaging using a Supra55VP field emission microscope equipped with a Secondary Electron Detector (SE2) at a voltage of 2 kV.

Tunable Resistive Pulse Sensing (TRPS)

TRPS measurements were conducted using a qNano instrument (Izon Science, MA, USA) with a NP300 nanopore as previously described by Pal and coworkers.[24] The nanopore was mounted on the cruciform at the top of the instrument body. Approximately 75ul RPMI-1640 medium was added to the lower electrode at the bottom side of the nanopore and 45ul of added to the topside of the nanopore in the fluid cell. The nanopore was then stretched to ~ 47 mm and pore was allowed to wet with the electrolyte. Following nanopore wetting the top fluid was replaced with 45ul of PP NPs suspended in RPMI-1640 medium. Nanopore stretch and voltage across the nanopore were optimized to obtain a current of ~110 nA and voltage of 0.36V across the nanopore. Multi-pressure sample analysis was conducted with three different pressure conditions of 2,4 and 7 millibar respectively using the variable pressure module (Izon Science, MA, USA). For each measurement 500 translocation events were measured and Izon Control Suite 3.2 was used for calculating size, size distribution and particles concentration.

Measurement of effective density of formed agglomerates using VCM

Because buoyant ENM agglomerates move toward rather than away from the rotor center during centrifugation, it was necessary to perform the VCM with the PCV tubes inverted in the centrifuge. In addition, because the top of the PCV tube is not normally tightly sealed, it was necessary to modify the PCV tube to withstand inverted centrifugation without leakage: The lower half of a standard polystyrene cryopreservation tube with tightly-sealing threaded cap and silicone washer was cut away, and the capped top half fixed within the upper lip of

the PCV tube with cyanoacrylate adhesive (superglue). Using the modified PCV tube in an inverted orientation, we were then able to collect and measure the buoyant agglomerates, and determine their effective density as previously described.[17]

In vitro dosimetric analysis

The DG model was utilized [15, 49] to calculate the concentration profiles across the well and the fraction of administered particles deposited to the cell surface as a function of time, $f_D(t)$, for test NP suspensions. The agglomerate hydrodynamic diameter, d_H , as measured by DLS, and the effective density of particle agglomerates suspended in cell culture media, as measured by VCM,[17] were used as inputs to the DG model to calculate the fate and transport of test NP suspensions.

Cell Culture

AM-HMDMs were prepared as previously described.[23, 46] Briefly, buffy coats were harvested from blood obtained from discarded platelet apheresis collars obtained from the Kraft Family Blood Donor Center at the Dana-Farber Cancer Institute (Boston, MA, USA), and enriched for monocytes using RosetteSep Monocyte Enrichment kit (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturers protocol. Monocytes were resuspended in RPMI supplemented with 10% heat-inactivated FBS, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin (Gibco-Life Technologies), 10 mM HEPES (Gibco-Life Technologies), and 20 ng ml⁻¹ human granulocyte/macrophage colony stimulating factor (GM-CSF, Peprotech, Rocky Hill, NJ, USA), and incubated in FEP VueLife® bags (2PF-0072, Saint-Gobain, Gaithersburg, MD, USA), which was incubated for 10-11 days at 37° C and 5% CO₂. Harvested AM-MDMs were resuspended at 0.4×10^6 cells ml⁻¹ in fresh complete media with GM-CSF, and dispensed into 96-well imaging plates (BD-353219, BD Biosciences, Franklin Lakes, NJ, USA) at 1×10^5 cells per well, and incubated at 37° C and 5% CO₂ for 48 hours prior to performing experiments.

Cell Exposure and Toxicological Assessment

Membrane integrity and metabolic activity was measured using the CytoTox-ONE Homogenous Membrane Integrity (LDH) and CellTiter 96 Aqueous One Solution (MTS) (Promega, Madison, WI) assays, respectively. Briefly, AM-HMDMs were seeded in 96-well plates (Corning Inc., New York, NY) and were maintained until 70-80% confluency. In the inverted cell culture exposure, fresh media (210 µl) was added to cells and 210 µl of 2× PP NP suspension diluted in RPMI-1640 supplemented with 3% FBS at various concentrations (0, 10, 100, 250 µg/ml) were administered. The 96-well plate was then assembled with the gas permeable Teflon membrane fitted with rubber gaskets and modified plate cover, then placed in the inverted cell culture platform as described in Figure 4 and incubated for 24 hours. For conventional cell culture exposures, a total volume of 400 µl of fresh media and 2× PP NP suspension was used. Following exposure, supernatants were collected, placed in separate 96 well culture plates, and exposed cell surfaces were washed twice with 1× phosphate buffered saline (PBS). Supernatant plates were spun down at 250 ×g for 10 minutes and 100 µl of supernatant was collected and transferred to fresh 96-well plates containing 100 µl of LDH reagent. LDH plates were allowed to incubate at room temperature for 30 minutes in the dark. Plates were read immediately following incubation

