Investigation of the Associations between a Nanomaterial’s Microrheology and Toxicology

Romi Singh Maharjan,* Ajay Vikram Singh, Javaria Hanif, Daniel Rosenkranz, Rashad Haidar, Amruta Shelar, Shubham Pratap Singh, Aditya Dey, Rajendra Patil, Paolo Zamboni, Peter Laux, and Andreas Luch

ABSTRACT: With the advent of Nanotechnology, the use of nanomaterials in consumer products is increasing on a daily basis, due to which a deep understanding and proper investigation regarding their safety and risk assessment should be a major priority. To date, there is no investigation regarding the microrheological properties of nanomaterials (NMs) in biological media. In our study, we utilized in silico models to select the suitable NMs based on their physicochemical properties such as solubility and lipophilicity. Then, we established a new method based on dynamic light scattering (DLS) microrheology to get the mean square displacement (MSD) and viscoelastic property of two model NMs that are dendrimers and cerium dioxide nanoparticles in Dulbecco’s Modified Eagle Medium (DMEM) complete media at three different concentrations for both NMs. Subsequently, we established the cytotoxicological profiling using water-soluble tetrazolium salt-1 (WST-1) and a reactive oxygen species (ROS) assay. To take one step forward, we further looked into the tight junction properties of the cells using immunostaining with Zonula occluden-1 (ZO-1) antibodies and found that the tight junction function or transepithelial resistance (TEER) was affected in response to the microrheology and cytotoxicity. The quantitative polymerase chain reaction (q-PCR) results in the gene expression of ZO-1 after the 24 h treatment with NPs further validates the findings of immunostaining results. This new method that we established will be a reference point for other NM studies which are used in our day-to-day consumer products.

INTRODUCTION

The wide applicability of nanoparticles in consumer products necessitates their extensive study related to their safety and potential risk. At the nanoscale, matter has fundamentally different properties from bulk materials. As material is scaled down to nanoparticles, chemical properties, biological properties, optical properties, and electrical properties are different from their bulk counterpart. Such unique properties could vastly alter the toxicity profile, requiring additional safety assessment considerations as compared to larger or bulk counterparts of the same materials. Although animal models are still used for risk assessment experiments, based on the 3R (refine, reduce, and replace) principle, the development of alternative testing methods is emphasizing in vitro model experiments. Aside from acting as an alternative evaluation tool, in vitro assays also play a key role in understanding the mechanism of the biological activities of nanoparticles. There are many challenges associated with in vitro methodologies to ensure they are as robust and reliable as traditional in vivo approaches. However, by overcoming such issues and adopting new testing strategies, we are able to improve safety assessments and reduce in vivo experiments. One of the challenges is the influence of physicochemical properties as well as physical properties of nanosuspension in the in vitro experiments. Poor characterization of nanoparticle suspension may lead to misinterpretation of nanotoxicity results. Therefore, proper characterization of the nanoparticle suspension is of the utmost importance. Studies have already been carried out regarding the major properties of nanoparticle suspension, such as absorptive properties, release of metal ions, and stability of suspension, but the microrheological property of the nanoparticle suspension in the cell culture media has never been explored.

Rheological properties such as viscoelasticity have been found to be an essential characteristic of living tissues, structural proteins, and the extracellular matrix (ECM). Viscoelastic materials exhibit a response between the two
extremes of purely elastic where all of the input deformation energy can be “stored” and “recovered” during each cycle without any loss and purely viscous where all of the input deformation energy is dissipated or “lost” by internal friction in the system as it flows. In response to the application of an external stress, viscoelastic materials undergo deformation and stress relaxation in a time-dependent manner in response to a step deformation. While traditional rheological studies are more based on concentrated proteins, only little research has been carried out on dilute protein samples, due to the lack of adequate experimental techniques. Over the past decade, optical microrheological techniques like DLS microrheology have gained huge popularity for rheological characterization of various complex fluids as well as dilute proteins. In comparison to conventional mechanical rheometry, DLS microrheology can measure much less sample volume and can measure over a much wider frequency range. Since DLS microrheology uses Brownian motion of tracer particles, less stress is applied on fluid, and this is of critical importance for many biological samples that can exhibit significant strain sensitivity. DLS microrheology provides mean square displacement (MSD), which is the distance covered by nanoparticles with respect to movement of the tracer particle with time. By using MSD, linear viscoelastic parameters for the complex fluid matrix are extracted through a generalized Stokes–Einstein relationship. Although different studies have been conducted on the microrheological properties of various proteins, complex fluids, and biological systems, microrheological studies explicitly on the nanoparticle suspensions in the cell culture medium have never been addressed. The stability of any suspension under study greatly depends on its rheological property. Toxicological investigations on nanoparticles require a comprehensive physicochemical characterization including the rheological properties of the nanoparticle suspension. Hence, the main aim of this study is to investigate the microrheological properties of two model nanoparticles of cerium dioxide (CNP) and dendrimers in cell culture media and to know their subsequent toxicological profiles.

The reasons for including cerium dioxide nanoparticles and dendrimers as our model nanoparticles is due to the fact that these represent two physicochemically different NPs. CNPs are metallic oxide nanoparticles, whereas dendrimers are organic nanoparticles. Apart from that, their vast applicability in the consumer products makes them nanoparticles of interest. CNPs are used commercially in numerous industries, such as petroleum refining (as a catalyst for cracking), coatings, polishing agents (for glass mirrors, plate glass, television tubes, ophthalmic lenses, precision optics, and electronic wafers), and fuel cells. They are extensively used in consumer products such as semiconductors, as diesel fuel additives, and as additives in cigarettes. It has been demonstrated that they can protect cell membranes from sources of oxidative stress (hydrogen peroxide, ultraviolet rays, and ionizing radiation), buffer reactive oxygen species, and thus decrease damage to cellular biomolecular structures, resulting from oxidative stress, making them promising antioxidant agents for treating oxidative stress-related diseases. Combined with their catalytic activity and electrochemical characteristics, their properties can be used to create highly sensitive, third-generation biosensors as well as quenchers in fluorescent biosensors. Dendrimers on the other hand show a wide range of medicinal and practical applications such as in photo-

**EXPERIMENTAL SECTION**

**Cell Culture.** Madin-Darby Canine Kidney (MDCK) cells (ATCC cat. no.: CCL-34) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) (PAN-Biotec GmbH, Germany), 1% penicillin/streptomycin (PAN-Biotec GmbH, Germany), and 1% l-glutamine (PAN-Biotec GmbH, Germany). Cells were passaged two times per week.

**Dynamic Light Scattering (DLS) and Zeta Potential.** The hydrodynamic diameter and the zeta potential were determined using a Zetasizer Nano ZS from Malvern (Malvern Panalytical, Worcestershire, England) was used.

**Data Collections, QSAR Model, and Statistical Analysis.** There are many versatile in silico methods, and quantitative structure–activity relationship (QSAR) models have been developed to generate consensus predictions for the various physicochemical properties including water solubility (log S) and lipophilicity (log P<sub>o/w</sub>). For data collection for the nanomaterials used in this study, we selected the third generation PAMAM dendrimer from the freely accessible web resource end point and from the PubChem database (accessed 10th October 2021). The cerium oxides were selected from the PubChem database (accessed 10th October 2021). The quasi-SMILES for this study was derived from the PubChem database, and their symbols are analogues of the simplified molecular input line entry system (SMILES). The quasi-SMILES required for QSAR modeling of NMs represents the available ecotoxic information with similar lines of symbols. We calculated the mean of log S, log P<sub>o/w</sub>, and other physicochemical properties such as distribution coefficient (log D) and topological polar surface area (TPSA), using the SILICOS-IT program based on topological and molecular descriptors, as explained in previous studies.

We further performed statistical analysis to determine if a difference between the different groups for each category of NMs (dendrimer and CNP) existed.

