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Investigation of Twenty Metal, Metal Oxide, and Metal Sulfide Nanoparticles' Impact on Differentiated Caco-2 Monolayer Integrity

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Abstract

The use of engineered nanomaterials (ENMs) in foods and consumer products is rising, increasing the potential for unintentional ingestion. While the cytotoxicity of many ENMs has been investigated, less attention has been given to adverse impact on the intestinal barrier integrity. Chronical disruption of gastrointestinal integrity can have far reaching health implications.

Using fully differentiated Caco-2 cells, the perturbation of intestinal barrier function and cytotoxicity were investigated for 20 metal, metal oxide, and metal sulfide ENMs. Caco-2 cells were exposed to 50 µg/mL ENMs for 24 hours. ENM formulations were characterized at 0 and 24 hours, and *In Vitro* Sedimentation, Diffusion and Dosimetry Modeling was applied to calculate the effective dose of exposure during 24 hours. The apparent permeability coefficient (P_{app}) was determined for fluorescent labeled dextran (3,000 Da) and tight junction integrity was evaluated by immunofluorescence microscopy. Cytotoxicity was investigated by determining lactate dehydrogenase release (LDH) and cell metabolic activity (tetrazolium based MTS) assays.

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Four ENMs led to significantly increased P_{app} (15.8% w/w% Ag-SiO₂ nanoparticle (NP), 60 nm CdS NP, 100 nm V₂O₅ flakes, and 50 nm ZnO NP), while one ENM (20 nm MgO NP) decreased P_{app} . With the exception of CdS NP, significantly increased P_{app} was not connected with cell cytotoxicity. The calculated effective dose concentration was not correlated with increased P_{app} .

Our results illustrate that while many metal, metal oxide, and metal sulfide ENMs do not adversely affect monolayer integrity or induce cytotoxicity in differentiated Caco-2 cells, a subset of ENMs may compromise the intestinal integrity. This study demonstrated the use of differentiated Caco-2 monolayer and P_{app} as an endpoint to identify and prioritize ENMs that should be investigated further. The interaction between ENMs and the intestinal epithelium needs to be evaluated to understand potential intestinal barrier dysfunction and resulting health implications.

Keywords

Engineered Nanomaterials; ENM Dosimetry; Cytotoxicity; Intestinal Integrity; Caco-2 monolayer

1.0. Introduction

As the field of nanotechnology evolves, there is a need for comprehensive biological response profiles to guide the development of safe and sustainable use of nanomaterials. In the past two decades, the number of consumer products containing engineered nanomaterials (ENMs) has increased substantially, including those within the food and agriculture sectors (Bergin et al. 2013, Bouwmeester et al. 2014, EFSA 2015). According to several consumer product databases and inventories, there are now hundreds of commercially available food and beverage products that contain ENMs (Nanotechnologies 2015, Counsil et al. 2016, CFS 2017). It has been estimated that the daily number of ingested metal or metal oxide particles amounts to 10¹², comprised mainly of titanium dioxide (TiO₂) and silicon dioxide (SiO₂) (Lomer et al. 2002). Food grade TiO₂ is a food additive used as a whitening agent that has been found in food at concentrations between 0.02 – 9.0 mg TiO₂/g product (Peters et al. 2014), and the estimated daily consumption of TiO₂ ranges from 2 mg/kg body weight (bw) for children between age 2.5–4.5 years to 0.2 mg/kg bw for adults (Weir et al. 2012). That ingested particles cross the intestinal barrier has been demonstrated by the detection of TiO₂ particles in 7 out of 15 adult (age 56 – 104) human liver samples (collected post mortem) (Heringa et al. 2018). While TiO₂ and SiO₂ nanoparticles (NP) are the most prevalent in food, a range of other ENMs are also used as e.g. food supplements, antimicrobial agents, and colorant (Table 1). The level and diversity of ingested ENMs are therefore likely to keep rising, and more knowledge is needed to estimate the interaction between ENMs and the intestinal tract.

The gastrointestinal tract is a selective barrier between the lumen and the body, and responsible for nutrient absorption as well as providing protection against harmful pathogens and xenobiotics. Disruption of the gastrointestinal tract health and homeostasis can lead to many chronic diseases. Increased intestinal permeability, called “Leaky Gut Syndrome”, happens when the intestinal tight junctions (TJs) do not work properly (as reviewed (Kiefer et al. 2004, Liu et al. 2005, Hollander et al. 2019)). TJs connect the intestinal epithelial cells and control passive diffusion of ions and other small solutes paracellularly, maintaining any

gradient created by the active transport pathways associated with the transcellular route (as reviewed (Mitic et al. 1998, Anderson et al. 2009)). Disruption of TJs therefore leads to decrease in protection against xenobiotics and pathogens present in the lumen. Chronic disruption of gastrointestinal integrity has been associated with a predisposition to developing autoimmune diseases, liver dysfunction, arthritic and other degenerative diseases (Meletis 1998, Arrieta et al. 2006, Fukui 2016, Konig et al. 2016).

In vitro models of the intestinal tract have been used to investigate ENM translocation across cell monolayers and cytotoxic effects (Zha et al. 2008, Esch et al. 2012, Fisichella et al. 2012, Loo et al. 2012, Piret et al. 2012, Gerloff et al. 2013, Gitrowski et al. 2014, Ude et al. 2017, Ye et al. 2017, Vila et al. 2018a, Vila et al. 2018b). A well-established model for the intestinal barrier is the epithelial colorectal adenocarcinoma Caco-2 cells. Fully differentiated Caco-2 cells resemble mature enterocytes, expressing an organized brush border with a dense network of functional TJs (Hilgendorf et al. 2000, Carr et al. 2012). Differentiated Caco-2 transwell (TW) cultures are the model of choice for uptake studies into the small intestine (Christensen et al. 2012), and are considered a good model for studying intestinal barrier function (Alvarez-Hernandez et al. 1991, Jarc et al. 2019). It has been established that Caco-2 cells need to grow on a semipermeable surface, such as a TW membrane, in order to fully differentiate into polarized enterocytes, exhibiting well-developed microvilli and polarized distribution of brush border enzymes and transporters (Hidalgo et al. 1989, Sambuy et al. 2005, Natoli et al. 2012). The use of undifferentiated cells is quicker and cheaper than using the differentiated Caco-2 model; however, differentiated cells more accurately mimic *in vivo* conditions and allow for studies of monolayer integrity.

There is an increasing incentive to reduce animal testing and develop more physiological relevant *in vitro* models. With the large number of diverse ENMs implemented in food and consumer products, where safety needs to be assessed, it is important to align nanosafety and toxicology studies to the 3Rs principles (Replacement, Reduction and Refinement of animal testing) (Burden et al. 2017). Initiatives in developing and validating 2-dimensional (2D) and 3-dimensional (3D) mono- and co-culture *in vitro* models have been expanding rapidly during the past decade and are showing great promise. However, dependent on the biological mechanism in focus, i.e. studying enterocyte integrity, transwell mono-culture may be the most suitable *in vitro* model.

