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Combinatorial approaches to evaluate nanodiamond uptake and induced cellular fate

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Abstract

Nanodiamonds (NDs) are an emerging class of engineered nanomaterials that hold great promise for the next generation of bionanotechnological products to be used for drug and gene delivery, or for bio-imaging and biosensing. Previous studies have shown that upon their cellular uptake, NDs exhibit high biocompatibility in various *in vitro* and *in vivo* set-ups. Herein we hypothesized that the increased NDs biocompatibility is a result of minimum membrane perturbations and their reduced ability to induce disruption or damage during cellular translocation. Using multi-scale combinatorial approaches that simulate ND-membrane interactions, we correlated NDs real-time cellular uptake and kinetics with the ND-induced membrane fluctuations to derive energy requirements for the uptake to occur. Our discrete and real-time analyses showed that the majority of NDs internalization occurs within 2 h of cellular exposure, however, with no effects on cellular viability, proliferation or cellular behavior. Furthermore, our simulation analyses using coarse-grained models identified key changes in the energy profile, membrane deformation and recovery time, all functions of the average ND or ND-based agglomerate size. Understanding the mechanisms responsible for ND-cell membrane interactions could possibly advance their implementation in various biomedical applications.

Keywords

nanodiamonds; coarse grained simulation; cell-based sensing

Introduction

Nanodiamonds (NDs) are an emerging class of engineered carbon-based nanomaterials known for their narrow size distribution and high surface area, light weight, high adsorption capability, chemical and thermal stability, as well as biocompatibility [¹, ²]. Their ability to

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allow multi-surface functionalization by bio-conjugation with nucleic acids, proteins and therapeutic agents [3, 4] make NDs viable candidates for a variety of applications in the biomedical, pharmaceutical, and industrial fields [5]. In particular, the inexpensive and large-scale production of NDs through detonation of carbon-containing explosives could lead to the formation of the next generation of smart systems to be used as drug delivery platforms in cancer treatment, as carriers of genetic material and proteins, or as bio-imaging probes [6]. However, before such large-scale implementation could be achieved, ND uptake, internalization, interactions and ultimately induced cellular fate need to be fully assessed.

Previous studies have showed that NDs have minimum toxicity when exposed to different cellular systems; for instance, Liu *et al* showed that exposure of human lung epithelial cells A549 and HFL-1 normal fibroblasts to ND concentrations between 0.1 and 100 $\mu\text{g ml}^{-1}$ did not affect cellular viability nor altered protein expression profiles [7]. Complementarily, studies of cellular morphological changes and DNA fragmentation in macrophages and HT-29 human colorectal adenocarcinoma cells respectively did not show cell transformation [8] nor significant reactive oxygen species generation [7, 9]. Additionally, Schrand *et al* showed that toxicity of NDs was significantly lower when compared to the toxicity of other carbon-based nanomaterials, with the order of toxicity decreasing from single-walled (SW) to multi-walled carbon nanotubes (MWCNTs), to carbon black and NDs [9] respectively. Furthermore, *in vivo* investigations using animal models complemented *in vitro* analyses, with histopathological and ultrastructural images of biological tissues showing that NDs did not induce pulmonary toxicity when administered to mice by intratracheal instillation [8]. Similarly, subcutaneous exposure, intraperitoneal injection or oral administration for different periods (from 10 d up to 3 months) in both mice and rats did not lead to inflammatory or adverse immune reactions [10] nor weight changes of mice and rats, nor of their offsprings internal organ (e.g., lungs, kidneys, and heart) [8, 11]. While the majority of studies reported high biocompatibility of NDs *in vivo*, some concentration and functionalities of the NDs were shown to induce thromboembolism in mice [12].