DLS microrheology is a passive technique in which the dispersed probe or tracer particles are tracked in a complex fluid in order to determine its local and bulk rheological properties. Similar to mechanical rheometry, a strain is applied to the system through Brownian motion of the probe particle, and the change in position of the probe particle is used to measure deformation. Such a microrheological technique permits access to very high-frequency or short-time dynamics of even very dilute samples with less sample volume.

Micro rheology is a new measurement type available to users of the Zetasizer Nano ZS and ZSP. It allows the measurement of the viscoelastic modulus of samples within the linear...
viscoelastic region. Microrheology measurements require a software key to access the software features and functionality.

The protocol for DLS optical microrheology was followed as per the instructions of the manufacturer, Malvern Panalytical (Worcestershire, England). Temperature was set to 25 °C and pH to 7.5 for the whole experiment. Polystyrene beads of 1.5 μm diameter (Polysciences, Inc., Warrington, United States) were used as tracer particles. According to the standard operating procedure of Malvern Panalytical for microrheology measurement, tracer compatibility with the sample and tracer concentration required were subsequently checked before measuring the microrheology. To check the tracer compatibility with NPs, the zeta potential of polystyrene beads was measured in 1 mL of continuous phase solution (here DMEM medium with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine). Then, a small volume of NPs was added, and zeta potential was remeasured. If the zeta potential values differ significantly, it implies the interaction between tracer particles and NPs, resulting in adsorption and aggregation between them. The zeta potential in the presence of both tracer and sample particles should be within the set limit of ±5 mV. After that, the tracer concentration was determined by following the instructions of the software. Initially 5 μL of tracer particles (polystyrene beads) is added to 1 mL of the complete DMEM media in a disposable cuvette, and then the scattering intensity is measured. After each reading, the software recommends how much of the tracer particle should be further added to get a relative scattering intensity of approximately 95% for tracer particles as compared to the NPs in the sample.

**Transepithelial Electrical Resistance (TEER).** The Millicell ERS-2 (electrical resistance system, STX2 electrode) (Merk, Darmstadt, Germany) was used to measure the transepithelial resistance (TEER) or barrier function of the Millicell ERS-2 (electrical resistance system, STX2 electrode) (Merk, Darmstadt, Germany) for 15 min at room temperature. They were then permeabilized with 0.5% triton X-100 (Merck, KGaA, Darmstadt, Germany) in TBS for 10 min and thereafter washed with 0.025% triton X-100 in TBS. Subsequently, a blocking step was performed with PBS containing 10% FCS. The cells were then treated with anti-ZO-1 antibody (Cat # A32728, Alexa Fluor 647, Rockford, USA), which was diluted 1:200 in TBS and then incubated for 45 min in the dark at room temperature. After that, the cells were washed with PBS three times and counterstained with Hoechst (1:1000 times diluted in TBS). Samples were then analyzed by a confocal laser scanning microscope (LSM 700, Zeiss). Microscopic images of the fixed samples were acquired using a 63× objective. The images were processed in Fiji.

**WST-Assay.** The water-soluble tetrazolium salt-1 (WST-1) assay is the cell viability assay for analyzing the number of viable cells by the cleavage of tetrAZolium salts added to the culture medium. The tetrazolium salts (slightly red) are cleaved to formazan (yellow) by cellular enzymes called mitochondrial dehydrogenase. The increase in the number of cells increases the overall activity of mitochondrial dehydrogenase in the sample, as a result of which the amount of formazan dye formed is also increased. Since the enzyme is produced by metabolically active cells, this assay thus quantifies the metabolically active viable cells. The WST-assay was performed according to the protocol of Sigma-Aldrich (Cat # S015944001 Roche, Basel, Switzerland) after treatment with different concentrations of NPs. According to the protocol provided by Invitrogen (Thermo Fisher Scientific, Waltham, Massachusetts, USA), a final concentration of 5 μM of CellROX reagent was prepared. An amount of 20 μL of CellROX deep red reagent (Cat # C10422, Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) after treatment with different concentrations of NPs. According to the protocol provided by Invitrogen (Thermo Fisher Scientific, Waltham, Massachusetts, USA), a final concentration of 5 μM of CellROX reagent was prepared. An amount of 20 μL of CellROX deep red reagent was mixed with 10 mL of complete DMEM medium without phenol red containing 10% WST reagent (Roche, Basel, Switzerland) was added into each well. Then the 96-well plate was incubated for half an hour in the incubator (37 °C, 5% CO₂), and the absorbance was measured with a plate reader (BioTek Instruments, Inc., Vermont, USA) at 450 and 630 nm (reference wavelength). Eight technical replicates were taken.

**ROS-Assay.** The level of intracellular reactive oxygen species (ROS) generation was determined using a CellROX deep red reagent (Cat # C10422, Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) after treatment with different concentrations of NPs. According to the protocol for ROS quantification in the freshly prepared medium without phenol red (PAN Biotech GmbH, Aidenbach, Germany). The old medium in a 96-well plate was removed, and the cells were washed three times with PBS. Then, 100 μL of the diluted CellROX deep red reagent was added to each well. Care was taken to avoid bubbles while pipetting, so negative pipetting was performed. The 96-well plate was kept in the incubator for half an hour at 37 °C, with 5% CO₂. The reactive oxygen species were measured by reading the fluorescence of the solutions kept in a 96-well plate using the plate reader (BioTek Instruments, Inc., Vermont,
USA) at 640 nm (excitation) and 665 nm (emission) wavelengths. Calculations were performed using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA).

**Figure 1.** Schematic showing the different steps of the characterization: (A) *in silico* analysis, (B) DLS microrheology-based viscoelasticity quantification, and (C) *in vitro* assays for the subsequent analysis of relevant toxicology parameters in detail.

**RT-qPCR.** The cells were seeded at the seeding density of 300 000 in 6-well plates. When the cells were confluent, they were treated with all three concentrations of CNP and...
dendrimers and incubated for 24 h. The cells were detached from wells using trypsin (500 μL), and 1 mL of DMEM was used to stop the reaction. The cell pellet was generated by centrifuging the cells containing 1.5 mL of Eppendorf for 6 min at 4 °C and 9000g (rcf). Then the RNA extraction was performed using the protocol as described by the manufacturer (Qiagen GmbH, Hilden, Germany). The concentration of RNA was measured using the NanoDrop spectrophotometer with the software NanoDrop 1000 3.7.1 (peQLab, Biotechnology GmbH, Erlangen, Germany). After that, cDNA was generated using the protocol of a reverse transcriptase (RT) kit (Applied Biosystem, Thermo Fischer Scientific, USA). There were two technical replicates, so for three different concentrations of each nanoparticle and two repeats of control, a master mix of 14 reactions was prepared (1 extra for having extra volume, so 15 total reactions). An amount of 500 ng of RNA mixed with 5 μL of double distilled (dd) water and 5 μL of the master mix was added in each flat cap strip (Nerbe plus GmbH and Co., Winsen, Germany). The strips were vortexed in a microcentrifuge (VWR-mini Star, Korea) shortly before starting the PCR. The cDNA was synthesized using PCR (PeQLab, Biotechnology GmbH). An amount of 40 μL of RNase-free water was added in cDNA and stored at −20 °C. Finally, for RT-qPCR sample preparation, a 96-well plate from the PCR cool block was taken. HPRT was used as a reference gene. A fast SYBR green master mix was made for each gene where the PCR cool block was taken. HPRT was used as a reference gene.

**RESULTS AND DISCUSSION**

**QSAR Analysis to Select the Optimum Physicochemical Parameters for the Target Formulations.** We connect the in silico methods, optical microrheology, and toxicological profiling of two classes of NMs demonstrated in Figure 1 by showing the different steps of the sequential workflow.