For testing ENM transport across the intestinal tract *in vitro* co-culture models including Caco-2, human mucus producing goblet cells (HT29-MTX) (Lesuffleur et al. 1993, Walter et al. 1996) and/or human B-lymphocytes (Raji B), which convert a portion of the Caco-2 cells to resemble Peyer's patch follicle-associated epithelium (FAE) microfold (M) cells (Lo et al. 2004, Beloqui et al. 2017), have been utilized. These cell models have been used to evaluate the role of the mucus layer in ENM interaction with the intestinal tract *in vitro* (Loo et al. 2012, Walczak et al. 2015). The M-cells playing a central role in the intestinal translocation and transport of ENMs have been included in studies focusing on ingested ENM uptake (Loo et al. 2012, Walczak et al. 2015). It is also worth noting that these co-culture models have lower transepithelial electrical resistance (TEER) measurements than the Caco-2 mono-culture, indicating that both HT29-MTX and Raji B cells impact the TJs of

the monolayer (Araujo et al. 2013). While this has been argued to more closely resemble the human intestinal tract than the Caco-2 mono-culture, it may pose a challenge when investigating ENM interaction with enterocyte integrity. Additionally, the Caco-2 monolayer model resembles the enterocyte in the small intestinal tract where the mucus layer is considerably thinner than that of the large intestinal tract, and M-cells are located in Peyer's patches at the base of the epithelium and not in the microvilli. It is therefore important to recognize that the intestinal tract has a considerable variation in its anatomy which must be taken into consideration when selecting an *in vitro* model. Furthermore, these complex cell models are often expensive, with low through-put and might not aid in answering the biological function or mechanism in question. There is therefore still a need for established and well-characterized *in vitro* models, including the human enterocyte monolayer model, in evaluating the potential health risk of ingesting ENMs, and investigating a fundamental question such as enterocyte health and integrity.

Another aspect of *in vitro* evaluation of ENM exposure is simulated gastrointestinal digestion, first presented as an *in vitro* digestion model for bioaccessibility of toxins (Versantvoort et al. 2005), and later adopted in nanotoxicity studies (Peters et al. 2012, Lichtenstein et al. 2015, Bove et al. 2017, DeLoid et al. 2017b). While *in vitro* digestion provides valuable insight in the potential physiochemical changes of ENMs, it also raises questions of what food matrix to use. As illustrated in the literature, the presence of a food matrix, and the type of food matrix, dramatically changes the outcome of the ENM *in vitro* digestion (Peters et al. 2012, Lichtenstein et al. 2015, Kästner et al. 2017, McClements et al. 2017). Assumptions of orogastric transition time, medication, and underlying medical conditions, are all important aspects to address and need to be systematically investigated in future research of ENM ingestion studies.

This study was conducted as part of National Institute of Environmental Health Sciences (NIEHS) Nanomaterials Health Implications Research (NHIR) Consortium and the investigated ENMs were provided by the NIEHS-NHIR Consortium. Herein we compare the impact of 20 metal, metal oxide, and metal sulfide ENMs, selected by NIEHS NHIR, on intestinal integrity *in vitro* using the well-established Caco-2 model of fully differentiated cells resembling small intestinal enterocytes. In this study we tested a dose of 50 µg/ml ENM. Determination of the most realistic dosage is challenging since no actual data of ENM concentration in the intestinal tract is available. We based our dose selection on literature of what ENM concentrations are present in food. E.g. food grade TiO₂ has been found in food at a concentration between 0.02 – 9.0 mg TiO₂/g product (Peters et al. 2014). Therefore, 50 µg/ml for TiO₂ E171 may be substantially lower than the concentration in food. We do acknowledge that this dose might not be as realistic for other tested ENMs (e.g. V₂O₅ flakes, ZnS NP, and WO₃ NP). However, since the aim of this study is to provide a comparison between 20 ENMs, we have decided to test the same concentration for all ENMs, at the same time modeling the effective dose. We investigated the cell layer integrity following *in vitro* exposure, using fluorescence labeled dextran, and cytotoxic response by lactate dehydrogenase (LDH) release and mitochondrial activity assays.

2.0. Materials and Methods

2.1. Engineered Nanomaterial Formulation and Characterization

Twenty metal, metal oxide, and metal sulfide ENMs were selected by NIEHS NHIR. The ENMs were synthesized or procured and comprehensively characterized by the Consortium Engineered Nanomaterials Resource and Coordination Core (ERCC) as part of the NIEHS NHIR Consortium (Table 1). Eighteen of the ENMs were provided as powders (20 nm Ag NP, 3.8 w/w% Ag-SiO₂ NP, 15.9 w/w% Ag-SiO₂ NP, 30 nm Al₂O₃ NP, 60 nm CdS NP, 10 nm CeO₂ NP, 30 nm CeO₂ NP, 50 nm CuO NP, 10 nm Fe₂O₃ NP, 100 nm Fe₂O₃ NP, 20 nm MgO NP, 15 nm SiO₂ NP, 25 nm TiO₂ P25 NP, 100 nm TiO₂ E171 NP, 100 nm V₂O₅ flakes, 15 nm WO₃ NP, 50 nm ZnO NP, and 100 nm ZnS NP). Two ENMs were provided as suspension in citrate buffer (20 nm Ag NP-C and 15 nm Au NP-C) at a concentration of 50 µg/mL. Of the 20 ENMs, ten were synthesized by the precision flame spray pyrolysis (FSP) approach (Beltran-Huarac et al. 2018) (20 nm Ag, 3.8 w/w% Ag-SiO₂, 15.9 w/w% Ag-SiO₂, 30 nm Al₂O₃, 10 nm CeO₂, 30 nm CeO₂, 10 nm Fe₂O₃, 100 nm Fe₂O₃, 15 nm SiO₂, and 15 nm WO₃), two ENMs were synthesized by a hydrothermal method (Ahn et al. 2018) (20 nm Ag NP-C and 15 nm Au NP-C). Eight ENMs were purchased from various sources: 50 nm CuO NP (Sigma-Aldrich), 60 nm CdS NP (NanoShel LLC), 20 nm MgO NP (Strem Chemicals, Inc.), TiO₂ Degussa P25 (Acros Organics.), TiO₂ E171 (PRECHEZA, packed by Pronto Foods Co.), 100 nm V₂O₅ flakes (NanoShel LLC), 50 nm ZnO NP (Meliorum Technologies, Inc.), and 100 nm ZnS NP (NanoShel LLC). For those materials where transmission electron microscopy (TEM) or Brunauer-Emmett-Teller (BET) characterization were available in the literature the information was captured in Table 1. Formulation for ENMs provided as powders was performed at a concentration of 0.5 mg/mL following published protocols (DeLoid et al. 2017a, Cohen et al. 2018). The exception was 20 nm MgO which was only formulated in media by vortexing, since MgO will react with water if energy is added to the solution. In short, 0.5 mg/mL ENM dosing solutions were formulated in filtered deionized water (dH₂O), sonicated in a cup horn sonicator (Ultrasonic Liquid Processor S-4000, Misonix Inc., Farmingdale, NY) for 2 minutes and vortexed for 30 seconds, repeatedly. The hydrodynamic diameter was measured by Dynamic Light Scattering (DLS, Malvern Zetasizer Nano-ZS, Malvern Panalytical, Westborough, MA) every 2–5 minutes, until the change in hydrodynamic diameter was less than 5%. The critical delivered sonication energy (DSE_{cr}) for each ENM required to achieve a suspension with the smallest possible hydrodynamic diameter and polydispersity index (Pdi) and highest stability over 24 hours was determined (DeLoid et al. 2017a). For ENMs suspended in citrate buffer, centrifugation (19,000xg) for 10 minutes was used to concentrate the ENMs, which were subsequently diluted in either dH₂O or growth media. Zeta potential (Malvern Zetasizer Nano-ZS, Malvern Panalytical, Westborough, MA) was measured using disposable Folded Capillary Zeta Cells (Malvern Panalytical, Westborough, MA) in dH₂O and cell growth media at 0 and 24 hours.