Although these results show that the toxicity of NDs has been extensively investigated, there is limited available information that revealed NDs dynamics upon cellular uptake or translocation. For instance, previous research indicated that NDs are uptaken through an energy-dependent clathrin-mediated endocytotic pathway [13, 14] at a higher cellular uptake rate than the rate of other carbon-based nanomaterials (i.e., SW-, MWCNTs or graphene for instance) [15]; however, such studies failed to demonstrate why the proposed pathway was the preferred mechanism. Given that the realtime uptake and internalization of NDs rely on the average particle size as well as on ND interaction with the proteins in cellular media [16, 17], understanding the mechanisms responsible for ND-cell membrane interactions as well as possible ND-induced membrane damage will provide fundamental knowledge to help explain their observed biocompatibility and thus ensure further implementation as smart drug delivery nanovehicles [16].

Herein, we hypothesize that the increased biocompatibility of NDs is due to minimum membrane damage induced by their uptake. Using combinatorial platforms that simulate ND interactions with the cellular membrane and cells as primary transducers, we unravel NDs' real-time uptake, translocation, as well as induced-cellular fate in model cellular systems

[18] to provide novel scientific insights that help advance their implementation in biomedical applications.

Materials and methods

ND preparation

Detonation NDs powder was purchased from Plasmachem (GmbH, Germany) and grinded using a planetary ball mill (Fritsch Pulverisette 7 premium line, Germany) and zirconia oxide balls (100 μm of diameter, Germany) at 7000 U min⁻¹, two times for 30 min each, with a 10 min break in between. The resulting grinded powder was suspended in Milli-Q water to obtain a 1 wt% solution; subsequently, the solution was sonicated in pulsed mode in an ultrasonic homogenizer (Bandelin Sonoplus HD 2200; control unit GM 2200, Germany) at a cycle rate of 50% and the pH was adjusted to 7.4.

Chemical and physical characterization of NDs

Fourier transform infrared spectroscopy was performed on a Smart OMNI-Transmission module (Thermo Scientific, Nicolet IZ10, Germany); at least 32 scans per sample in the range of 400–4000 cm⁻¹ and at a resolution of 4 cm⁻¹ were acquired and analyzed using the manufacturer software (Omnicon, Germany). The size and morphology of the NDs were evaluated using scanning (SEM) and transmission electron (TEM) microscopy. SEM investigations were performed on a Carl Zeiss 1.500tes Gemini SEM (GmbH, Germany); for this, a droplet of a 0.1 wt% NDs suspension was dispersed in water and dried on a silicon wafer. TEM images were obtained on a Carl Zeiss LIBRA[®] 200 (GmbH, Germany) by placing small amounts of a 1 wt% NDs suspension on a clean copper-grid with a carbon-film with holes (400 mesh, Plano Germany). Analyses of NDs aggregation and zeta potential were performed using dynamic light scattering (DLS) (Delsa[™] Nano Particle Analyzer Beckman Coulter, Germany). In each experiment, suspensions of NDs (100 $\mu\text{g ml}^{-1}$) were prepared in Milli-Q water or Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) supplemented with 5% Fetal Bovine Serum (FBS, Invitrogen, USA). For each analysis, four replicates were performed for a total of 150 measurements, at 20 °C and considering a 1.33 refractive index for water and a 1.34 for DMEM respectively [19].

Cell culture

Immortalized human bronchial epithelial cells (BEAS-2B) were purchased from American Type Culture Collection (ATCC, USA) and cultured in DMEM media with 5% FBS, 0.1% L-glutamine and 1% penicillin/streptomycin (Invitrogen, USA). Cells were passaged regularly using 0.25% trypsin (Invitrogen, USA) and maintained in a humidified atmosphere at 37 °C and 5% CO₂ respectively.

Uptake of NDs

BEAS-2B cells were seeded overnight in a 12-well plate (Fisher, USA) at a density of 2.5×10^5 cell ml⁻¹. The cells were subsequently treated with 400, 200, 100, 50, and 25 $\mu\text{g ml}^{-1}$ ND suspensions in DMEM media with 5% FBS for 1 h, 2 h, 3 h or 24 h respectively. Following the NDs treatment, the cells were washed with phosphate buffer saline (PBS, Lonza, USA), trypsinized, resuspended in DMEM, centrifuged at room temperature and

1200 rpm for 5 min. The samples were further washed with PBS, fixed with 4% paraformaldehyde (Fisher Scientific, USA) for 15 min at room temperature, and then washed again with PBS several times to remove any of the fixative.