at an excitation wavelength of 560 nm and emission of 590 nm using a fluorescent microplate reader (Molecular Devices). Fresh media containing MTS reagent was added to the exposed and washed cells for 1 hour and read at 490 nm. Media only and PP NP controls were performed to ensure reagent integrity, which showed no interaction with reagent substrates. Each assay was performed in triplicate.

Measurement of Reactive Oxygen Species

It is well known that many nanomaterials produce reactive oxygen species that can lead to deleterious effects in cells. Using the CellROX® Green ROS Detection Assay (Invitrogen), we assessed the ability of PP NPs to generate reactive oxygen species in living cells. AM-HMDMs were exposed to PP NPs for 24 h using conventional and inverted cell culturing systems. Following aspiration of PP NP suspensions and two washes with 1× PBS, CellROX Green diluted in complete media (5 µM) was added to cells for 30 minutes at 37°C. Fluorescent measurements were then performed using a fluorescent plate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA) using excitation and emission pair of 485/520 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Grassian VH, Haes AJ, Mudunkotuwa IA, Demokritou P, Kane AB, Murphy CJ, Hutchison JE, Isaacs JA, Jun Y-S, Karn B, Khondaker SI, Larsen SC, Lau BLT, Pettibone JM, Sadik OA, Saleh NB, Teague C. *Environmental Science: Nano*. 2016 DOI 10.1039/C5EN00112A.
2. Demokritou P, Gass S, Pyrgiotakis G, Cohen JM, Goldsmith W, McKinney W, Frazer D, Ma J, Schwegler-Berry D, Brain J, Castranova V. *Nanotoxicology*. 2013; 7(8):1338–50. DOI 10.3109/17435390.2012.739665. [PubMed: 23061914]
3. Pirela SV, Miousse IR, Lu X, Castranova V, Thomas T, Qian Y, Bello D, Kobzik L, Koturbash I, Demokritou P. *Environmental health perspectives*. 2015 DOI 10.1289/ehp.1409582.
4. Pyrgiotakis G, Blattmann CO, Demokritou P. *ACS sustainable chemistry & engineering*. 2014; 2(7):1681–1690. DOI 10.1021/sc500152g. [PubMed: 25068097]
5. Sotiriou GA, Watson C, Murdaugh KM, Darrah TH, Pyrgiotakis G, Elder A, Brain JD, Demokritou P. *Environmental science. Nano*. 2014; 1(2):144–153. DOI 10.1039/c3en00062a.
6. Watson C, Ge J, Cohen J, Pyrgiotakis G, Engelward BP, Demokritou P. *ACS nano*. 2014; 8(3):2118–33. DOI 10.1021/nn404871p. [PubMed: 24617523]
7. Lu X, Miousse IR, Pirela SV, Melnyk S, Koturbash I, Demokritou P. *Nanotoxicology*. 2015:1–11. DOI 10.3109/17435390.2015.1025115.
8. Gass S, Cohen JM, Pyrgiotakis G, Sotiriou GA, Pratsinis SE, Demokritou P. *ACS sustainable chemistry & engineering*. 2013; 1(7):843–857. DOI 10.1021/sc300152f. [PubMed: 23961338]
9. Pyrgiotakis G, Blattmann CO, Pratsinis S, Demokritou P. *Langmuir*. 2013; 29(36):11385–95. DOI 10.1021/la4019585. [PubMed: 23978039]