The criteria for ENM solution stability, at a concentration of 50 µg/mL in media, was a change of the hydrodynamic diameter less than 30% in the time course of 24 hours. ENM formulation stability was determined in dH₂O and cell growth media at 0 and 24 hours.

2.2. In Vitro Sedimentation, Diffusion and Dosimetry (ISDD) Modeling

The effective dose for each ENM was calculated following the protocol published by DeLoid et al. (2017a). In short, the effective density for each ENM was determined in growth media using the Volumetric Centrifugation Method (DeLoid et al. 2017a). ENM pellet volume was measured using TPP Packed cell volume (PCV) tubes and an Easy-read measuring device for PCV tubes (TPP Techno Plastic Products, Midwest Scientific, Valley Park, MO). The concentration of each metal in the supernatant after centrifugation was determined by Inductively coupled plasma - optical emission spectrometry (ICP-OES) (Varian 710 ICP-OES Axial Spectrometer, Walnut Creek, CA). We were not able to measure the concentration of Va in the supernatant due to instrument errors, and therefore assumed that it was zero in the calculation of the effective density. The dynamic viscosity of cell culture media was measured using a size 25 Cannon-Fenske tube viscometer (Sigma-Aldrich, St. Louis, MO). After calculating the effective density for each of the 20 ENMs in growth media, the effective dosimetry for each of the 20 ENMs was determined by computational modeling using the Volumetric Centrifugation Method – *In vitro* Sedimentation, Diffusion and Dosimetry (VCM-ISDD) or distorted grid (DG) model, published by DeLoid et al. (2017a).

2.3. Cell Culture

Human intestinal epithelial Caco-2 cells (ATCC HTB-37®, ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY), 1% non-essential amino acids (NEAA; Gibco, Life Technologies, Grand Island, NY) and 100 U penicillin/streptomycin (P/S; Gibco, Life Technologies, Grand Island, NY). Cells were maintained at 37 °C in 5% humidified CO₂, at a concentration of 3×10^4 cell/mL and passaged twice a week by washing with pre-warmed phosphate-buffered saline (PBS; Gibco, Life Technologies, Grand Island, NY) and detached using 0.25% trypsin-EDTA (Gibco, Life Technologies, Grand Island, NY). Caco-2 cells were used in experimental cultures between passage numbers 8–35.

TW cultures were performed in 24-well TW plates with polycarbonate membrane, 3.0 µm pores, and a surface area of 0.33 cm² (Corning, Tewksbury, MA). TW plates were seeded at a concentration of 1×10^5 cells/cm². Media was replaced every 48 hours between days 1–8 and then every day until exposure on day 16. Transepithelial electrical resistance (TEER) was measured using an Epithelial Voltohmmeter EVOM2 with an ENDOHM-6G Cell Culture Cup Chambers (World Precision Instruments, Sarasota, FL) prior to ENM exposure. The TEER values for cell monolayers were subtracted by the value of a blank TW without cells and multiplied by the TW membrane surface area of 0.33 cm². Only TW cultures with a TEER value $> 260 \Omega \cdot \text{cm}^2$ were used. Fully differentiated Caco-2 TW cultures were exposed to ENM at a concentration of 50 µg/mL on the apical side, representing the intestinal lumen, for 24 hours.

2.4. Caco-2 Cell Monolayer Permeability Assay

Following 24 hours exposure to 50 µg/mL ENMs, the cell monolayer permeability in the apical to basolateral direction was measured using Alexa Fluor 488 (AF488)-labeled dextran

(3,000 Da) (ThermoFisher Scientific, Oak Ridge, TN) (Strauman et al. 2010). Fifty (50) µg/mL AF488-dextran in P-buffer (donor solution) was added to the apical compartment of the TW and P-buffer alone (receiver solution) was added to the basolateral chambers (Matsuzawa et al. 2005). After 3 hours of incubation with the AF488-dextran, fluorescence was measured in the apical and basolateral solution using a plate reader (CLARIOstar, BMG LABTECH, Cary, NC). The AF488-dextran concentration was extrapolated from the standard curves using Prism GraphPad 7.4 (GraphPad Software, San Diego, CA). Standard curve with 9 concentrations of AF488-dextran in P-buffer (0.0, 0.006, 0.031, 0.078, 0.156, 1.25, 10, 50, and 100 µg/mL) was run in duplicate.

The Apparent Permeability Coefficient (P_{app} [cm/s]) for AF488-dextran was calculated as described in the literature (Ampasavate et al. 2002); P_{app} (cm/s) = $X/(A \cdot t \cdot C_d)$, X is the amount of substance (mol) in the receiver chamber at time t (s), A is the diffusion area (0.33 cm²), and C_d is the concentration of the substance in the donor chamber (mol/cm³). The inverse of the TW membrane permeability was subtracted from the inverse of the permeability calculated above to find the cell monolayer permeability only (P_{app}). Triton X-100 (Sigma-Aldrich, St. Louis, MO.), 0.1% in cell media, was used as a positive control. All studies were conducted in at least biological duplicates and experimental triplicates.

2.5. Fluorescence Microscopy

Cell monolayers were fixed in 4% paraformaldehyde for 20 minutes at room temperature. They were permeabilized with 0.05% Triton-X (Acros Organics, NJ) for 5 minutes at 4°C and blocked in 10% Normal Goat Serum (NGS; Jackson ImmunoResearch, West Grove, PA) in PBS and then incubated with primary antibody for the intracellular TJ protein ZO-1 (ZO-1-1A12, Thermo Fisher, Waltham, MA) in 1% NGS overnight at 4 °C. This was followed by incubation with secondary antibodies (Alexa Fluor 488 A1101, Invitrogen, Carlsbad, CA) in 1% Bovine Serum Albumin (BSA, Sigma-Aldrich, St. Louis, MO) for 1 hour. Nuclei were labeled using 1:500 Hoechst 33342 staining (Life Technologies, Grand Island, NY). Fluorescence was visualized by Zeiss Observer Z1 3D inverted microscope with three channels. To assess cell health, 50 nuclei were counted at random in eight images of each exposure, noting the healthy and condensed or fragmented nuclei. A healthy nucleus was classified as having a clear boundary and an oblong morphology. Fragmented nuclei were classified as having a decreased intensity compared to healthy neighboring nuclei and a lack of clear boundaries, while condensed nuclei were classified as having a rounded, spherical morphology with above an 2-fold increase in intensity compared to healthy neighboring nuclei (Supp. Figure 3). Nuclei that looked to be condensed because they were undergoing cell division were not considered. The percentage of condensed cells was calculated for each image. Triton X-100 (Sigma-Aldrich, St. Louis, MO.), 0.1% in cell media, was used as a positive control.