Fluorescence activated cell sorting (FACS) was performed on a BD LSR fortessa flow cell analyzer (BD Biosciences, USA); the forward scatter (FSC) and side scatter (SSC) were used to gate the live cells population and at least 10 000 events were recorded for each sample. Data was analyzed and plotted using FlowJo v7.2.5 software and NDs uptake was determined by averaging the FITC signal of the exposed cells at 488 nm excitation and 515 nm emission.

Electric cell impedance sensing (ECIS)

Real-time analysis of the BEAS-2B cellular behavior before and after exposure to NDs was performed using an (ECIS 96W, Applied Biophysics) platform. In each experiment, 96W10+ array (Applied Biophysics, USA) with 20 gold electrodes in inter-digitated finger configuration was employed to provide concomitant measurements of 2000–4000 cells per well in real-time and at multiple frequencies. For this, the array holder was placed in a humidified incubator at 37 °C and 5% CO₂ to ensure optimal conditions for cell growth. Prior to any experiment, the electrodes were stabilized for 3 h in 200 μ l serum free DMEM; subsequently, BEAS-2B cells were added at a density of 2.5×10^5 cell ml⁻¹ in a volume of 200 μ l/well. Cells were allowed to settle and grow over the electrodes until a confluent monolayer was formed [20]. Upon monolayer formation, the cells were treated with 400, 200, 100, 50, and 25 μ g ml⁻¹ NDs suspended in DMEM media with 5% FBS and their cellular behavior was subsequently monitored for 48 h.

Cell viability

To determine the effect of NDs on cellular viability, BEAS-2B cells were seeded overnight in a 96-well plate (Fisher, USA) at a density of 1×10^5 cell ml⁻¹ and treated with 400, 200, 100, 50, and 25 μ g ml⁻¹ ND suspensions for 24 h. Subsequently, 10 μ l of tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, known as WST-1 (Roche, USA), was added to each well and incubated for 2 h. Changes in cell viability were based on the cellular ability to cleave WST-1 salt to formazan by enzyme dehydrogenase [21]. The amount of formazan dye formed was assessed using a BioTek 96 plate reader (BioTek, USA) and measuring the absorbance of the sample at 450 nm. Relative cellular viability was calculated as the ratio of absorbance signal as derived from the NDs exposed cells to the absorbance signal of control (i.e., untreated cells).

Cell cycle analysis

To study the effects of different ND doses on cell cycle progression, BEAS-2B cells were seeded overnight in 12 well plate at a density of 2.5×10^5 cells/well, and exposed to 400, 200, 100, 50 and 25 μ g ml⁻¹ NDs suspended in DMEM media with 5% FBS for 24 h. Subsequently, the cells were trypsinized, collected, washed twice with PBS, centrifuged at 1500 rpm for 6 min and fixed overnight at -20 °C in 2 ml 70% ethanol (Sigma, USA). Upon time elapsed, the cells were again washed in PBS, resuspended in 0.2% tween 20 (Sigma, USA) for 15 min, treated with 10 μ l 0.05% RNase (Sigma, USA) for 15 min and, lastly,

stained with 30 μ l propidium iodide (Sigma, USA). The DNA content was determined using BD LSR Fortessa Flow cell analyzer. FSC and SSC were used for gating to include the majority of live cell population; 20 000 events were collected for each sample using the BD FACS Diva software (Verity Software House, USA) and analyzed using FlowJo software V10.0.7 (Tree Star Inc., USA) to measure their DNA content.

Simulation setup

To evaluate the uptake of NDs, we used coarse-grained (CG) representation and the GROMACS software package (v4.5.5) [22]. The CG representation was developed using the MARTINI four-to-one mapping scheme [23]. Atomistic models of NDs (3, 5, and 7 nm in diameter) were generated using the diamond lattice (with Fd3m space group and face-centered cubic lattice) in CrystalMaker software. Coarse grained ND beads were given standard C1 bead type and a zero net surface charge. Subsequently, the constructed ND was energy minimized and equilibrated with explicit MARTINI water (isobaric-isothermal ensemble at 300 K and 1 atm pressure).