10. Fadeel B. Journal of internal medicine. 2013; 274(6):578–80. DOI 10.1111/joim.12137. [PubMed: 24102766]
11. Desai N. The AAPS journal. 2012; 14(2):282–95. DOI 10.1208/s12248-012-9339-4. [PubMed: 22407288]
12. Pyrgiotakis G, McDevitt J, Bordini A, Diaz E, Molina R, Watson C, Deloid G, Lenard S, Fix N, Mizuyama Y, Yamauchi T, Brain J, Demokritou P. Environmental science. Nano. 2014; 2014(1): 15–26. DOI 10.1039/c3en00007a. [PubMed: 26180637]
13. Pyrgiotakis G, McDevitt J, Gao Y, Branco A, Eleftheriadou M, Lemos B, Nardell E, Demokritou P. Nanomedicine : nanotechnology, biology, and medicine. 2014; 10(6):1175–83. DOI 10.1016/j.nano.2014.02.016.
14. Pyrgiotakis G, Vasanthakumar A, Gao Y, Eleftheriadou M, Toledo E, DeAraujo A, McDevitt J, Han T, Mainelis G, Mitchell R, Demokritou P. Environ Sci Technol. 2015; 49(6):3737–45. DOI 10.1021/es505868a. [PubMed: 25695127]
15. Cohen J, DeLoid G, Pyrgiotakis G, Demokritou P. Nanotoxicology. 2013; 7(4):417–431. DOI doi: 10.3109/17435390.2012.666576. [PubMed: 22393878]
16. Cohen J, DeLoid G, Pyrgiotakis G, Demokritou P. Nanotoxicology. 2012 DOI 10.3109/17435390.2012.666576.
17. DeLoid G, Cohen J, Darrah TH, Pyrgiotakis G, Wohlleben W, Demokritou P. Nature Communications. 2014 in press.
18. DeLoid G, Cohen JM, Darrah T, Derk R, Rojanasakul L, Pyrgiotakis G, Wohlleben W, Demokritou P. Nat Commun. 2014; 5:3514. DOI 10.1038/ncomms4514. [PubMed: 24675174]
19. Cai WB, Yang HL, Zhang J, Yin JK, Yang YL, Yuan LJ, Zhang L, Duan YY. Scientific reports. 2015; 5:13725. DOI 10.1038/srep13725. [PubMed: 26333917]
20. Suzuki R, Maruyama K. Methods in molecular biology (Clifton, N.J.). 2010; 605:473–86. DOI 10.1007/978-1-60327-360-2_33.
21. DasSarma S, Karan R, DasSarma P, Barnes S, Ekulona F, Smith B. BMC biotechnology. 2013; 13:112. DOI 10.1186/1472-6750-13-112. [PubMed: 24359319]
22. Schmit VL, Martoglio R, Scott B, Strickland AD, Carron KT. Journal of the American Chemical Society. 2012; 134(1):59–62. DOI 10.1021/ja208463f. [PubMed: 22077992]
23. Bello D, Martin J, Santeufemio C, Sun Q, Lee Bunker K, Shafer M, Demokritou P. Nanotoxicology. 2012:989–1003. DOI 10.3109/17435390.2012.689883. [PubMed: 22551088]
24. Pal AK, Aalaei I, Gadde S, Gaines P, Schmidt D, Demokritou P, Bello D. ACS nano. 2014; 8(9): 9003–15. DOI 10.1021/nn502219q. [PubMed: 25093451]
25. Growney DJ, Fowler PW, Mykhaylyk OO, Fielding LA, Derry MJ, Aragrag N, Lamb GD, Armes SP. Langmuir. 2015; 31(32):8764–8773. DOI 10.1021/acs.langmuir.5b01651. [PubMed: 26203669]
26. Walter J, Thajudeen T, Su S, Segets D, Peukert W. Nanoscale. 2015; 7(15):6574–6587. DOI 10.1039/C5NR00995B. [PubMed: 25789666]
27. Pal AK, Bello D, Cohen J, Demokritou P. Nanotoxicology. 2015; 9(7):871–85. DOI 10.3109/17435390.2014.986670. [PubMed: 25672815]
28. Cohen JM, Teeguarden JG, Demokritou P. Particle and fibre toxicology. 2014; 11:20. DOI 10.1186/1743-8977-11-20. [PubMed: 24885440]
29. Hinderliter PM, Minard KR, Orr G, Chrisler WB, Thrall BD, Pounds JG, Teeguarden JG. Particle and fibre toxicology. 2010; 7(1):36. DOI 10.1186/1743-8977-7-36. [PubMed: 21118529]
30. DeLoid GM, Cohen JM, Pyrgiotakis G, Pirela SV, Pal A, Liu J, Srebric J, Demokritou P. Particle and fibre toxicology. 2015; 12:32. DOI 10.1186/s12989-015-0109-1. [PubMed: 26497802]
31. Sotiriou GA, Singh D, Zhang F, Wohlleben W, Chalbot M-CG, Kavouras IG, Demokritou P. Environmental Science: Nano. 2015; 2(3):262–272. DOI 10.1039/C4EN00210E. [PubMed: 26200119]
32. Sotiriou GA, Singh D, Zhang F, Chalbot MC, Spielman-Sun E, Hoering L, Kavouras IG, Lowry GV, Wohlleben W, Demokritou P. Journal of hazardous materials. 2016; 305:87–95. DOI 10.1016/j.jhazmat.2015.11.001. [PubMed: 26642449]