2.6. Toxicity Assay

After 24 hours of ENM exposure, the apical and basolateral media were collected for lactate dehydrogenase (LDH) release measurements. LDH assays (TOX7, Sigma-Aldrich, St. Louis, MO) were performed according to the manufacturer's protocol to measure LDH released to the media. Briefly, 100 µL of media from the apical and basolateral

compartments of the TW plate were analyzed to assess cell viability as a function of cell membrane integrity. It was not possible to gain meaningful LDH data for 15 nm Au NP-C and 20 nm Ag NP-C suspended in citrate buffer, since the absorbance of even small amounts of buffer interfered with the wavelength used in the colorimetric assay (adsorption at wavelength 490–690 subtracted growth media background; 15 nm Au NP-C: 0.254 ± 0.0145 and 20 nm Ag NP-C: 2.06 ± 0.0106).

Prior to the MTS assay the monolayer was washed twice with PBS to remove media and non-attached ENMs. MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assays (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI) were performed according to the manufacturer's protocol. Briefly, the cell reagent solution was added to the cells, and metabolic activity determined by colorimetric measurement of MTS, which is reduced to colored formazan by viable, metabolic active cells.

All studies were conducted in at least biological duplicates and experimental triplicates. Data was expressed as percentage of their representative controls. Triton X-100 (Sigma-Aldrich, St. Louis, MO.), 0.1% in cell media, was used as a positive control.

2.7. Data Analysis

Data are expressed as mean $\pm$ standard deviation. Statistical analysis was performed by Student's t-test (Prism, GraphPad 7.4, GraphPad Software, San Diego, CA). A P-value <0.05 was considered significant.

3.0. Results

3.1. ENM Characterization and Stability

The DSE_{cr} (J/mL) needed to achieve the most monodisperse and stable ENM suspension possible varied considerably between the 20 metal, metal oxide, and metal sulfide ENMs, with 30 nm Al_2O_3 NP (407 J/mL), 15 nm SiO_2 NP (795 J/mL), and 25 nm TiO_2 P25 (908 J/mL) requiring the lowest amount of energy, and 15 nm WO_3 NP (2,130 J/mL), 60 nm CdS NP (3,446 J/mL), and 100 nm V_2O_5 flakes (3,636 J/mL) requiring the highest amount (Table 2). The criteria for ENM formulation stability was $<30\%$ change of the hydrodynamic diameter over 24 hours in cell culture media. The hydrodynamic diameter of 13 ENMs changed $<30\%$ in media during 24 hours (20 nm Ag NP, 20 nm NP-C, 15.9% Ag- SiO_2 NP, 15 nm Au NP-C, 10 nm CeO_2 NP, 30 nm CeO_2 NP, 10 nm Fe_2O_3 NP, 100 nm Fe_2O_3 NP, 20 nm MgO NP, 15 nm SiO_2 NP, 25 nm TiO_2 P25, 15 nm WO_3 NP, and 50 nm ZnO NP). The hydrodynamic diameter for two ENMs decreased $>30\%$ over 24 hours (3.8% Ag- SiO_2 NP and 100 nm V_2O_5 flakes), while the hydrodynamic diameter of five ENMs increased $>30\%$ in cell media (30 nm Al_2O_3 NP, 60 nm CdS NP, 50 nm CuO NP, 100 nm TiO_2 E171 and 100 nm ZnS NP). Increasing the DSE_{cr} did not result in more stable or monodisperse suspensions for 30 nm Al_2O_3 NP, 60 nm CdS NP, 50 nm CuO NP, 100 nm TiO_2 E171, and 100 nm ZnS NP and they were tested *in vitro* as listed in Table 2. Likewise, increasing DSE_{cr} did not decrease the hydrodynamic diameter for 100 nm Fe_2O_3 NP, 100 nm V_2O_5 flakes, or 60 nm CdS NP which all had hydrodynamic diameters considerably larger than the

reported diameter measured by TEM. The Zeta-potential for the 20 ENMs were similar in media both at 0 and 24 hours, between -10 mV to -15 mV, which were in contrast to the Zeta-potential in water that ranged from positive surface charge of 51 mV (30 nm CeO₂ NP) to negative surface charge of -51 mV (20 nm Ag NP-C), suggesting that the cell media components (e.g. proteins) shields the original ENM surface charge (Mortensen et al. 2013).

3.2. In Vitro Sedimentation, Diffusion and Dosimetry Modeling

ISDD modeling was used to calculate the effective dose for the ENMs over the time course of 24 hours, and showed that the concentration and fraction of ENMs in the growth media immediately above the Caco-2 cell monolayer (expressed as the volume between the cell monolayer and $10\text{ }\mu\text{m}$ above) varied by orders of magnitudes between the ENMs (Figure 1 and Supp Table 1). The ENMs with the highest calculated mean concentration at the bottom of the transwells during 24 hours were 10 nm Fe₂O₃ NP (22.8 mg/mL), 100 nm Fe₂O₃ NP (13.9 mg/mL), and TiO₂ E171 (12.1 mg/mL), while ENMs with the lowest mean concentrations were 3.5 w/w% Ag-SiO₂ NP (0.0370 mg/mL), 60 nm CdS NP (0.0411 mg/mL), and 15 nm WO₃ NP (0.0540 mg/mL). The ISDD modeling also demonstrated the difference in ENM settling over 24 hours, with 20 nm Ag NP-C, 15.8 w/w% Ag-SiO₂ NP, and 15 nm SiO₂ NP rapidly reaching a plateau, while 30 nm CeO₂ NP and 50 nm CuO NP showed an almost linear settling profile (Figure 1).

3.3. ENM Impact on Intestinal Integrity

The impact of 24 hours exposure to the 20 metal, metal oxide, and metal sulfide ENMs on the integrity of the Caco-2 cell monolayer was investigated using 3,000 Da AF488-dextran and by immunofluorescence staining of TJ (Supp Figures 2 and 3). P_{app} for AF488-dextran increased significantly following exposure to 15.8% Ag-SiO₂ NP, 60 nm CdS NP, 50 nm CuO NP, 50 nm ZnO NP, and 100 nm V₂O₅ flakes compared to control, while MgO NP significantly decreased P_{app} for AF488-dextran (Figure 3). Immunofluorescence staining did not show major reorganization of ZO-1, suggesting that TJ remained intact, for the ENMs that led to increased P_{app} for AF488-dextran, but small areas of weak and inconsistent staining could be observed. CuO NP exposure led to diffused ZO-1 staining with very little well organized TJ between the cells. Interestingly, CuO NP did not lead to a significant increase in P_{app} for AF488-dextran although a more disperse location of ZO-1 proteins was evident.

3.4. ENM Cytotoxicity

Cytotoxicity of the 20 metal, metal oxide, and metal sulfide ENMs were assessed by measuring LDH released to the apical and basolateral side (Figure 2A–B) and MTS (Figure 2C). While LDH measures cell membrane integrity, MTS detects the mitochondrial and metabolic activity of the cells. MTS as a percent of control was significantly increased for 20 nm Ag NP. Ag NP has been shown to interfere with MTS assay, having a positive effect by the direct reaction of Ag NP with MTS (Liang et al. 2015), which suggests a possible interference between 20 nm Ag NP and the MTS assay.