The CG lipid bilayer used for the analysis was made up of neutral distearoylphosphatidyl choline (DSPC) molecules and negatively charged distearoylphosphatidyl glycerol (DSPG) lipid molecules. The DSPC:DSPG ratio was maintained at 3:1 to resemble a net negative charge of mammalian cell membranes [24]. The electroneutrality of the total system was achieved by adding the required number of sodium counterions as determined by the number of DSPG lipid molecules being available. The bilayer was allowed to self-assemble without any constraints in a user-designed simulation box with a size of $16.0 \times 16.0 \times 30$ nm³. In all simulations the center-of-mass (COM) of the ND was placed 7.0 nm along the +z-axis above the lipid bilayer as the starting structure for the simulation; the entire simulation box was solvated with CG water beads of which 10% were anti-freeze type.

All initial systems were energy minimized using the steepest descent algorithm followed by isochoric–isothermal (NVT) and isobaric–isothermal (NPT) equilibration simulations for 100 ns each at 300 K. The production run simulations were performed using the Berendsen barostat [25] and temperature coupling at 300 K. The equilibrated systems were subsequently subjected to umbrella sampling pull simulations for 3.6 ns and over a distance of approximately 13 nm by applying a constant force of $1000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ to the COM of the ND to pull it through the lipid bilayer. In each case, the geometries of the system were saved at regular time intervals to generate over 174 configuration windows with each window being analyzed to determine the distance between the COM of the ND and the mid-plane of the lipid bilayer. Out of the initial set of configurations, uniformly separated windows with 0.2 nm spacing were selected. For each of the selected windows, NPT equilibration was performed for 5 ns followed by 100 ns of production run with no geometric constraints. At the end of the production run the potential of mean force (PMF) values were obtained for the translocation process by using the weighted histogram analysis method [26]. The activation barrier for each translocation simulation was calculated by taking a difference between the maximum and the minimum energy points of the PMF curve. The rate constants were calculated by using the first order transition state theory;

subsequently, the rate constant was used to calculate the half-lives and characteristic lifetime of the translocation process [17].

To mimic the experimental serum protein in medium, bovine serum albumin (BSA), (PDB: 4F5S) protein [27] was used. High resolution BSA crystal structure was obtained from the protein data bank and CG using the available MARTINI scripts. The secondary structure of the protein and the bead assignment were based on standard MARTINI coarse grained methods [23]. The interactions between coarse grained BSA and NDs of sizes 3, 5, and 7 nm were investigated in three separate simulations. In each case, the NDs were introduced in a well-equilibrated system of BSA molecules with explicit water and positively charged counter ions (Na^+) at 300 K. The counter ions were added to maintain the electroneutrality of the simulation system because BSA is a negatively charged protein under the physiological conditions.

Statistical analysis

All results are presented as mean $\pm$ standard deviation. Uptake, cellular viability and cell cycle experiments were performed in duplicates and repeated at least three times, for a total minimum of six replicates per each concentration and each time point being investigated. ECIS experiments were performed in duplicates and repeated at least four times per each dose being tested. Two-way analysis of variance and unpaired two-tailed student's T-test were performed using SigmaPlot 10.0 (Systat Software Inc.); differences were considered significant for $p^* < 0.05$. Each CG simulation was repeated two times to sample over the starting configurations and the initial conditions of the system. The random number generator was initialized with different seeds in each simulation. The statistical errors in the PMF curves were estimated using the bootstrap analysis available in GROMACS. The size analysis of equilibrated ND particle after BSA corona formation was performed by averaging over a multiple interparticle distances of farthest, diametrically opposite CG beads of BSA proteins. The average values of the diameter and standard deviation are reported.