We used a very different class of nanomaterials to correlate the microrheological changes when they are mixed into the biological medium. Since we rely on viscoelastic measurement using dynamic light scattering (DLS) in aqueous media and a subsequent cytotoxicity investigation, it is useful to have the solubility and lipophilicity of the two types of materials in priori. Further optimizing the physicochemical parameters in silico additionally facilitates the ease of handling and the preparation formulation for the toxicological analysis, as recently machine learning advances boosted the toxicology analysis in vitro.55–57 After analyses, we compared the predicted values with optimum solubility and lipophilicity, as shown in Table 1 (log S as the first priority and then log P, TPSA, and log D). For the selection, we considered the optimum solubility value with moderate lipophilicity, TPSA, and log D to be suitable for the formulation of the microrheology and toxicological profiling. We present in Table 1 only eight representative compounds with minimum, moderate, optimal, and suboptimal log S values after analysis of the list of dendrimers and CNP quasi-SMILES. Once the best compounds for each NM were selected, which were Den S1 and CNP2 (Table 1), we looked at the Sigma-Aldrich chemical information database based on their CAS number or quasi-SMILE to purchase and began with experimental analysis for viscoelastic and toxicology measurements58 (accessed 5 November 2021).

**Nanoparticle Size Distribution and Zeta Potential.** The hydrodynamic size and aggregation behavior through zeta potential of CNP and dendrimers at different concentrations were characterized using DLS. With the increase in concentration, there is a gradual increase in the hydrodynamic size denoted by the Z-average (d/nm) as well as polydispersity index (PI) and a decrease in the diffusion coefficient for both CNP and dendrimers, as shown in Table 2. The diffusion coefficient is directly proportional to the concentration gradient between the particle along with its corona and the solvent. This concentration gradient is high in the case of low concentrated suspension, due to which a consequent decrease in PI, and CNP quasi-SMILES. For the selection, we considered the optimum solubility value with moderate lipophilicity, TPSA, and log D to be suitable for the formulation of the microrheology and toxicological profiling. We present in Table 1 only eight representative compounds with minimum, moderate, optimal, and suboptimal log S values after analysis of the list of dendrimers and CNP quasi-SMILES. Once the best compounds for each NM were selected, which were Den S1 and CNP2 (Table 1), we looked at the Sigma-Aldrich chemical information database based on their CAS number or quasi-SMILE to purchase and began with experimental analysis for viscoelastic and toxicology measurements58 (accessed 5 November 2021).

**Nanoparticle Size Distribution and Zeta Potential.** The hydrodynamic size and aggregation behavior through zeta potential of CNP and dendrimers at different concentrations were characterized using DLS. With the increase in concentration, there is a gradual increase in the hydrodynamic size denoted by the Z-average (d/nm) as well as polydispersity index (PI) and a decrease in the diffusion coefficient for both CNP and dendrimers, as shown in Table 2. The diffusion coefficient is directly proportional to the concentration gradient between the particle along with its corona and the solvent. This concentration gradient is high in the case of low concentrated suspension, due to which a consequent decrease in PI, and CNP quasi-SMILES. For the selection, we considered the optimum solubility value with moderate lipophilicity, TPSA, and log D to be suitable for the formulation of the microrheology and toxicological profiling. We present in Table 1 only eight representative compounds with minimum, moderate, optimal, and suboptimal log S values after analysis of the list of dendrimers and CNP quasi-SMILES. Once the best compounds for each NM were selected, which were Den S1 and CNP2 (Table 1), we looked at the Sigma-Aldrich chemical information database based on their CAS number or quasi-SMILE to purchase and began with experimental analysis for viscoelastic and toxicology measurements58 (accessed 5 November 2021).

| SN | sample name | Z-average (d/nm) | polydispersity index (PI) | diffusion coefficient (μm/s) | zeta potential (mV) |
|----|-------------|-----------------|--------------------------|-----------------------------|-------------------|
| 1  | CNP 10X     | 316.7           | 0.261                    | 1.35                        | 0.409             |
| 2  | CNP 100X    | 411             | 0.284                    | 1.04                        | −9.39             |
| 3  | CNP 1000X   | 451.1           | 0.475                    | 0.945                       | −0.521            |
| 4  | dendrimers 1X | 25.18          | 0.434                    | 28.1                        | −13.3             |
| 5  | dendrimers 5X | 34.37          | 0.503                    | 12.4                        | −3.82             |
| 6  | dendrimers 10X | 44.42         | 0.544                    | 9.6                         | −14.2             |

*For CNP 10X, 100X, and 1000X represent 10 μg/mL, 100 μg/mL, and 1000 μg/mL, respectively. For dendrimers, 1X, 5X, and 10X represent 1%, 5%, and 10% of the stock solution (10% weight in methanol) of dendrimers, respectively.

**Table 1. In Silico Determination of the Physicochemical Properties Like Solubility (log S), Lipophilicity (log P), Diffusion Coefficient (log D), and Topological Polar Surface Area (TPSA) of Different Dendrimers and CNPs**

| sample | log S (log mol/L) | log P (log mol/L) | log D (log L/kg) | TPSA |
|--------|-----------------|-----------------|-----------------|------|
| Den S1 | −0.11           | −4.67           | −4.097          | 167  |
| Den S2 | 0.394           | −8.763          | −6.578          | 576.8|
| Den S3 | −0.71           | −3.899          | −3.683          | 226.9|
| Den S4 | 4.242           | −20.673         | −14.521         | 1036.6|
| CNP1  | −4.783          | −0.3636         | −0.424          | 704.6|
| CNP2  | 0.688           | −0.206          | −0.36           | 172.1|
| CNP3  | −0.806          | −1.6494         | −0.247          | 316.2|
| CNP4  | 0.033           | −0.2401         | −0.254          | 79.8  |
| optimal range | −4−0.5 | 0−3 | 1−3 | 0−140 |

"Den S(1−4): dendrimer samples (1−4). CNP (1−4): cerium dioxide (1−4)."
Figure 2. Size distribution of different concentrations of cerium dioxide nanoparticles (A, B, C) and dendrimers (D, E, F) in DMEM media. A, B, and C represent 10 μg/mL, 100 μg/mL, and 1000 μg/mL concentrations of CNP, respectively, and D, E, and F represent 1%, 5%, and 10% (1x, 5x, and 10x) of the stock solution (10% weight in methanol) of dendrimers, respectively.

Figure 3. Mean square displacement (A, B) and viscoelastic moduli (C, D) of different concentrations of (A, C) cerium dioxide and (B, D) dendrimers in DMEM media. For CNP, 10x, 100x, and 1000x represent 10 μg/mL, 100 μg/mL, and 1000 μg/mL, respectively. For dendrimers 1x, 5x, and 10x represent 1%, 5%, and 10% of the stock solution (10% weight in methanol) of dendrimers, respectively.
in the diffusion coefficient is seen with the increase in concentration (Table 2). On the other hand, the increase in the concentration increases the PI, which indicates the polydispersity of the suspension and broadness of the size distribution. This trend is clearly seen in Figure 2, where 10× and 100× concentrations of CNP have well-defined narrow peaks, whereas 1000× concentration shows a broadness in peak along with an additional peak. This depicts the aggregation behavior of CNPs, which increases with increasing concentration. However, in the case of dendrimers, there is a shift of the Gaussian distribution peak to higher size with the increase in concentration, possibly due to the interaction with the organic particles in DMEM since dendrimers are also organic nanoparticles. Overall, the PI is well below 0.7, which is regarded as highly polydisperse, and since our PI values are around 0.5 or below, they meet the standards for DLS measurements. Apart from that the zeta potential values for CNP at different concentrations are recorded between 0 and −9.4, which show more aggregation and less stability. Comparatively, the dendrimers show zeta potential between −3 and −14.2 at three different concentrations, which is relatively stable compared to CNPs. The XRD data show the crystalline nature of cerium oxide, and a mixed redox population of cerium dioxide with high Ce³⁺ concentration was observed (Supporting Information Figure 1).