LDH in the apical media, expressed as percentage of control, was significantly ($P<0.05$) increased for 60 nm CdS NP, 100 nm Fe₂O₃ NP15, and 15 nm SiO₂ NP, while significantly

decreased LDH was measured for 50 nm CuO NP, 15 nm WO₃ NP, and 100 nm ZnS NP. In the basolateral media, LDH were significantly increased for 60 nm CdS NP and decreased for 50 nm CuO NP. Metal ions have been demonstrated to interfere with the LDH enzyme activity (Han et al. 2011, Ong et al. 2014), so results for ENMs with high dissolution rate in media should be analyzed with care (e.g. CuO NP, WO₃ NP, and ZnS NP). That CuO NP resulted in decreased LDH in both apical and basolateral media suggests that Cu ion was translocated to the basolateral side of the cell monolayer. CdS NP caused an increase in LDH release both in the apical and basolateral media, suggesting that the cell membrane might be damaged throughout the cell monolayer, and have a more severe cytotoxic response than SiO₂ NP and 100 nm Fe₂O₃ NP where an increased in LDH were only measured on apical side of the monolayer.

As an assessment of cell viability, nuclei morphology (health, condensed, or fragmented) were evaluated (Figure 3D) showing a significant increase in number of condensed or fragmented nuclei following exposure to 20 nm Ag NP, 20 nm Ag NP-C, 60 nm CdS NP, 10 nm CeO₂ NP, 30 nm CeO₂ NP, 10 nm Fe₂O₃ NP, 30 nm Fe₂O₃ NP, and 10 nm V₂O₅ flakes. These findings suggest that the ENMs cause an increase in cell necrosis or apoptosis.

Collectively, the data demonstrated that 60 nm CdS NP exposure results in a cytotoxicity response in differentiated Caco-2 cells, and suggests that 20 nm Ag NP, 20 nm Ag NP-C, 10 nm CeO₂ NP, 30 nm CeO₂ NP, 10 nm Fe₂O₃ NP, 30 nm Fe₂O₃ NP, 15 nm SiO₂ NP, and 10 nm V₂O₅ flakes impact cell health to a lesser extent.

4.0. Discussion

Considerable effort has been made in the field of nanotoxicology and nanotechnology to generate hazard rankings, safety and risk assessments in order to provide stakeholders and policy makers with robust risk governance tools to ensure safe and sustainable use of ENMs in food and consumer products. However, the large number of available ENMs, *in vitro* and *in vivo* models, biological assays, and endpoints often make it challenging to compare the biological interactions between different ENMs for a specific biological system based on the literature. While it should be acknowledged that a wide array of additional parameters, such as co-culture *in vitro* models and simulated digestion, have been applied in the literature in an effort to make the *in vitro* assays more physiological relevant, a basic comparison of metal, metal oxide, and metal sulfide ENMs' impact on enterocyte monolayer integrity is lacking. It must also be mentioned that while *in vitro* cell models, like the Caco-2 cell monolayer, are valuable for assessing and prioritizing ENMs for further investigation, they may not be directly translatable to human conditions. The electrical resistance of human small intestinal tissue, collected from patients with pancreatic cancer (duodenum) and obese patients undergoing gastric bypass Roux-en-Y surgery (jejunum), measured in a Ussing chamber approximate 40 Ω cm² (Sjoberg et al. 2013), which is in contrast with the TEER value of differentiated Caco-2 monolayer. It is therefore possible that the human intestinal tract might have higher sensitivity to ENM exposure than what is suggested by the Caco-2 monolayer model. Here, we focused on collecting data for 20 metal, metal oxide, and metal sulfide ENMs that can provide basic knowledge needed to compare multiple ENMs.

In the scientific community's collected effort to generate comprehensive response profiles for ENMs with diverse physiochemical properties (PCPs), it is important to ensure that the tested ENM formulations are as monodisperse and stable as possible. Taking the necessary steps to ensure stable and well characterized ENM suspensions helps limit variabilities between studies. Influenced by their PCPs, including size, surface properties, and metal, ENMs tend to aggregate and/or settle in suspensions. In addition to careful preparation of ENM suspensions to ensure monodispersity and stability, characterization of ENM for the duration of testing is equally important. Here we prepared 20 metal, metal oxide, and metal sulfide ENMs using the two published ENM formulation protocols (DeLoid et al. 2017a, Cohen et al. 2018), characterize the ENM in suspension over 24 hours, and used ISDD modeling to calculate effective dose for each ENM (DeLoid et al. 2017a). While most of the ENMs were stable in media for the duration of 24 hours, three metal oxide and the two metal sulfide ENMs were not. ENMs aggregation and settling is not always preventable without coating or the addition of surfactants, which in turn will impact the ENMs' PCP and might not reflect the desired material properties.

The ISDD model does not capture the change in hydrodynamic diameter over the time course of 24 hours, but relies on the volume-weighted hydrodynamic diameter at time 0 hours, so both the DLS characterization of the ENM formulation and the ISDD modeling provide important data when interpreting the biological effect. For example, 10 nm Fe₂O₃ NP had the highest calculated mean effective dose (22.8 mg/mL) at the bottom of the transwell, but the change in hydrodynamic diameter in media was <30% during the course of 24 hours. On the other hand, CdS NP hydrodynamic diameter increased >30% during 24 hours in media, but had the second lowest modeled mean concentration at the bottom of the transwell (0.0411 mg/mL). The hydrodynamic diameter for the two ENMs were comparable, illustrating the need for both measuring the ENMs in the suspension during the course of the study, and model the effective dose in order to better interpret the biological *in vitro* findings.

The cytotoxic response in undifferentiated and differentiated Caco-2 cells varies significantly, showing that fully differentiated Caco-2 are less susceptible to adverse outcome of ENM exposure (Gerloff et al. 2013, Vila et al. 2018a, Vila et al. 2018b). This suggests that using undifferentiated Caco-2 cells for evaluating the biological interaction between ENMs and the intestinal tract may lead to false positive findings of adverse effects, complicating the process of prioritizing which ENMs needing further investigation in animal models. We therefore utilized the well-established model of fully differentiated Caco-2 cells resembling enterocytes of the small intestinal tract to evaluate the impact of the 20 ENMs selected by the NIEHS NHIR Consortium on enterocyte integrity. Differentiated Caco-2 monolayer exposed to 15.8% w/w% Ag-SiO₂ NP, CdS NP, ZnO NP, and 100 nm V₂O₅ flakes showed significantly increased P_{app} of AF488-dextran. While, the increased P_{app} was significantly higher for the four ENMs, the immunofluorescence staining did not show major reorganization or disruption of ZO-1, however small areas of weak and inconsistent staining could be observed. Immunofluorescence staining showed that CuO NP led to a more disperse location of the intracellular TJ protein ZO-1, without changing permeability. The settling profile for CuO NP was almost linear, and while the P_{app} was not impacted, the TJ complex appeared less structured. To ensure that the increased P_{app} of AF488-dextran

was not due to cytotoxic effects following ENM exposure, cell membrane integrity (LDH release), metabolic activity (MTS assay), and evaluation of nuclei morphology were utilized. The only ENM causing increased P_{app} which also resulted in a cytotoxic response was CdS NP, with increased level of LDH release measured in both apical and basolateral media, suggesting that the observed P_{app} is not only due to an increase in the paracellular route. However, more research is needed to further address this. Interestingly, CdS NP have the second lowest effective dose (0.0411 mg/mL), demonstrating that relatively high effective dose is not necessarily linked to biological effect. This is further supported by the observation that 10 nm Fe_2O_3 with the highest effective dose did not cause any change in P_{app} but did show an increase in number of condensed or fragmented nuclei. In contrast, 100 nm Fe_2O_3 , with the second highest effective dose (13.9 mg/mL), resulted in a low, but significant, increase in LDH level to the apical side of the monolayer as well as increase in number of condensed or fragmented nuclei. All ENMs were administered the same dose of 50 μ g/mL, but the ISDD modeled delivered dose varied with almost three orders of magnitude between the 20 ENMs (Supp Table 1), demonstrating the importance of calculating the delivered dose in *in vitro* assays to understand the interaction between ENMs and cells. Our findings illustrate the necessity to model the delivered dose when comparing multiple ENMs, but also that high delivered dose is not necessarily linked to high biological impact. Together these results suggest that further investigations are needed to understand the underlying mechanisms and long-term consequences of exposure.