Results

Physico-chemical characterization of NDs

Comprehensive analyses of NDs physico-chemical properties were performed using infrared spectroscopy and TEM. Spectroscopy results identified O-related groups (i.e., OH, COOH, and O-CH groups respectively) on the NDs surface (figure 1(a)) as resulted from their manufacturing process and oxidation of sp^2 carbon during detonation soot purification [28]. Specifically, the peak at 3350 cm^{-1} was attributed to the O-H stretching vibration, while the peak at around 1750 cm^{-1} was associated with the CO stretching vibration [18]. In addition, the small peak observed at around 1600 cm^{-1} was a result of the O-H bending vibration, with the peaks between 1000 and 1250 cm^{-1} being associated with the C-O stretching vibrations [29]. Electron microscopy investigations showed that NDs had planar structures that ranged from 3 to 7 nm in size, with an average of 5 nm (figure 1(b)). Furthermore, the NDs formed primary agglomerates (conglomerates of individual NDs; figure 1(b) insert) which ranged from 30 to 100 nm and were comparable to previous studies [30, 31].

Dispersity analyses performed using DLS (figure 1(c)) showed that NDs dispersed in water had an average size of about 63.6 ± 10.3 nm while the ones dispersed in cellular media reached 89.6 ± 12.8 nm. Their agglomeration is a result of the O-balance present at their synthesis as demonstrated by the negative zeta potential (-28.2 ± 2.4 mV). The larger size of NDs in media was presumably due to additional serum proteins adsorbed onto their surfaces and the formation of protein corona as demonstrated by the change in zeta potential (-93.35 ± 5.5 mV).

To explain the agglomeration and the changes in dispersity, we performed computational analyses (figure 1(d)). We considered neutral NDs and subsequently evaluated their agglomeration in the presence of serum proteins, with BSA being the dominant one. The consideration for neutral NDs as compared to anionic ones come from previous studies that showed that larger protein concentrations in the media would lead to overall minor variations in zeta potentials to influence particle cellular uptake [32, 33]. Complementarily, the consideration for BSA was based on previous studies showing that concentrations of up to 27 mg ml^{-1} BSA are present in certified FBS, resulting in an approximate 1.35 mg ml^{-1} in DMEM media containing 5% FBS (v/v) [34]. Our approach also took into account that most cellular membranes are negatively charged thus further influencing the local environment of NDs and their uptake.

The BSA protein being aspherical and with cross-sectional lengths of 9.1 nm, 6.4 nm and 5.1 nm respectively, is comparable with or larger than individual NDs used in this work. Analysis showed that when the BSA was interacted with the neutral NDs, an individual BSA-ND complex was formed, all within 200 ns of the simulation, with the complex formation being mainly driven by van der Waals and hydrophobic interactions between the neutral ND and negatively charged BSA molecules respectively. Results also showed that only a fraction of the BSA surface could contribute to the formation of the complex. As such, a stable nonuniform monolayer of BSA molecules would be observed for all ND sizes being tested with the monolayer leading to an increase in the hydrodynamic radius of the NDs or resulting complexes (figure 1(d)). For instance, the snapshot of 3 nm ND showed a non-uniform protein corona being formed onto its surface, with about three BSA molecules being adsorbed as blobs with pockets to lead to a complex of a 8.6 ± 2.1 nm diameter. Non-uniform protein coronas were also observed for both 5 and 7 nm ND sizes resulting in 13.1 ± 3.2 and 15.7 ± 4.9 nm diameters, respectively. In control, analyses with negatively charged particles (supporting information; S1) also led to a non-uniform protein corona formation as resulted from a maximum adsorption of about four BSA molecules onto larger particles. Taking into account that the stoichiometry of the BSA-ND complex is sensitive to several physico-chemical properties such as NDs size, ND surface charge, protein-protein interactions, charge on the protein, as well as the pH of the medium, the variation in protein corona stoichiometry is minimal relative to the protein corona formed for the neutral particle and represents the result of the continuous physical exchange at ND interfaces [35].