Microrheological Characterization. A wide range of microrheological studies have been carried out, such as high-throughput viscosity measurement of proteins,⁵⁹ viscoelasticity of other complex fluids,⁶⁰ and biological samples.⁶¹ However, the influence of the nanoparticle concentration on the microrheology of the cell culture media like DMEM has never been studied. In order to effectively use optical microrheological techniques, one of the main challenges is to determine which tracer particles to use to probe the rheological response. Since the hydrodynamic size of the CNP is in the range of 400 nm and that of dendrimers is in the range of 40 nm, a relatively larger tracer particle is required, which could dominate the light scattering of the NPs. Therefore, polystyrene beads with a diameter of 1.5 μm (Polysciences, Inc., Warrington, United States) were used to employing stress on the NPs. Before the measurement of the microrheology of NPs, the compatibility of the nanoparticles and the tracer particles was checked for each concentration by determining the zeta potential of the tracer particles in DMEM media with and without the NPs. It was observed that the zeta potential in the presence of both a tracer and NPs at different concentrations was within the set limit of 5 mV, which could confirm the compatibility of polystyrene beads and NPs used for the experiment. Apart from that, the concentration of polystyrene beads was used in such a way that their particle size distribution (PSD) accounted for at least 95% of the total area.

Microrheological properties like mean square displacement and viscoelasticity have important contributions to the aggregation properties.⁶² They measure the spatial extent covered by random motion. Insights into the motion of the nanoparticles can be quantitatively gained through the MSD, which can be obtained from the electric field autocorrelation function.⁶¹ Since the temperature (25 °C) as well as the pH (7.4) of DMEM media were kept constant, the NP suspension in DMEM acted as a nonviscous system, although DMEM has 10% fetal bovine serum which can act as a cocktail of proteins. The protein is not denatured at 25 °C and therefore does not have large length-scale structures. As depicted by Figure 3(A,B) and the Supporting Information Figure 2, both the MSD and correlation coefficient comply with each other. Figure 3 shows that the MSD is inversely proportional to the viscoelastic modulus, which is in agreement with the generalized Stokes–Einstein relation.⁶² It is evident from Figure 3 that for CNP an increase in concentration decreases the viscoelastic modulus, and the MSD is captured for a relatively shorter time window, which can be due to the aggregating behavior of CNPs with the increasing concentration. However, in the case of dendrimers, an increase in concentration tends to increase the viscoelasticity, and the MSD is recorded for a relatively longer time frame as compared to CNPs. Also, the MSDs for dendrimers run close to each other and even overlap with each other toward the end. This is because the dendrimers and DMEM media are both in the organic phase and increase in concentration, which increases the total viscoelasticity of the whole system. However, at the highest concentration (10×), the dendrimers show a sharp decline after reaching the peak. This could be due to the interaction of dendrimers with the other organic
molecules of the DMEM media. Nevertheless, in the case of CNPs the lowest concentration seems to contribute to the overall viscoelasticity due to the fact that there is less aggregation of 10× CNPs and that there is a relatively stable suspension. However, as the concentration increases, there is aggregation of the CNPs, and this results in the decrease in the overall viscoelasticity. In the case of 1000×, there is a sharp decline since the CNPs aggregate quickly at this concentration.

**Metabolic Activity Analysis.** The epithelial kidney cells MDCK were chosen for the experiment as the model cell lines as the kidney plays an important role in renal clearance, which makes it a favorable place for accumulation and toxicity of foreign bodies including nanoparticles. Due to easy cultivation, maintenance of the functional membrane transporters and cytochromes makes them the mostly used cell line for *in vitro* studies. The MDCK cells in the submersed culture were exposed to three different concentrations of both CNPs and dendrimers for 24 h, after which the influence on the metabolic activity (WST-1) and generation of ROS was investigated. As shown in Figure 4A, the highest concentration (1000×) of CNP shows the maximum viability as compared to the positive control (1% FBS) and other concentrations of CNPs and dendrimers. This could be because CNPs are known as nanoenzymes (catalysts), and they have an effect on cell proliferation. In a study carried out by Wang and his research group, the effect and mechanisms of CNP on osteoblasts, the cell proliferation, cellular uptake, endocytosis mechanism, cell cycle, and cell adhesion forces were analyzed. The results showed that CNP promoted the proliferation of a primary osteoblast as well as increased the cell adhesion force, whereas another study carried out by Zhang et al. suggests that the concentration of Ce⁶⁺ and the time of culture had an effect on the proliferation, differentiation, adipocyte transdifferentiation, and mineralization function of primary osteoblasts. Apart from that, according to the studies done by Dai and his co-workers, the aggregation potential increases with increasing concentration and changing variables. This could act as a factor influencing the availability of CNPs to the cells in submerged culture with the increase in concentration. However, our results contradict the study conducted by Sauer et al. in which they reported the cytotoxic effect of the CNP on the rat lung cells at the concentration of 1000 μg/mL and no toxicity below this concentration. Since the CNP showed the absorbance at the wavelength of the WST-1 measurement (Supporting Information Figure 3), the cells exposed to CNPs were washed twice before the exposure of the WST-1 reagent, but the complete removal of the CNP was not possible for 100 μg/mL and 1000 μg/mL in order to prevent the washaway of cells. Some part of the absorbance for 100 and 1000 μg/mL may be contributed by the leftover CNP. In the case of dendrimers, as depicted in Figure 4A, the percentage viability or metabolic activity decrease with the increase in concentration. Poly(amido amine) (PAMAM) dendrimers are characterized by concentration- and generation-dependent toxicity. Molecular interactions between negatively charged cell membranes and positively charged dendrimers explain the cytotoxicity of cationic dendrimers. As a result of such interactions, nanopores are formed in the cell membrane, which cause damage, leakage of cellular content, and subsequently cell death. However, studies show that the dendrimers terminated with neutral or anionic groups seem to be much less toxic than cation dendrimers. A new insight was shed by a study carried out by Mukherjee et al. in which they disclosed an indirect impact of PAMAM dendrimers on cell viability. They suggested that the indirect mechanism of generation-dependent PAMAM cytotoxicity results from the depletion of medium components due to the absorption of proteins from media by dendrimers. This could also explain the decrease in cell viability with an increase in dendrimer concentration, as shown in Figure 4A. Our dendrimer results comply with the study conducted by Malik et al. in which carboxylate PAMAM dendrimers showed a hemolytic effect after 24 h. Although a significant decrease in cell viability should have corresponded to severe changes in cellular processes, such as massive ROS generation or vice versa, the WST-1 result does not exactly correspond to the ROS results for both CNP and dendrimers. Nevertheless, as compared to the control, the reactive oxygen species (ROS) is shown to be high in all the treated samples (Figure 4B). Correlating viscoelasticity with the metabolic changes of the cell, we see a clear trend for both categories of NMs with subsequent changes in metabolic activity. 