Fully differentiated Caco-2 cells have frequently been used in the literature to evaluate the interactions between NPs and the intestinal tract *in vitro*. Our findings for 20 nm Ag NPs were confirmed by the published work by Vila and coworkers (Vila et al. 2018a) reporting that 8 nm Ag NP did not induce a cytotoxic response in differentiated Caco-2 cells after 24 hours exposure to 50 μ g/mL nor did exposure lead to significant changes in apical to basolateral permeability of Lucifer Yellow (LY, 444 Da). The study did find that Ag NP caused oxidative DNA damage (Vila et al. 2018a), while our study showed an increase in percentage of condensed or fragmented nuclei morphology. Also, for CeO_2 NP (10 nm and 30 nm) our findings were in agreement with published results for 70 nm CeO_2 , which found no cytotoxicity or perturbation of Caco-2 monolayer integrity (Vila et al. 2018b). Furthermore, when differentiated Caco-2 monolayers were exposed for 24 hours to 4 nm CeO_2 NP, no morphological changes were observed in the microvilli (Cabellos et al. 2019). Comparison between our results for 50 nm CuO NP and published reports for CuO NP studies is less straight forward. Ude et al., (2017) tested 10 nm CuO NP using differentiated Caco-2 transwells, and reported that 14.2 μ g/mL did not affect viability after 24-hour exposure (Ude et al. 2017). However, exposure to 10 nm CuO NP did reduce the integrity of immunofluorescence stained TJ (Ude et al. 2017). Also, 24-hour exposure of 25 μ g/mL 12 nm rod-shape CuO NP, and 50 nm spherical CuO NP did not increase P_{app} of LY in Caco-2 monolayer (Piret et al. 2012). While 25 μ g/mL of 12 nm rod-shape CuO NP decreased cell viability after 24 hours, the same concentration of 50 nm CuO NP did not (Piret et al. 2012). In our studies, testing 50 μ g/mL 50 nm CuO NP we saw no cytotoxic effects or increase in P_{app} of AF488-dextran compared to control after 24 hours of exposure, but we did observe a dispersion of the intracellular TJ protein ZO-1 location. Collectively, the two reports and our results suggest that the size and shape of CuO NP plays a critical role in the interaction with

Caco-2 monolayer, and that while 50 nm CuO NP show no cytotoxic effect after 24 hours, the ENM might still impact TJ's organization which should be investigated further.

We found no adverse effects induced by TiO₂ P25 or TiO₂ E171, despite their relatively high effective dose (5.57 and 12.1 mg/mL, respectively). In the literature, TiO₂ P25 was observed in intracellular vesicles in fully differentiated Caco-2 cells by electron microscopy after 24 hours of exposure (Gitrowski et al. 2014). Together with our results, these data suggest that TiO₂ NPs do not adversely impact differentiated Caco-2 cells, but uptake might still happen depending on TiO₂ NP PCPs.

Ag-SiO₂ NP (15.8% w/w%), 60 nm CdS NP, 100 nm V₂O₅, and 50 nm ZnO NP were the only ENMs of the 20 tested metal, metal oxide, and metal sulfide ENMs that increased P_{app} for AF488-dextran. The finding that 15.8% w/w Ag-SiO₂ NP, 60 nm CdS NP, 100 nm V₂O₅ flakes, and 50 nm ZnO NP adversely impact the integrity of the enterocyte monolayer suggests that they could also have an adverse effect in the intestinal tract *in vivo* without causing acute toxicity and should therefore be investigated further. To the authors knowledge, the effects on cytotoxicity and monolayer integrity of Ag-SiO₂ NP, Al₂O₃ NP, CdS NP, Fe₂O₃ NP, MgO NP, V₂O₅ flakes, WO₃ NP, ZnO NP, and ZnS NP ENMs have not previously been reported in the literature. Investigating the monolayer integrity of fully differentiated Caco-2 cells is a functional and sensitive endpoint analysis that provides an important insight into the interaction between ingested ENMs and the intestinal barrier which plays an important role in human health.

5.0. Conclusion

This study provides a comparison between 20 metal, metal oxide, and metal sulfide ENMs' impact on differentiated Caco-2 monolayer integrity. While the majority of the investigated ENMs did not cause cytotoxicity or impacted cell monolayer integrity, we did find that some ENMs adversely impact the monolayer integrity. As the differentiated Caco-2 cells resemble the enterocytes of the small intestinal tract, our findings suggest that ingestion of ENMs may result in perturbation of intestinal integrity potentially compromising the homeostasis and health of the intestinal tract, and that long-term exposure might lead to intestinal barrier dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Transwell cultures of intestinal enterocytes were exposed to 50 µg/mL ENM for 24 h
- *In Vitro* Sedimentation, Diffusion and Dosimetry Modeling was conducted for all ENMs
- The permeability coefficient (P_{app}) for dextran was increased by four ENMs
- P_{app} for dextran was decreased by MgO NP
- With the exception of CdS NP, increased P_{app} was not connected with cytotoxicity

Fig. 1.

Effective dosimetry concentration (blue line) and fraction (red line) calculated for each of the 20 Metal, Metal Oxide and Metal Sulfide ENMs at the bottom of the transwell proximal to the Caco-2 cell monolayer using the In Vitro Sedimentation, Diffusion and Dosimetry Model. The mean ENM concentration throughout the 24 h exposure is indicated with a blue dotted line for each ENM, and the calculated mean concentration is listed in the box on each graph (A–T).

Fig. 2.

Cytotoxicity of the 20 metal, metal oxide, metal sulfide ENMs were investigated following 24 hours exposure to 50 $\mu\text{g/mL}$ ENM by A-B) LDH assay of apical and basolateral media, C) MTS expressed as percentage of corresponding vehicle control, and D) condensed or fragmented (C/F) nuclei as a percentage of total counted nuclei. Increased LDH release suggests damage of the cell membrane. Significantly increased level of LDH release and cell undergoing necrosis or apoptosis are marked with “*” and significantly decreased level of LDH is marked with “#”, “***” in the MTS assay indicate significant increase in % of control which is possible due to 20 nm Ag NP interference with the MTS assay. The positive control 0.1% Triton X was significant different from the control for all four measurements.

Fig. 3.

Apparent permeability coefficient (P_{app}) of AF488-dextran (3,000 Da) and tight junction (ZO-1) staining following 24 hours exposure to 50 μ g/mL ENM with their individual controls. P_{app} increase following exposure to 15.8% Ag-SiO₂ NP, 60 nm CdS, 50 nm ZnO NP, and 100 nm V₂O₅ flakes, while 20 nm MgO NP significantly decreased P_{app} . Tight junctions showed minimal disruption for 15.8% Ag-SiO₂ NP, 60 nm CdS NP, 50 nm ZnO NP, and 100 nm V₂O₅ flakes, but for 50 nm CuO NP, ZO-1 staining was very diffused and very little organized ZO-1 was observed in distinct band between the cells, as seen for the control.