ND uptake and induced cellular biocompatibility

We confirmed the uptake of NDs using their intrinsic photoluminescence and standard *in vitro* FACS analysis. Briefly, human lung epithelial cells (BEAS-2B) were exposed to NDs

dispersed in cellular media containing BSA for 1 h, 2h, 3 h and 24 h respectively; BEAS-2B previously served as a suitable model system for *in vitro* testing of uptake of carbon nanotubes [18], titanium dioxide [36], and silver nanoparticles [37] just to name a few. Figure 2(a) shows that NDs cellular uptake was function of both the concentration and the exposure time, with analyses revealing that cells exposed to concentrations of 400, 200 and 100 $\mu\text{g ml}^{-1}$ NDs had significantly higher fluorescence intensity relative to their control counterparts (i.e., unexposed cells). Fluorescent signals of cells exposed to 50 and 25 $\mu\text{g ml}^{-1}$ NDs were however not significantly different from their control counterparts. Control experiments did not identify large conglomerates or particles thus confirming that the NDs uptake was in its majority.

The uptake did not influence the cell structure or morphology; this was confirmed experimentally using realtime non-invasive ECIS experiments. Specifically, we used the insulating nature of the plasma membrane and ECIS to provide sensitive analysis of membrane fluctuations or cytoskeleton reorganization [38–41]. The ECIS was previously employed to study cellular viability, proliferation [42], motion [40], morphology [39] and attachment [20] post exposure to different toxins [42], therapeutic agents or viral vectors [42], and nanomaterials of carbon [43]. Figure 2(b) shows representative ECIS measurements of the normalized resistance values for control and NDs exposed cells performed for 48 h at 4 kHz frequency; the selected operational frequency was previously shown to allow for optimal measurement of the cellular membrane integrity, cellular adhesion and cell-cell interactions over gold electrodes [38, 41].

Our results revealed that the resistance curves of the NDs-exposed cells were similar to those of the control cells and showed no changes in the morphology or integrity of the cellular monolayer. Furthermore, analyses showed that there were no quantifiable differences in the fluctuations of the resistance curves, which confirmed that the uptake of NDs did not lead with observable membrane changes or cellular-induced micromotion onto electrodes [40, 44] (figure 2(c)).

Upon confirming NDs uptake, we validated their induced biocompatibility through analysis of cellular activity and cell cycle progression respectively. Cellular activity is regulated by different organelles such as the mitochondria, plasma membrane, as well as by cell–cell interactions and is a reflection of the overall cell fate [45]. Cell cycle controls proliferation, differentiation, and apoptosis processes [46], with changes in the cell cycle phases being associated with cyto- and/or genotoxic effects observed upon exposure to different analytes, as well as cellular transformation to a malignant phenotype [47].

Cellular activity analyses at discrete time points using colorimetric detection based on WST-1 assay [21] showed that 24 h exposure to different concentrations of NDs did not induce significant effects on cell viability (figure 3(a)), thus confirming previous studies [13, 48]. Complementary, cell cycle analyses performed by measuring the percentage of the DNA content showed that NDs exposure did not alter cell cycle progression nor interfered with cellular proliferation (figure 3(b)).

Discussion

We used experimentally validated multiscale modeling and a cell-based sensing platform to evaluate real-time uptake of detonation NDs. The consideration for NDs came from their potential applicability for the delivery of genes and drugs as resulted from their ease of biomolecular functionalization and slow release capabilities [5, 30]. Our multi-scale strategy involved first characterizing the physico-chemical properties of user-produced NDs, secondly validating their uptake in model cellular systems, and thirdly providing our hypothesis to explain their increased biocompatibility in such systems.

Our analyses showed that even though a narrow size distribution for the synthesized NDs was recorded, upon their dispersion in cellular media their agglomeration state has changed. The change in the agglomeration state was presumably due to the attachment of the proteins present in the media. Specifically, previous studies showed that the composition and size of such proteins could lead to protein corona formation, with the size of the corona being dependent on the concentration, the affinity, as well as the interaction of these proteins with the interfaces they are exposed to [49, 50].