**Characterization of Barrier Function with Trans-epithelial Resistance (TEER) Measurement and Tight Junction Staining and ZO-1 Gene Expression through q-PCR.** Unlike endothelial cells, epithelial cells like MDCK cells are able to produce tight junction proteins and form a monolayer. The barrier function of the *in vitro* MDCK cell monolayer was characterized using TEER measurements and immunostaining analysis of the tight junction protein zonula occludens-1 (ZO-1). An electrical resistance measurement across a cellular monolayer using TEER ensures the integrity and permeability of a monolayer. For this, MDCK cells were cultured in the 12-well hanging inserts in the submerged culture, which was exposed to various concentrations of CNPs and dendrimers, and the subsequent effect on barrier function was measured using TEER after the first, third, and seventh days of exposure. A total of two biological replicate and three technical replicates were considered during the measurement. As shown in the figure, the maximum TEER measurements were recorded at the third day after the exposure of the MDCK cells with various CNP and dendrimer concentrations. According to Lars et al. the resistance values as well as time required to achieve the high TEER value differ from cell line to cell line along with the nutrient content and the cell culture conditions. The TEER values show the maximum at the third day for each sample, including in the control without treatment with NPs. In the case of CNPs, the resistance shows a dose-dependent increase which could be due to the aggregating nature of CNPs. Apart from that, CNPs also show the cell proliferating effect which may have caused more proliferation and more tight junctions with the increase in the concentration. In the case of the sample treated with a 10× dendrimer, a low resistance of 40.52 Ω·cm² is measured, rendering the high permeability after 24 h exposure. On the
Third day, the average TEER measurement was observed to be 129.43 Ω* cm⁻². Since the measurements are taken at only three different days, the exact days and exact highest TEER values were not recorded, but the trend is compliant with the findings of Cho et al., which stated the highest TEER value for the MDCK cell line to be at the fifth day. The literature describes two different strains of MDCK: strain I generates an epithelium with transepithelial resistance (TEER) above 1000 Ω* cm⁻², and strain II generates an epithelium with TEER of 100 Ω* cm⁻² or less. Since all the values of TEER generated were in the range of around 100 Ω* cm⁻², the strain of MDCK we have might be strain II. For TEER measurements performed manually with a Millicell ERS-2, the electrode position has a significant impact on the resistance value. One explanation for the large standard deviation obtained in our experiments could be this factor. Such problems can be mitigated by the use of integrated electrodes in organ on chip systems for the TEER measurements. Incorporating immobilized TEER electrodes directly within the chip model and in close proximity to the cellular monolayer will not only reduce the contribution of electrical resistance from the cell culture medium but also reduce any motion-related signal noise.

ZO-1 proteins are well expressed by MDCK cell lines. The molecular characterization of ZO-1 proteins as well as their function in the assembly of a tight junction in the MDCK cell line are well studied. The immunostaining of the tight junction protein was performed after 24 h of exposure of the MDCK cells with the different concentrations of CNP and dendrimers. As per the TEER values, the cells exposed with 10X dendrimers showed the least TEER measurement after 24 h. This is in agreement with the ZO-1 staining which shows the distorted ZO-1 formation with the cells in the patch rather than the confluent (Figure 6) and also the reduced confocal microscopy signal intensities for both ZO-1 staining and DAPI staining as shown in Figure 5B. This shows the cytotoxic nature and loss of viability caused by the dendrimers at higher concentrations. The measured ZO-1 mRNA by using the qPCR method as shown in Figure 5C further validates the immunostaining results. As seen in Figure 5C, the exposure of MDCK cells with various concentrations of CNP and dendrimers tends to influence the expression of ZO-1 genes differently (Figure 7). 10X CNPs and all three concentrations of dendrimers show a slightly more than 1-fold and 100× CNP by a 2.7-fold increase of ZO-1 expression, whereas 1000× CNP exposure tends to downregulate the ZO-1 expression. The overexpression of the ZO-1 gene as compared to control may be explained as a defense mechanism of the cells to the external stimuli.

In our previous study, we established a machine-learning-based graph modeling and correlation approach using a tight junction protein ZO-1-mediated alteration in the cell phenotype to quantify and propose it as indices of cell–NM interactions. We found that the phenotypic variation such as cell shape and nucleus area in the epithelial cell is determined by the physicochemical properties (e.g., shape, size, zeta potential, concentration, diffusion coefficients, polydispersity, and so on) of the different classes of nanomaterials, which critically regulate the intracellular uptake or cell membrane interactions when exposed to the epithelial cells at sublethal concentrations. By analyzing the intrinsic and extrinsic properties of the representative nanomaterials (NMs) using optical (dynamic light scattering, NP tracking analysis) methods, a set of nanodescriptors related to cell–NM interactions via phenotype adjustments were created. In relation with toxicology, we established a machine-learning algorithm fitting correlation function, which was used to successfully predict cell and nuclei shapes and polarity functions as phenotypic markers for five different classes of organic and inorganic nanoparticles.
controls which are not exposed to any NPs. The concentrations 1, 5, and 10 μg/mL of dendrimers, respectively.

The second row represents ZO-1 staining, and the third row represents DAPI staining. The first column represents the controls which are not exposed to any NPs. The concentrations 1x, 5x, and 10x represent 1%, 5%, and 10% of the stock solution (10% weight in methanol) of dendrimers, respectively.

Figure 6. Confocal images of cells after 24 h exposure with different concentrations of dendrimers. The cells were immunostained with the ZO-1 antibody conjugated with Alexa fluor 647 and finally stained with DAPI. The first row depicts the overlay of both ZO-1 staining and DAPI staining. The second row represents ZO-1 staining, and the third row represents DAPI staining. The first column represents the controls which are not exposed to any NPs. The concentrations 1x, 5x, and 10x represent 1%, 5%, and 10% of the stock solution (10% weight in methanol) of dendrimers, respectively.

Figure 7. Confocal images of cells after 24 h exposure with different concentrations of CNPs. The cells were immunostained with the ZO-1 antibody conjugated with Alexa fluor 647 and finally stained with DAPI. The first row depicts the overlay of both ZO-1 staining and DAPI staining. The second row represents ZO-1 staining, and the third row represents DAPI staining. The first column represents the control sample which is not exposed to any NPs. The concentrations 10x, 100x, and 1000x represent 10 μg/mL, 100 μg/mL, and 1000 μg/mL of CNPs, respectively.

CONCLUSION

By taking the growing applicability of NPs in consumer products into consideration, in this study we established a new method based on DLS micro rheology to analyze the MSD and viscoelasticity of two model NPs that are dendrimers and CNPs at three different concentrations. MSD results showed that they were inversely proportional to the viscoelastic modulus which infers that our results are in agreement with the generalized Stokes–Einstein relation. The decrease in viscoelastic modulus with the increase in concentration suggests the aggregating potential of CNP, whereas the increasing viscoelasticity with the increasing concentration of dendrimers reflects on the organic nature of the dendrimers. Further investigation into the toxicological profile showed that dendrimers show cytotoxic effects with the increasing concentrations and are in agreement with TEER, ZO-1 immunostaining, and ZO-1 expression experiments. However, in the case of CNPs due to its high aggregating nature, WST and TEER results at higher concentrations seem to interfere, and further investigation into this matter is required. The inverse correlation in viscoelasticity and the metabolic nature of cerium dioxide versus dendrimers might arise due to their different category of material property (inorganic vs organic). This study emphasizes the importance of micro rheology for the NP characterization and its influence on the toxicological profile. Moreover, our approach is based on the difference in the concentration of two different model nanoparticles. The findings in this study can support further micro rheological studies by changing several parameters like temperature, pH, different biological media, and more. Further research in this field can help in the development of in vivo methods for the safety and risk assessment of nanoparticles and additionally narrow the gap between in vivo and in vitro models.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00472.

List of additional Supporting Information with the XRD data, autocorrelation function of different concentrations of NMs in DMEM media, and absorbance of the different concentrations of CNPs. Dendrimers at the same wavelength of the WST-1 assay are supplied (PDF)

AUTHOR INFORMATION

Corresponding Author

Romi Singh Maharjan — German Federal Institute for Risk Assessment (BfR), Department of Chemical and Product Safety, 10589 Berlin, Germany; Email: ajay-vikram.singh@bf.r.bund.de

Authors

Ajay Vikram Singh — German Federal Institute for Risk Assessment (BfR), Department of Chemical and Product Safety, 10589 Berlin, Germany; orcid.org/0000-0002-9875-7727

Javaria Hanif — University of Potsdam, Department of Food Chemistry, 14476 Potsdam, Germany

Daniel Rosenkranz — Klinikum Oldenburg, University Medical Center Oldenburg, Institute for Clinic Chemistry and Laboratory Medicine, 26133 Oldenburg, Germany

Rashad Haidar — German Federal Institute for Risk Assessment (BfR), Department of Chemical and Product Safety, 10589 Berlin, Germany

Amruta Shelar — Department of Technology, Savitribai Phule Pune University, Pune 411007 MH, India

Shubham Pratap Singh — Faculty of Informatics, Otto von Guericke University, Magdeburg 39106, Germany

Aditya Dey — Faculty of Informatics, Otto von Guericke University, Magdeburg 39106, Germany

https://doi.org/10.1021/acsomega.2c00472

ACS Omega 2022, 7, 13985–13997
Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c00472

Author Contributions
Conceptualization, A.V.S. and R.S.M.; data curation, H.J.; R.S.M., A.S., R.P., S.P.S., R.H.; and A.V.S.; writing—original draft preparation, R.S.M. and A.V.S.; writing—review and editing, R.S.M., P.Z., A.V.S., P.L., and A.L.; graphic design and visualization, R.S.M., A.S., D.R., A.D., and S.P.S.; supervision, R.S.M., P.Z., A.V.S., P.L., and A.L.; funding acquisition, A.L. All authors have read and agreed to the published version of the manuscript.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
This work was supported by BfR SFP 1322-725 and BfR SFP 1322-735 (A.V.S.).