33. Sayes CM, Reed KL, Warheit DB. Toxicological sciences : an official journal of the Society of Toxicology. 2007; 97(1):163–80. DOI 10.1093/toxsci/kfm018. [PubMed: 17301066]
34. Prasad RY, McGee JK, Killius MG, Suarez DA, Blackman CF, DeMarini DM, Simmons SO. Toxicology in vitro : an international journal published in association with BIBRA. 2013; 27(6): 2013–21. DOI 10.1016/j.tiv.2013.07.005. [PubMed: 23872425]
35. Caracciolo G, Palchetti S, Colapicchioni V, Digiaco L, Pozzi D, Capriotti AL, La Barbera G, Laganà A. Langmuir. 2015; 31(39):10764–10773. DOI 10.1021/acs.langmuir.5b02158. [PubMed: 26378619]
36. Konduru NV, Jimenez RJ, Swami A, Friend S, Castranova V, Demokritou P, Brain JD, Molina RM. Particle and fibre toxicology. 2015; 12:31. DOI 10.1186/s12989-015-0106-4. [PubMed: 26458946]
37. Lynch I, Cedervall T, Lundqvist M, Cabaleiro-Lago C, Linse S, Dawson KA. Advances in colloid and interface science. 2007;134–135. 167–74. DOI 10.1016/j.cis.2007.04.021.
38. Lynch I, Dawson KA, Linse S. Science's STKE : signal transduction knowledge environment. 2006; 2006(327):pe14. DOI 10.1126/stke.3272006pe14. [PubMed: 16552091]
39. Lynch I, Salvati A, Dawson KA. Nature nanotechnology. 2009; 4(9):546–7. DOI 10.1038/nnano.2009.248.
40. Cho WS, Thielbeer F, Duffin R, Johansson EM, Megson IL, MacNee W, Bradley M, Donaldson K. Nanotoxicology. 2014; 8(2):202–11. DOI 10.3109/17435390.2013.773465. [PubMed: 23379633]
41. Bhattacharjee S, Ershov D, Islam MA, Kampfer AM, Maslowska KA, van der Gucht J, Alink GM, Marcelis ATM, Zuilhof H, Rietjens IMCM. RSC Advances. 2014; 4(37):19321–19330. DOI 10.1039/C3RA46869K.
42. Karlsson HL, Cronholm P, Gustafsson J, Möller L. Chemical research in toxicology. 2008; 21(9): 1726–1732. DOI 10.1021/tx800064j. [PubMed: 18710264]
43. Fahmy B, Cormier SA. Toxicology in vitro : an international journal published in association with BIBRA. 2009; 23(7):1365–71. DOI 10.1016/j.tiv.2009.08.005. [PubMed: 19699289]
44. Lu X, Mioussé IR, Pirela SV, Moore JK, Melnyk S, Koturbash I, Demokritou P. Nanotoxicology. 2015;1–11. DOI 10.3109/17435390.2015.1108473.
45. Lu X, Mioussé IR, Pirela SV, Melnyk S, Koturbash I, Demokritou P. Nanotoxicology. 2016; 10(2): 140–50. DOI 10.3109/17435390.2015.1025115. [PubMed: 25938281]
46. Project on Emerging Nanotechnologies. 2013 ed.. Woodrow Wilson International Center for Scholars; 2011. <http://www.nanotechproject.org/inventories/consumer/>
47. Lough GC, Schauer JJ, Park JS, Shafer MM, Deminter JT, Weinstein JP. Environmental Science & Technology. 2005; 39(3):826–836. DOI 10.1021/es048715f. [PubMed: 15757346]
48. Pal AK, Watson C, Pirela SV, Singh D, Chalbot M-CG, Kavouras I, Demokritou P. Toxicological Sciences. 2015 DOI 10.1093/toxsci/kfv095.
49. Cohen JM, Derk R, Rojanasakul L, Godleski J, Kobzik L, Brain J, Demokritou P. Nanotoxicology. 2014 in press, DOI:10.3109/17435390.2013.879612.

Figure 1. Behavior of buoyant NPs in media

a.) Primary particles in culture media may form particle agglomerates, which are protein coronae wrapped bundles of particles with intra-agglomerate media within the bundles. **b.)** In in vitro cellular systems, particle agglomerates may display different behavior based on their average hydrodynamic diameter and effective density. Non-buoyant nanoparticles possessing higher effective density than culture media ($\rho_{EV} > \rho_m$) will eventually settle at the bottom of the well. Particles with effective densities lower than the cell culture media ($\rho_{EV} < \rho_m$) will exhibit buoyancy, which may alter particle deposition and dose-response relationship. Overtime, the concentration of the buoyant nanoparticles will accumulate at or near the top of the well and decrease at the bottom of the well over time using conventional cell culture techniques.