The detailed analysis of the interactions between the ND and a representative protein present in the media, namely BSA, showed non-specific binding of its amino acid residues onto the ND surface. Furthermore, the asymmetry of the protein sterically seemed to hinder the addition of other protein molecules in exposed NDs pockets. As such, the remaining BSA surface area would presumably be available to facilitate the agglomeration of the NDs as evident from the experimental DLS data. In particular, using the value ratios of individual NDs and their average size with and without the protein corona, the packing density would be function of the dispersion media, i.e. water or media respectively, with the number of NDs being packed in the media presumably being due to the formation of thin electric dipole layers of the protein corona that will affect the hydrodynamic diameter of the NDs [51].

Our analysis showed that the majority of the NDs uptake occurred within the first 2 h of exposure, with cells subsequently reaching a 'saturation' plateau. In particular, mathematical modeling of the fluorescent signal of cells exposed to NDs at concentrations above 200 $\mu\text{g ml}^{-1}$ showed that uptake followed a three-parameters exponential curve according to the following equation:

$$I(t)=A[1 - e^{k(t_c-t)}],$$

where $I(t)$ represents the mean fluorescence intensity at the particular time, A is the saturation value of mean fluorescence intensity, k is the rate constant, and t_c is the lag in particle diffusion and targeting. The model also showed that about 95% of the NDs were internalized within the 1.71 h of incubation ($R^2 = 0.95$) with such analysis being consistent with previously published results investigating NDs uptake in a serum-rich medium [52].

While a clear discrepancy was recorded in the literature on the uptake and induced cellular fate upon other carbon-based nanomaterials such as SW-, and MWCNTs, with the

conflicting data being attributed to the differences in the preparation procedures, intrinsic purities and activities of such nanomaterials, our study confirms the high cellular biocompatibility observed upon NDs uptake [53]. Specifically, previous studies showed that NDs functionalized with O-containing groups (either -OH or -CO₂H groups) might lead to complex membrane-particle interactions [54] and their non-specific binding or clustering on the cationic sites of the plasma membrane, with subsequent endocytosis through a temperature-, energy-, and clathrin-dependent pathway [54–57]. The uptake of NDs is in contrast with both the uptake of SW- and MWCNTs respectively shown to lead to membrane damage, association with cytoskeleton structures and increased disruption of cellular integrity and organelles upon their translocation [18, 58].

Since our results showed that the NDs uptake did not change cell activity nor affect its cycle, we hypothesized that the observed biocompatibility is a result of minimal membrane damage induced upon their uptake. Our hypothesis takes into account the role of the cellular membrane to serve as a ‘gate’ that regulates cell activity and structure through direct association with cellular organelles and cytoskeletal components. In particular, if the NDs would have had disrupted the membrane, such disruption would have had resulted in ‘membrane pinches’ possibly accompanied by cytoskeletal reorganization to affect the overall integrity of the cell and lead to apoptosis or necrosis [24, 34, 45]. Indeed, previous membrane passive penetration mechanisms were shown to affect the cell fate by leading to the formation of holes and a significant membrane disruption. [59, 60]. In contrast, energy-dependent cellular uptake was not shown lead to such effects.

To validate our hypothesis we performed additional CG computational analyses. Our results showed that the size of the NDs or induced conglomerates played a critical role in the internalization process, with the maxima and minima in their energy profiles providing direct evidence of the lack of membrane disruption. Specifically, the PMF profiles for the three representative sizes of NDs showed that key features such as the distance between the COM of ND and membrane mid-plane were reduced. Further, we found that as the 3 nm NDs get closer to the cellular membrane, the uptake energy increases as less favorable interactions between the uncharged NDs and the negatively charged polar head groups of the lipid membrane would take place (figure 4(a)). However, past the lipid heads and as the NDs get embedded in the membrane, the hydrophobic lipid tails interact favorably to stabilize the individual particles leading to energies as high as 60 kJ mol⁻¹. In contrast, the simulations showed that the 5 nm NDs had no minima when embedded in the lipid chains since the hydrophobic thickness of the membrane was smaller than the diameter of the ND (figure 4(b)). A further increase in diameter to 7 nm respectively would have led to pronounced deformations of the membrane structure causing an increase of about 14-fold in the energy barrier relative to the 5 nm ND (figure 4(c)). However, the lack of membrane fluctuations recorded by ECIS confirmed that there were no changes on the membrane integrity thus validating our above proposed hypothesis.