REFERENCES
(1) Singh, A. V.; Laux, P.; Luch, A.; Sudrik, C.; Wiehr, S.; Wild, A. M.; Santamouaro, G.; Bill, J.; Sitti, M. Review of emerging concepts in nanotoxicology: Opportunities and challenges for safer nanomaterial design. Toxicol mech and methods. 2019, 29 (5), 378–387.
(2) Hade, F., An introduction to nanomaterials. In Environ. Nanotechnol.; Springer: 2018; pp 1–58.
(3) Josue Varghese, R.; Sakho, E. H. M.; Parani, S.; Thomas, S.; Olufemfam, O. S.; Wu, J. Chapter 3 - Introduction to nanomaterials: synthesis and applications. In Nanomat. j. Solar Cell Appl.; Thomas, S., Sakho, E. H. M., Kalarikkal, N., Olufemfam, O. S., Wu, J., Eds.; Elsevier: 2019; pp 75–95.
(4) Singh, A. V.; Romeo, A.; Scott, K.; Wagener, S.; Leibrock, L.; Laux, P.; Luch, A.; Kerkar, P.; Balkrishnan, S.; Dakua, S. P.; Park, B.-W. Emerging Technologies for In Vitro Inhalation Toxicology. Adv. Healthc Mater. 2021, 10 (18), 2100633.
(5) Singh, A. V.; Maharjan, R. S.; Kromer, C.; Laux, P.; Luch, A.; Vats, T.; Chandrasekar, V.; Dakua, S. P.; Park, B.-W. Advances in Smoking Related In Vitro Inhalation Toxicology: A Perspective Case of Challenges and Opportunities from Progresses in Lung-on-Chip Technologies. Chem. Res. Toxicol. 2021, 34 (9), 1984–2002.
(6) Hassan, S.; Singh, A. V. Biophysicochemical perspective of nanoparticle compatibility: a critically ignored parameter in nanomedicine. J. Nanosci Nanotechnol. 2014, 14 (1), 402–14.
(7) Horie, M.; Kato, H.; Iwashashi, H. Cellular effects of manufactured nanoparticles: effect of adsorption ability of nanoparticles. Arch toxicol 2013, 87 (5), 771–781.
(8) Horie, M.; Kato, H.; Fujita, K.; Endoh, S.; Iwashashi, H. In Vitro Evaluation of Cellular Response Induced by Manufactured Nanoparticles. Chem. Res. Toxicol. 2012, 25 (3), 605–619.
(9) Shelar, A.; Singh, A. V.; Maharjan, R. S.; Laux, P.; Luch, A.; Gemmati, D.; Tisato, V.; Singh, S. P.; Santilli, M. F.; Shelar, A.; Chaskar, M.; Patil, R. Sustainable Agriculture through Multi-disciplinary Seed Nanopriming: Prospects of Opportunities and Challenges. Cells 2021, 10 (9), 2428.
(10) Wörle-Knirsch, J. M.; Pulskamp, K.; Krug, H. F.Oops They Did It Again! Carbon Nanotubes Hao Scientists in Viability Assays. Nano Lett. 2006, 6 (6), 1261–1268.
(11) Horie, M.; Nishio, K.; Fujita, K.; Endoh, S.; Miyauchi, A.; Saito, Y.; Iwashashi, H.; Yamamoto, K.; Murayama, H.; Nakano, H.; Nanashima, N.; Niki, E.; Yoshida, Y. Protein Adsorption of Ultrafine Metal Oxide and Its Influence on Cytotoxicity toward Cultured Cells. Chem. Res. Toxicol. 2009, 22 (3), 543–553.
(12) Cerdavelli, T.; Lynch, I.; Lindman, S.; Berggaard, T.; Thulin, E.; Nilsson, H.; Dawson, K. A.; Linse, S. Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. Proc. Natl. Acad. Sci. U. S. A. 2007, 104 (7), 2050–2055.
(13) Horie, M.; Nishio, K.; Endoh, S.; Kato, H.; Fujita, K.; Miyauchi, A.; Nakamura, A.; Kinugasa, S.; Yamamoto, K.; Niki, E.; et al. Chromium (III) oxide nanoparticles induced remarkable oxidative stress and apoptosis on culture cells. Environ. toxicol. 2013, 28 (2), 61–75.
(14) Teeguarden, J. G.; Hindelratter, P. M.; Orr, G.; Thrall, B. D.; Pounds, J. G. Particokinetics In Vitro: Dosimetry Considerations for In Vitro Nanoparticle Toxicity Assessments. Toxicol. Sci. 2007, 95 (2), 300–312.
(15) Kato, H.; Fujita, K.; Horie, M.; Suzuki, M.; Nakamura, A.; Endoh, S.; Yoshida, Y.; Iwashashi, H.; Takahashi, K.; Kinugasa, S. Dispersion characteristics of various metal oxide secondary nanoparticles in culture medium for in vitro toxicology assessment. Toxicol in Vitro. 2010, 24 (3), 1009–1018.
(16) Joshi, K. M.; Shelar, A.; Kasabe, U.; Nikam, L. K.; Pawar, R. A.; Sanghahiti, J.; Kale, B. B.; Singh, A. V.; Patil, R.; Chaskar, M. G. Biofilm inhibition in Candida albicans with biogenic hierarchical zinc-oxide nanoparticles. Mater. Sci. Eng. C 2021, 112592.
(17) Singh, A. V.; Kishore, V.; Santamouaro, G.; Yasa, O.; Bill, J.; Sitti, M. Mechanical Coupling of Puller and Pusher Active Microswimmers Influences Motility. Langmuir. 2020, 36 (19), 5435–5443.
(18) Sanjeevi, R. A viscoelastic model for the mechanical properties of biological materials. J. Biomech. 1982, 15 (2), 107–109.
(19) Chaudhuri, O.; Cooper-White, J.; Janmey, P. A.; Mooney, D. J.; Shenoy, V. B. Effects of extracellular matrix viscoelasticity on cellular behaviour. Nature. 2020, 584 (7822), 535–546.
(20) Inoue, H.; Matsumoto, T. Viscoelastic characterization of solid-like structure in aqueous colloids of globular proteins. Colloids Surf, A Physicochem. Eng. Asp. 1996, 109, 89–96.
(21) Lefebvre, D.; Renard, D.; Sánchez Gimeno, A. Structure and rheology of heat-set gels of globular proteins. 1. Bovine serum albumin gels in isoelectric conditions. Rheol. Acta 1998, 37, 345–357.
(22) Waigh, T. A. Advances in the micro rheology of complex fluids. Rep. Prog. Phys. 2016, 79 (7), 074601.
(23) Weils, D.; Mason, T. G.; Teitell, M. A. Bio-micro rheology: a frontier in micro rheology. Biophys. J. 2006, 91 (1), 4296–305.
(24) Amin, S.; Rega, C. A.; Jankevis, H. Detection of viscoelasticity in aggregating dilute protein solutions through dynamic light scattering-based optical micro rheology. Rheologica acta 2012, 51 (4), 329–342.
(25) Amin, S.; Blake, S.; Kennel, R. C.; Lewis, E. N. Revealing New Structural Insights from Surfactant Micelles through DLS, Micro rheology and Raman Spectroscopy. Mater. 2015, 8 (6), 3754–3766.
(26) He, F.; Becker, G. W.; Litowski, J. R.; Narhi, L. O.; Brems, D. N.; Razinkov, V. I. High-throughput dynamic light scattering method for measuring viscosity of concentrated protein solutions. Anal biochem 2010, 399 (1), 141–143.
(27) Mason, T. G.; Weitz, D. A. Optical Measurements of Frequency-Dependent Linear Viscoelastic Moduli of Complex Fluids. Phys. Rev. Lett. 1995, 74 (7), 1250–1253.
(28) Larsen, T. H.; Furst, E. M. Micro rheology of the Liquid-Solid Transition during Gelation. Phys. Rev. Lett. 2008, 100 (14), 146001.
(29) Jung, H.; Kittelson, D. B.; Zachariah, M. R. The influence of a cerium additive on ultrafine diesel particle emissions and kinetics of oxidation. Combust. flame. 2005, 142 (3), 276–288.
(31) Ivanov, V. K.; Shcherbakov, A. B.; Usatenko, A. Structure-sensitive properties and biomedical applications of nanodispersed cerium dioxide. Russ. Chem. Rev. 2009, 78 (9), 855.
(32) Reed, K.; Cormack, A.; Kulkarni, A.; Mayton, M.; Sayle, D.; Klaessig, F.; Studler, B. Exploring the properties and applications of nanoceria: is there still plenty of room at the bottom? Env. Sci. Nano. 2014, 1 (5), 390–405.
(33) Chockalingam, R.; Amarakoon, V. R.; Giesche, H. Alumina/ cerium oxide nano-composite electrolyte for solid oxide fuel cell applications. J. Eur. Ceram. Soc. 2008, 28 (5), 959–963.