Table 1.

List of Metal, Metal Oxide and Metal Sulfide ENMs provided by the NIEHS NHIR ERCC.

Nanoparticle	Size (mean $\pm$ std.), TEM	Stabilizer	Source/Synthesis Method	Ref./Source	Food and Consumer Products	Ref./Source
20 nm Ag NP	25.6 $\pm$ 6.5 nm	None	NHIR/FSP ***	(Beltran-Huarac et al. 2018)	Food supplement and as an antimicrobial additive in food packaging	(Carbone et al. 2016, CFS 2019, Council et al. 2019, Nanotechnologies 2019)
20 nm Ag NP-C	21.3 $\pm$ 2.8 nm	Citrate	NHIR/ Hydrothermal	(Ahn et al. 2018, Zimmermann et al. 2019)		
3.5 w/w% Ag-SiO ₂ NP	Ag: 10.6 $\pm$ 7.1 nm SiO ₂ : 7.1 $\pm$ 0.4 nm *	None	NHIR/FSP	(Beltran-Huarac et al. 2018)	See 20 nm Ag NP and 15 nm SiO ₂ NP	(Carbone et al. 2016, CFS 2019, Council et al. 2019, Nanotechnologies 2019)
15.0 w/w% Ag-SiO ₂ NP	Ag: 7.9 $\pm$ 4.3 nm SiO ₂ : 6.1 $\pm$ 0.1 nm *	None	NHIR/FSP	(Beltran-Huarac et al. 2018, Cohen et al. 2018)		
30 nm Al ₂ O ₃ NP	28.1 $\pm$ 13.1 nm	None	NHIR/FSP	(Beltran-Huarac et al. 2018, Zimmermann et al. 2019)	Used in cosmetics and personal care products	(FutureMarkets 2013, Nanotechnologies 2019)
15 nm Au NP-C	n.d. **	Citrate	NHIR/ Hydrothermal	(Zimmermann et al. 2019)	Used in cosmetics and foods. Marketed as "antiaging" foods	(CFS 2019, Council et al. 2019, Nanotechnologies 2019)
10 nm CeO ₂ NP	10.5 $\pm$ 5.0 nm	None	NHIR/FSP	(Beltran-Huarac et al. 2018, Zimmermann et al. 2019)	Proposed as alternative to TiO ₂ in sunscreen	(Ortiz et al. 2019)
30 nm CeO ₂ NP	34.7 $\pm$ 22.9 nm	None	NHIR/FSP	(Stueckle et al. 2017, Beltran-Huarac et al. 2018)		
60 nm CdS NP	n.d.	None	Commercial; NA #	NHIR	Used in diagnosis and treatment of different cancers. Pigment in paint and plastics.	(Gupta 2019)
50 nm CuO NP	n.d.	None	Commercial; NA	NHIR	Supplemental form of copper in beverages, used in water filtration	(CFS 2019, Council et al. 2019, Nanotechnologies 2019)
10 nm Fe ₂ O ₃	9.9 $\pm$ 3.8 nm	None	NHIR/FSP	(Beltran-Huarac et al. 2018)	Added as food colorant, potential iron fortifier, food supplement	(EFSA 2014, CFS 2019, Council et al. 2019, Nanotechnologies 2019)
100 nm Fe ₂ O ₃	108.9 $\pm$ 47.5 nm	None	NHIR/FSP	(Beltran-Huarac et al. 2018)		
20 nm MgO NP	n.d.	None	Commercial; NA	NHIR	Food supplement. Considered for food packaging	(Swaroop et al. 2018, Nanotechnologies 2019)
15 nm SiO ₂ NP	15.4 $\pm$ 1.0 nm *	None	NHIR/FSP	(Beltran-Huarac et al. 2018)	Added to foods to improve flow, used as food supplement	(Dekkers et al. 2011, CFS 2019, Council et al. 2019)
TiO ₂ E171	n.d.	None	Pronto Food; NA	NHIR	Added to foods to bleach or brighten colors and/or to enhance flavor in nonwhite foods.	(Weir et al. 2012, CFS 2019, Council et al. 2019)
TiO ₂ P25	28.8 $\pm$ 11.1 nm	None	Acros ORGANICS; NA	(Ahn et al. 2018)	Used in sunscreen and cosmetics Batteries	

Nanoparticle	Size (mean $\pm$ std.), TEM	Stabilizer	Source/Synthesis Method	Ref./Source	Food and Consumer Products	Ref./Source
100 nm V_2O_5 NP	n.d.	None	Commercial; NA	NHIR		(Liu et al. 2018)
15 nm WO_3 NP	n.d.	None	NHIR/FSP	NHIR	Antibacterial properties	(Malarkodi et al. 2014, Duan et al. 2019)
50 nm ZnO NP	n.d.	None	Commercial; NA	NHIR	Used as food supplement and in food packaging. Used in sunscreens and cosmetics	(Galstyan et al. 2018, CFS 2019, Council et al. 2019, Nanotechnologies 2019)
100 nm ZnS NP	n.d.	None	Commercial; NA	NHIR	Semiconductor, antibacterial properties	(Malarkodi et al. 2014)

* Diameter measured by Brunauer-Emmett-Teller (BET)

**

n.d. = No data available

FSP = Precision flame spray pyrolysis

#

NA = Not available

Table 2.
Formulation and Characterization of the Investigated Metal, Metal Oxide and Metal Sulfide ENMs.