Snapshots further showed that if the uptake process would have occurred via only translocation a clear deformation in the cell membrane would have been observed (figure 5). The membrane recovery time would have had correlated directly with the damage caused by the ND translocation, with the membrane lipid self-assembling into a stable bilayer within

50 ns of the simulations for both the 3 and 5 nm NDs while the rate constant of the translocation was appreciable resulting in a half-life of 1.4 h for the 5 nm diameter particle (table 1).

Our study demonstrates that the lack of membrane disruption can be recorded through multi-scale combinatorial, non-destructive and high throughput velocity analyses, all performed in real-time and in contrast with previously destructive cellular endpoint assays. By unraveling the cellular biocompatibility on the basis of limited membrane and cellular disruption the current study has the potential to advance the implementation of NDs for the next generation of nanovehicles used for drug or gene delivery.

Conclusions

Our results correlate the free-energy barrier, half time and rate constant of NDs uptake with the average size of the NDs in order to explain their increased cellular biocompatibility. Furthermore, our discrete and real-time analyses of the cellular viability, proliferation and overall cellular behavior upon NDs uptake help explain the lack of membrane disruption and allow for high-throughput evaluation of the cell fate through meaningful combinatorial approach with potential to expand testing of other nanomaterial's biocompatibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Physico-chemical characterization of detonation nanodiamonds (NDs), (a) Fourier transform infrared spectra indicating the presence of O-rich groups. (b) Transmission and scanning electron microscope images (TEM and SEM in insert respectively) showing the planner structure and the primary agglomerates formed from NDs. (c) Dynamic light scattering (DLS) intensity distribution showing the average agglomerate diameter of NDs in water and cellular media.

Figure 2.

ND uptake and induced cellular biocompatibility following NDs exposure. (a) Normalized FITC signal of cells exposed to (25, 50, 100, 200, 400 $\mu\text{g ml}^{-1}$) NDs for 1 h, 2 h, 3 h and 24 h respectively. (b) Representative normalized resistance measurements of BEAS-2B cells upon their exposure to different concentrations of NDs, (c) quantifications of membrane fluctuations of cells exposed to NDs relative to unexposed cells. Differences were considered statistically significant for $p^* < 0.05$.

Figure 3.

(a) Viability analysis following exposure to NDs for 24 h relative to control (unexposed) cells. (b) Cell cycle analysis following 24 h exposure to NDs. All differences were considered statistically significant for $p^* < 0.05$.

Figure 4. Coarse-grained (CG) computational analysis of the potential of mean force (PMF) profiles for the 3 nm (b), 5 nm (c) and 7 nm (d) NDs as function of the distance between the center-of-mass (COM) of NDs and membrane mid-plane.

Figure 5. Snapshots revealing the translocation of NDs with different diameter (i.e., 3, 5 and 7 nm respectively) through the membrane lipid bilayer. The images showed the membrane disruption in response to the NDs.

Table 1

Calculated barrier heights, rate constants and half-lives for cellular uptake of NDs without BSA protein corona.

ND diameter (nm)	Barrier height (kJ mol ⁻¹)	<i>k</i> (h ⁻¹),	<i>t</i> _{1/2} (h)
3	60 ± 1	6.5 × 10 ⁵	1.5 × 10 ⁻⁶
5	91 ± 3	4.9 × 10 ⁻¹	1.4
7	1250 ± 12	NA ^a	NA ^a

^aNA—Not applicable. There appears to be a change in the cellular uptake mechanism for NDs with size greater than 5 nm.