(34) Kusmierek, E. A CeO2 semiconductor as a photocatalytic and photoelectrocatalytic material for the remediation of pollutants in industrial wastewater: A review. Catal. 2020, 10 (12), 1435.
(35) Meij, D.; Li, X.; Wu, Q.; Sun, P. Role of cerium oxide nanoparticles as diesel additives in combustion efficiency improvements and emission reduction. J. Energy Eng. 2016, 142 (4), 04015050.
(36) Nijag, J.; Ispas, C.; Andreescu, S. Mixed ceria-based metal oxides biosensor for operation in oxygen restrictive environments. Anal. Chem. 2008, 80 (19), 7626–7627.
(37) Liu, Y.; Huang, J.; Claypool, J. B.; Castano, C. E.; O’Keefe, M. J. Structure and corrosion behavior of sputter deposited cerium oxide based coatings with various thickness on Al 2024-T3 alloy substrates. Appl. Surf. Sci. 2015, 355, 805–813.
(38) Pandey, P. K.; Maheshwari, R.; Raval, N.; Gondaliya, P.; Kalia, K.; Tekade, R. K. Nanogold-core functionalized dendrimer for pulsatile chemo-, photothermal-and photodynamic-therapy of rheumatoid arthritis. J. Colloid Interface Sci. 2019, 544, 61–77.
(39) McMahon, M. T.; Bulte, J. W. Two decades of dendrimers as versatile MRI agents: a tale with and without metals. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 2016, 8 (3), e1406.
(40) Gorain, B.; Tekade, M.; Kesharwani, P.; Iyer, A. K.; Kalia, K.; Tekade, R. K. The use of nanoscaffolds and dendrimers in tissue engineering. Drug Discov Today. 2017, 22 (4), 652–664.
(41) Hu, J.; Hu, K.; Cheng, Y. Tailoring the dendrimer core for efficient gene delivery. Acta Biomater. 2016, 35, 1–11.
(42) Mullen, D. G.; McNerny, D. Q.; Desai, A.; Cheng, X.-m.; DiMaggio, S. C.; Kotlyar, A.; Zhong, Y.; Qin, S.; Kelly, C. V.; Thomas, T. P.; et al. Design, synthesis, and biological functionality of a dendrimer-based modular drug delivery platform. Bioconjug Chem. 2011, 22 (4), 679–689.
(43) Pires, D. E. V.; Blundell, T. L.; Ascher, D. B. pkCSM: Predicting Small-Molecule Pharmacokinetic and Toxicity Properties Using Graph-Based Signatures. J. Med. Chem. 2015, 58 (9), 4066–4072.
(44) Daina, A.; Michielin, O.; Zoete, V. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Sci. Rep. 2017, 7 (1), 42717.
(45) Kaminskas, L. M.; Pires, D. E. V.; Ascher, D. B. dendPoint: a web resource for dendrimer pharmacokinetics investigation and prediction. Sci. Rep. 2019, 9 (1), 15465.
(46) Singh, C.; Friedrichs, S.; Ceccone, G.; Gibson, N.; Jensen, K.; Levin, M.; Infante, H.; Carlander, D.; Rasmussen, K. Cerium Dioxide, NM-211, NM-212, NM-213. Characterisation and test item preparation. JRC Sci. Policy Rep. Eur. Comm. 2014.
(47) Kim, S.; Thiessen, P. A.; Bolton, E. E.; Chen, J.; Fu, G.; Gindulyte, A.; Han, L.; He, J.; He, S.; Shoemaker, B. A.; Wang, J.; Yu, B.; Zhang, J.; Bryant, S. H. PubChem Substance and Compound databases. Nucleic Acids Res. 2016, 44, D1202–D1213.
(48) Cheng, F.; Li, W.; Zhou, Y.; Shen, J.; Wu, Z.; Liu, G.; Lee, P. W.; Tang, Y. admetSAR: A Comprehensive Source and Free Tool for Assessment of Chemical ADMET Properties. J. Chem. Inf Model. 2012, 52 (11), 3099–3105.
(49) Kim, M.; Li, L. Y.; Grace, J. R. Predictability of physicochemical properties of polychlorinated dibenzo-p-dioxins (PCDDs) based on single-molecular descriptor models. Environ. Pollut. 2016, 213, 99–111.
(50) Daina, A.; Michielin, O.; Zoete, V. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Sci. Rep. 2017, 7, 42717–42717.
(51) Cai, P. C.; Krajina, B. A.; Kratochvil, M.; J.; Zou, L.; Zhu, A.; Burgener, E. B.; Bolyky, P. L.; Milla, C. E.; Webber, M. J.; Spakowitz, A. J.; et al. Dynamic light scattering microcrystallere for soft and living materials. Soft Matter 2021, 17 (7), 1929–1939.
(52) Singh, A. V.; Maharan, R.-s.; Kanase, A.; Siewert, K.; Rosenkranz, D.; Singh, R.; Laux, P.; Luch, A. Machine-Learning-Based Approach to Decode the Influence of Nanomaterial Properties on Their Interaction with Cells. ACS Appl. Mater. Interfaces. 2021, 13 (1), 1943–1955.
(53) Singh, A. V.; Rosenkranz, D.; Ansari, M. H. D.; Singh, R.; Kanase, A.; Singh, S. P.; Johnston, B.; Tentschner, J.; Laux, P.; Luch, A. Artificial Intelligence and Machine Learning Empower Advanced Biomedical Material Design to Toxicity Prediction. Adv. Intell Sys. 2020, 2 (12), 2000084.
(54) Singh, A. V.; Ansari, M. H. D.; Rosenkranz, D.; Maharan, R. S.; Kriegel, F. L.; Gandhi, K.; Kanase, A.; Singh, R.; Laux, P.; Luch, A. Artificial Intelligence and Machine Learning in Computational Nanotoxicology: Unlocking and Empowering Nanomedicine. Adv. Healthc Mater. 2020, 9 (17), 1901862.
(55) Yoon, M.; Campbell, J.; Andersen, M.; Clewell, H. Quantitative in vitro to in vivo extrapolation of cell-based toxicity assay results. Crit. Rev. Toxicol. 2012, 42, 633.
(56) Breedveld, V.; Pine, D. J. Micro rheology as a tool for high-throughput screening. J. Mater. Sci. 2003, 38 (22), 4461–4470.
(57) Schultz, K. M.; Baldwin, A. D.; Kück, K. L.; Furst, E. M. Rapid rheological screening to identify conditions of biomaterial hydrogelation. Soft Mater. 2009, 5 (4), 740–742.
(58) Amin, S.; Rega, C.; Jankevics, H. Detection of Viscoelasticity in Aggregating Dilute Protein Solutions through Dynamic Light Scattering Based Optical Microcrystallere. Rheol. Acta 2012, 51, 329–342.
(59) Banchio, A. J.; Nägele, G.; Bergenholtz, J. Viscoelasticity and generalized Stokes-Einstein relations of colloidal dispersions. J. Chem. Phys. 1999, 111 (18), 8721–8740.
(60) Mukherjee, S. P.; Davoren, M.; Byrne, H. J. In vitro mammalian cytotoxicological study of PAMAM dendrimers-towards quantitative structure activity relationships. Toxicol in Vitro. 2010, 24 (1), 169–177.
(61) Vrbova, M.; Dastychova, E.; Rousar, T. Renal cell lines for study of nephrotoxicity in vitro. Mil. Med. Sci. Lett. 2016, 85 (2), 69–74.
(62) Wang, F.; Wang, E.; Han, J.; Li, Y.; Jin, Y.; Lv, F.; Ren, C.; Liu, H.; Zhou, G. Cerium oxide nanoparticles promote proliferation of primary osteoblasts via cell cycle machinery in vitro. J. Nanopart. Res. 2021, DOI: 10.1007/s11051-020-05115-y.
(63) Zhang, J.; Cuiilan, L.; Yaping, L.; Jing, S.; Peng, W.; Keqian, D.; Yanyan, Z. Effect of cerium ion on the proliferation, differentiation and mineralization function of primary mouse osteoblasts in vitro. J. Rare Earths. 2010, 28 (1), 138–142.
(64) Dai, H.; Sun, T.; Han, T.; Li, X.; Guo, Z.; Wang, X.; Chen, Y. Interactions between cerium dioxide nanoparticles and humic acid: Influence of light intensities and molecular weight fractions. Environ. Res. 2021, 195, 110861–2021.
(68) Sauer, U. G.; Vogel, S.; Aumann, A.; Hess, A.; Kolle, S. N.; Ma-Hock, L.; Wohilleben, W.; Dammann, M.; Strauss, V.; Treumann, S.; Grüters, S.; Wiench, K.; van Ravenzwaay, B.; Landsiedel, R. Applicability of rat precision-cut lung slices in evaluating nanomaterial cytotoxicity, apoptosis, oxidative stress, and inflammation. *Toxicol. Appl. Pharmacol.* **2014**, *276* (1), 1–20.