Figure 2. Colloidal Characterization and Dose Metrics of PP NP Suspensions. Comparisons of size distributions of PP NP in RPMI containing 3% FBS using DLS and TRPS along with transmission electron micrograph showing morphology of PP NPs.

Figure 3. Fate and transport of PP NP using computational modeling

Using the DG fate and transport computational model the following dosimetrics were obtained. **a.)** The fraction deposited of PP NP at the bottom of the well. **b.)** The fraction deposited at the top of the well. **c.)** The concentration at the bottom of the well. **d.)** The concentration at the top of the well.

Figure 4. Inverted cell culture platform for the assessment of buoyant NPs

a.) Deconstructed schematic showing components of the inverted cell culture platform used in these studies. A standard 96-well cell culture plate (white) rests in the base of the platform. The cover assembly is designed to contain the media in each well independently during inversion, and to maintain sterile cell culture conditions. A gas-permeable Teflon membrane is used to cover the inverted wells and allow CO₂ exchange during incubation. Siliconized gaskets for each well are used to provide a seal and prevent leakage during inversion. **b.)** Side view schematic of the assembled inverted cell culture platform with the expanded view demonstrating the position of the cells in relationship to the buoyant NPs using the inverted cell culture system.

Figure 5. Buoyant NPs influence dose response

Adherent AM-HMDMs were incubated for 24 h. in either upright or inverted cell culture with varying concentrations of PP NPs, prior to MTS, LDH and ROS assay. **a**) Cell viability (MTS) in conventional and inverted culture system. **b**) Cytotoxicity (LDH) in conventional and inverted culture system. **c**) Confocal images of CellROX-stained cells from conventional and inverted systems. **d**) ROS generation (number of fluorescent specs per cell) for conventional and inverted systems. $C_0 \mu\text{g ml}^{-1}$: initial administered concentration; $\bar{C}_{bot} \mu\text{g ml}^{-1}$: mean concentration at well bottom (conventional culture exposure concentration); $\bar{C}_{top} \mu\text{g ml}^{-1}$: mean concentration at well top (inverted culture exposure concentration). Data represent mean $\pm$ standard deviation for 4 (MTS), 3 (LDH) or 2 (ROS) independent experiments. * = $p < 0.0$

Table 1

Characterization hydrodynamic diameter ($d_{(h, z-ave)}$), polydispersity (PDI), zeta potential (mV), and conductance (mS/cm) of PP NP in DI water at DSE_{cr} (critical delivered sonication energy) and after particle dispersion in RPMI 3% FBS using DLS and TRPS method. The DSE_{cr} utilized was 466 J/ml.

Method	Media	$d_{(h, z-ave)}$ (nm)	PDI	Zeta Potential (mV)	Conductance (mS/cm)	pH	Effective Density (g/cm ³)
DLS	DI water	544.7±3.5	0.27±0.02	-40.6±4.5	-0.25±0.2	n/a	n/a
	RPMI 3% FBS	479.2±40.2	0.58±0.05	-16.3±1.6	12.3±0.2	7.46	0.9
TRPS	RPMI 3% FBS	$d_{(h, z-ave)}$ (nm)	$d90/d10$ Ratio	Surface Charge (mV)	Conductance (mS/cm)	pH	Effective Density (g/cm ³)
		392.0±58	2.25	-19.0	12.3±0.2	7.46	0.9

Table 2**Dosimetry summary**

The administered concentration and the delivered dose metrics are determined across 24 h PP NP exposure using the size distribution determined by DLS.

	Administered Concentration ($\mu\text{g/ml}$)		
$C_0 \mu\text{g ml}^{-1}$	10	100	250
	Delivered Dose Metrics		
$\bar{C}_{bot} \mu\text{g ml}^{-1}$	2.572	25.72	64.30
$\bar{C}_{top} \mu\text{g ml}^{-1}$	182.1	1821	4553
$\bar{t}_{D,bot}$	0.0002	0.0002	0.0002
$\bar{t}_{D,top}$	0.0167	0.0167	0.0167
$\bar{M}_{bot} \mu\text{g cm}^{-2}$	0.003	0.026	0.064
$\bar{M}_{top} \mu\text{g cm}^{-2}$	0.182	1.821	4.553