ENM	DSE _{cr} (J/mL)	Deionized H ₂ O			Cell Media		
		Dynamic Light Scattering		Z-potential mV	Dynamic Light Scattering		Z-potential mV
		Hydrodynamic Diameter (nm)	PDI		Hydrodynamic Diameter (nm)	PDI	
20 nm Ag	1,209	0 h = 407 ± 64.6	0 h = 0.455	0 h = -16.4 ± 0.516	0 h = 945 ± 265	0 h = 0.792	0 h = -12.2 ± 0.219
		24 h = 437 ± 100	24 h = 0.567	24 h = -34.2 ± 0.3	24 h = 1,067 ± 530	24 h = 0.842	24 h = -13.0 ± 0.173
20 nm Ag-C	NA	0 h = 35.3 ± 0.286	0 h = 0.233	0 h = -51.4 ± 0.862	0 h = 97.1 ± 0.807	0 h = 0.269	0 h = -12.2 ± 1.17
		24 h = 31.2 ± 0.0361	24 h = 0.173	24 h = -50.8 ± 0.781	24 h = 88.1 ± 1.42	24 h = 0.251	24 h = -12.5 ± 1.62
3.8%Ag-SiO₂	1,357	0 h = 246 ± 25.51	0 h = 0.638	0 h = -33.7 ± 0.551	0 h = 244 ± 16.97	0 h = 0.507	0 h = -10.5 ± 0.571
		24 h = 325 ± 37.6	24 h = 0.37	24 h = -34.1 ± 0.7	24 h = 101.7 ± 21.89	24 h = 0.259	24 h = -13.1 ± 1.1
15.9%Ag-SiO₂	1,437	0 h = 79.3 ± 0.722	0 h = 0.294	0 h = -45.2 ± 0.404	0 h = 139 ± 1.36	0 h = 0.27	0 h = -10.6 ± 0.694
		24 h = 89.5 ± 1.27	24 h = 0.286	24 h = -47.7 ± 1.45	24 h = 137 ± 5.73	24 h = 0.503	24 h = -11.9 ± 0.603
30 nm Al₂O₃	407	0 h = 144 ± 2.07	0 h = 0.123	0 h = 47.1 ± 0.551	0 h = 115 ± 3.22	0 h = 0.412	0 h = -12.7 ± 0.987
		24 h = 145 ± 3.66	24 h = 0.0910	24 h = 37.9 ± 1.72	24 h = 193 ± 0.656	24 h = 0.318	24 h = -15.3 ± 1.42
15 nm Au-C	NA	0 h = 50.1 ± 1.45	0 h = 0.546	0 h = -47.3 ± 0.6	0 h = 155 ± 66.2	0 h = 0.375	0 h = -14.6 ± 1.82
		24 h = 44.6 ± 7.31	24 h = 0.445	24 h = -36.8 ± 2.57	24 h = 142 ± 7.24	24 h = 0.415	24 h = -16.6 ± 1.04
60 nm CdS	3,446	0 h = 831 ± 112	0 h = 0.589	0 h = -0.135 ± 0.0965	0 h = 1,369 ± 137.2	0 h = 0.934	0 h = -8.16 ± 1.12
		24 h = 1,662 ± 192	24 h = 0.499	24 h = -19.9 ± 0.666	24 h = 1,805 ± 201.3	24 h = 0.885	24 h = -13.9 ± 1.75
10 nm CeO₂	1,268	0 h = 107 ± 1.96	0 h = 0.303	0 h = 45.5 ± 0.252	0 h = 170 ± 4.85	0 h = 0.426	0 h = -12.5 ± 0.611
		24 h = 164 ± 29.4	24 h = 0.37	24 h = 16.7 ± 0.777	24 h = 184 ± 1.34	24 h = 0.396	24 h = -15.0 ± 0.231
30 nm CeO₂	1,022	0 h = 139 ± 2.30	0 h = 0.159	0 h = 51.3 ± 0.929	0 h = 183 ± 1.38	0 h = 0.156	0 h = -11.8 ± 1.12
		24 h = 157 ± 2.18	24 h = 0.187	24 h = 6.37 ± 0.614	24 h = 222 ± 2.65	24 h = 0.29	24 h = -14.8 ± 0.3
50 nm CuO	1,638	0 h = 533 ± 14.2	0 h = 0.255	0 h = -27.7 ± 0.462	0 h = 613 ± 4.37	0 h = 0.508	0 h = -10.7 ± 0.643
		24 h = 298 ± 33.3	24 h = 0.496	24 h = -29.0 ± 0.462	24 h = 1,043 ± 465	24 h = 0.762	24 h = -12.5 ± 0.0577
10 nm Fe₂O₃	1,460	0 h = 89.9 ± 1.40	0 h = 0.302	0 h = 13.0 ± 0.586	0 h = 1,177 ± 77.1	0 h = 0.324	0 h = -15.6 ± 0.656
		24 h = 3,438 ± 158	24 h = 0.323	24 h = -31.3 ± 0.265	24 h = 1,381 ± 26.3	24 h = 0.437	24 h = -16.4 ± 0.451
100 nm Fe₂O₃	2,093	0 h = 4,978 ± 1,424	0 h = 0.42	0 h = 0.446 ± 0.154	0 h = 5,386 ± 1,157	0 h = 0.661	0 h = -12.2 ± 0.351
		24 h = 6,869 ± 1,137	24 h = 0.517	24 h = -38.6 ± 0.814	24 h = 5,834 ± 955	24 h = 0.831	24 h = -13.8 ± 0.252
20 nm MgO	NA	N/A	N/A	N/A	0 h = 491 ± 111	0 h = 0.68	0 h = -16.0 ± 0.7
		N/A	N/A	N/A	24 h = 354 ± 27.2	24 h = 0.61	24 h = -17.9 ± 1.1

ENM	DSE _{cr} (J/mL)	Deionized H ₂ O				Cell Media			
		Dynamic Light Scattering		Z-potential mV	PDI	Dynamic Light Scattering		PDI	Z-potential mV
		Hydrodynamic Diameter (nm)				Hydrodynamic Diameter (nm)			
15 nm SiO₂	795	0 h = 135 ± 0.513	0 h = 0.513	0 h = -35.3 ± 2.57	0 h = 0.574	0 h = 134 ± 1.50	0 h = -10.3 ± 1.14		
		24 h = 186 ± 0.616	24 h = 0.184	24 h = -36.7 ± 1.07	24 h = 0.440	24 h = 99.0 ± 43.1	24 h = -11.6 ± 1.19		
TiO₂ P25	908	0 h = 293 ± 12.5	0 h = 0.367	0 h = 8.67 ± 0.905	0 h = 0.254	0 h = 294 ± 4.40	0 h = -11.4 ± 0.964		
		24 h = 663 ± 75.0	24 h = 0.785	24 h = 16.4 ± 0.361	24 h = 0.405	24 h = 377 ± 15.6	24 h = -12.7 ± 0.781		
TiO₂ E171	1,607	0 h = 292 ± 2.87	0 h = 0.254	0 h = 2.58 ± 1.59	0 h = 0.228	0 h = 539 ± 27.9	0 h = -14.5 ± 0.577		
		24 h = 308 ± 12.0	24 h = 0.297	24 h = 1.88 ± 1.51	24 h = 0.338	24 h = 1,353 ± 69.5	24 h = -15.1 ± 0.361		
100 nm V₂O₅	3,636	0 h = 1,291 ± 270	0 h = 0.426	0 h = -28.2 ± 0.808	0 h = 0.952	0 h = 3,711 ± 1.678	0 h = -10.1 ± 0.936		
		24 h = 2,538 ± 33.2	24 h = 0.721	24 h = -37.0 ± 0.208	24 h = 0.985	24 h = 1,381 ± 298	24 h = -9.7 ± 0.864		
15 nm WO₃	2,130	0 h = 108 ± 33.5	0 h = 0.222	0 h = -59.7 ± 1.16	0 h = 0.472	0 h = 296 ± 244	0 h = -11.6 ± 0.764		
		24 h = 145 ± 70.9	24 h = 0.314	24 h = -42.9 ± 1.7	24 h = 0.405	24 h = 268 ± 171	24 h = -12.7 ± 0.737		
50 nm ZnO	1,262	0 h = 259 ± 2.88	0 h = 0.245	0 h = -20.2 ± 0.416	0 h = 0.25	0 h = 278 ± 61.6	0 h = -8.53 ± 0.191		
		24 h = 3,444 ± 86.2	24 h = 0.237	24 h = -7.81 ± 0.744	24 h = 0.268	24 h = 299 ± 3.06	24 h = -12.1 ± 0.265		
100 nm ZnS	1,217	0 h = 377 ± 21.8	0 h = 0.288	0 h = -0.117 ± 0.0436	0 h = 0.313	0 h = 392 ± 12.3	0 h = 0.168 ± 0.0552		
		24 h = 3,174 ± 23.1	24 h = 0.422	24 h = -10.5 ± 0.1	24 h = 0.399	24 h = 546 ± 20.3	24 h = -12.3 ± 0.95		

N/A = not applicable, not sonicated