(69) Jevprasesphant, R.; Penny, J.; Jalal, R.; Atwood, D.; McKeown, N.; D’emanuele, A. The influence of surface modification on the cytotoxicity of PAMAM dendrimers. *Int. J. Pharm.* **2003**, *252* (1–2), 263–266.

(70) González-Mariscal, L.; Islas, S.; Contreras, R. G.; García-Villegas, M. R.; Betanzos, A.; Vega, J.; Díaz-Quióno, A.; Martín-Orozco, N.; Ortiz-Navarrete, V.; Ceréjido, M.; Valdés, J. Molecular Characterization of the Tight Junction Protein ZO-1 in MDCK Cells. *Exp. Cell Res.* **1999**, *248* (1), 97–109.

(71) Janszewska, A.; Lazzniewska, J.; Trzepiński, P.; Marcinkowska, M.; Klajnert-Maculewicz, B. Cytotoxicity of Dendrimers. *Biomolecules* **2019**, *9* (8), 330.

(72) Ciołkowski, M.; Petersen, J. F.; Ficker, M.; Janszewska, A.; Christensen, J. B.; Klajnert, B.; Bryszewska, M. Surface modification of PAMAM dendrimer improves its biocompatibility. *Nanomedicine* **2012**, *8* (6), 815–817.

(73) Malik, N.; Wiwattanapatree, R.; Klopsch, R.; Lorenz, K.; Frey, H.; Weener, J.; Meijer, E.; Paulus, W.; Duncan, R. Dendrimers:: Relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of 125I-labelled polyamidoamine dendrimers in vivo. *J. Controlled Release* **2000**, *65* (1–2), 133–148.

(74) Amin, R.; Artmann, T. A.; Artmann, G.; Lazarovici, P.; Lelkes, P. I. In Permeability of an in vitro model of blood brain barrier (BBB), 13th International Conference on Biomedical Engineering; Springer: 2009; pp 81–84.

(75) Leibrock, L.; Wagener, S.; Singh, A. V.; Laux, P.; Luch, A. Nanoparticle induced barrier function assessment at liquid-liquid and air-liquid interface in novel human lung epithelia cell lines. *Toxicol. Res.* **2019**, *8* (6), 1016–1027.

(76) Gagnon, C.; Bruneau, A.; Turcotte, P.; Pilote, M.; Gagné, F. Fate of Cerium Oxide Nanoparticles in Natural Waters and Immunotoxicity in Exposed Rainbow Trout. *J. Nanomed. Nanotechnol.* **2018**, DOI: 10.4172/2157-7439.1000489.

(77) Vikram Singh, A.; Sigloch, H.; Laux, P.; Luch, A.; Wagener, S.; Tentschert, J. Peter Laux, Andreas Luch, Sandra Wagener, Jutta Tentschert., Micro/nanoplastics: an emerging environmental concern for the future decade. *Frontiers Nanosci. Nanotechnol.* **2021**, *7*, 1–2.

(78) Cho, M. J.; Thompson, D. P.; Cramer, C. T.; Vidmar, T. J.; Scieszka, J. F. The Madin Darby Canine Kidney (MDCK) Epithelial Cell Monolayer as a Model Cellular Transport Barrier. *Pharm. Res.* **1989**, *6* (1), 71–77.

(79) Richardson, J. C. W.; Scaler, V.; Simmons, N. L. Identification of two strains of MDCK cells which resemble separate nephron tubule segments. *Biochim. Biophys. Acta* **1981**, *673*, 26–36.

(80) Simmons, N. L. Ion transport in ‘tight’ epithelial monolayers of MDCK cells. *J. Membr. Biol.* **1981**, *59* (2), 105–114.

(81) Louvard, D. Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. *Proc. Natl. Acad. Sci. USA* **1980**, *77* (7), 4132–4136.

(82) Henry, O. Y. F.; Villenave, R.; Crone, M. J.; Leineweber, W. D.; Benz, M. A.; Ingber, D. E. Organs-on-chips with integrated electrodes for trans-epithelial electrical resistance (TEER) measurements of human epithelial barrier function. *Lab Chip* **2017**, *17* (13), 2264–2271.

(83) Srinivasan, B.; Kolli, A. R.; Esch, M. B.; Abaci, H. E.; Shuler, M. L.; Hickman, J. J. TEER Measurement Techniques for In Vitro Barrier Model Systems. *J. Lab Autom.* **2015**, *20* (2), 107–126.

(84) McNeil, E.; Capaldo, C. T.; Macara, I. G. Zonula occludens-1 function in the assembly of tight junctions in Madin-Darby canine kidney epithelial cells. *Mol. Bio Cell.* **2006**, *17* (4), 1922–1932.

(85) Singh, A. V.; Maharjan, R.-S.; Kanase, A.; Stiewert, K.; Rosenkranz, D.; Singh, R.; Laux, P.; Luch, A. Machine-learning-