Deagglomeration and characterization of detonation nanodiamonds for biomedical applications

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ABSTRACT

Detonation nanodiamonds (DNDs) are usually small particles of 4–5 nm, but in aqueous suspension, DNDs form agglomerates in sizes larger than 1 μm. We propose the use of Bead Assisted Sonic Disintegration and a carboxylation procedure, to reduce DNDs aggregates sizes to approximately 100 nm. High cost zirconium beads have been substituted by silica beads synthetized in our laboratory and less-time consuming conditions were standardized. Techniques as Dynamic Light Scattering (DLS), Fourier Transform InfraRed Spectroscopy (FTIR), Transmission Electron Microscopy (TEM) and X-ray Photoelectron Spectroscopy (XPS), have been used to characterize the resulting diamond nanoparticles. While the incubation of Red Blood Cells with partially disaggregated DNDs was used to study whether these nanodiamonds impact in a living system. Our results show the absence of a negative effect in cell viability as well as no differences between Raman spectra of hemoglobin (Hb), from control and cell + DNDs conditions.

Introduction

Nowadays detonation nanodiamonds with sizes between 4 and 6 nm, are very attractive for nanotechnology. Among several important applications, their narrow size distribution together with biocompatibility and chemical inertness, potentiate the use of DNDs in biomedical research. DNDs have a wide range of possibilities for surfaces functionalization (Krueger and Boedeker, 2008) allowing its combination with antibodies, and other biomolecules, with implications in cell imaging and drug delivery (Mochalin et al., 2012). However, the use of DNDs in biomedicine has been limited due to their tendency to form aggregates, and the impossibility of standard methods, like sonication and centrifugation, to break the micro-sized agglomerates.

To overcome this limitation, during the last decade, some works have used different methods to achieve a complete, or considerable, disaggregation. Kruger and colleagues obtained primary particles by using of stirred-media milling with micron-sized ceramic beads, from DNDs agglomerates of 100–200 nm. They used a self-made vertical stirred mill and spherical silica beads added to DNDs. After milling, beads separation, and sonication, a clear colloid with less than 10 nm particles was obtained. Although this process was efficient breaking the agglomerates, it started from relatively short aggregates and some silica contamination was generated (Kruger et al., 2005). Aleksenskiy et al. (2011) performed a similar procedure using zirconium beads instead of silica. The result showed a considerable reduction of the agglomerates and, also, the presence of contamination with zirconium dioxide. To avoid the contamination, these authors proposed a second and more extensive method of disaggregation, using strong acids for treatment of DNDs and annealing at 450°C. This method rendered particles of 70–80 nm, with a group of small units (less than 10 nm in size) that was separated by centrifugation.

The procedure developed by Pentecost et al. (2010) sought to overcome the contamination problems using dry media assisted attrition milling, with non-contaminating compounds as sodium chloride or sucrose and stainless steel grinding balls. Nevertheless, contamination with iron is possible and must be removed using an acid treatment. After 5 h of milling and a subsequent pH adjustment, the size of the agglomerates was reduced to less than 100 nm with predominant presence of particles around 10 nm. This could be one of the most efficient procedures but the
requirement of specific equipment and relative long times of milling might be drawbacks.

Ozawa et al. (2007) employed two main methods to disaggregate DNDs. First, the stirred media milling assisted with zirconium beads. Using this method, particles with less than 10 nm in size after 100 min of milling were obtained; but contamination with zirconium was found at the end of the process. The second method used was the sonication of DNDs in water, adding zirconium beads. This process, called Bead Assisted Sonic Disintegration (BASD), rendered all the particles with less than 100 nm and the largest population nearly the size of the primary particles, after 2 h of sonication. This is a simple procedure which greatly reduces contamination and allows to obtain diamond nanoparticles in sizes applicable to nano-biomedicine.

The work of Hsin et al. (2011) and others listed by Mochalin et al. (2012) used some of the methods described above, with similar results. In general, every deagglomeration technique has pros and cons, but: generation of contamination or not, the size of the final particles, and the desired application for nanodiamonds, could be the main three aspects to take into account when the most convenient method should be chosen.

The use of DNDs in biomedicine generally involves their incorporation by living cells through mechanisms as endocytosis. Internalization of DNDs with an average size of 46 nm (Faklaris et al., 2009) and 100 nm (Liu et al., 2009) have been observed before, and the process could be dependent of parameters as shape, size, aggregation and surface characteristics of DNDs and others as incubation time and cell line in study (Kaur and Badea, 2013). A common way to increase the nanodiamonds affinity for proteins and to facilitate endocytosis is the modification of DNDs surfaces by carboxylation. This reaction not only renders a high affinity for proteins but also allow conjugation with bio-molecules as DNA and antibodies (Liu et al., 2007). Consequently, surface modification and specifically carboxylation of DNDs, can be a mandatory step when working with living systems. Some proved abilities of nanodiamonds as the enhancement of therapeutic efficacy of anthracyclines and improvement of MRI contrast and fluorescence, show a promissory prospect for DNDs in biomedicine (Lin et al., 2012; Man and Ho, 2012).

In this work we applied the BASD technique used by Ozawa and colleagues, but the procedures followed in both cases were different. We used silica beads instead of zirconium, different sonication times were applied and other general conditions were also changed. As was mentioned before, nanodiamonds in sizes between 40 and 100 nm have been found to internalize cells, considering this, we established as the objectives of our work: 1) to find a relatively easy and fast method to obtain DNDs de-agglutinated to less than 100 nm and 2) to prove biocompatibility of these de-agglutinated DNDs.

While the sizes of primary particles (4–5 nm) were not obtained, as it was by the procedure applied in Ozawa’s work, we find our method to be a fast and accessible alternative for experiments in which bigger nanodiamonds can be used for biological applications.

Techniques as DLS, FTIR, TEM and XPS have been used to characterize partially disaggregated diamond nanoparticles. On the other hand, the incubation of Red Blood Cells with carboxylated DNDs was used to study whether these nanodiamonds impact in a living system. Our results show the absence of a negative effect of carboxylated nanodiamonds on cell viability and Hb Raman spectra between control and cell+DND conditions.

**Experimental procedures**

Nanodiamonds Nanopure-G01, 4 wt%, were obtained from Plasmachem GmbH, USA. Silica beads of 500 μm in size were prepared using Stober method, similar as described by Rao et al. (2005). Briefly, 2.8 mL of TEOS were added to a recipient containing 54.8 mL of ethanol in a sonication bath. After 20 min, 4.9 mL of 28% ammonium hydroxide were added as a catalyst to promote the condensation reaction. Sonication was continued for a further 12 h to get a white turbid suspension. All chemical reagents were obtained from Sigma-Aldrich Company, USA.

Bead Assisted Sonic Disintegration, was accomplished similar to reported by Ozawa et al. (2007). A volume of diamond powder in water was mixed with silica beads solution at a ratio 1:1 and 1:4 (Table 1). The mixture was sonicated at amplitude 60% in ice by using a sonicator equipped with a horn-type sonotrode (Qsonica, Q700: 700 W, 20 kHz). The conditions were adjusted to obtain an efficient disruption at approximately 100 nm and the silica particles were later separated by centrifugation. Nanodiamonds size distribution was analyzed by DLS, using 632 nm laser wavelength, with a Zetasizer nano ZS (Malvern Instruments Ltd.), as well as the zeta potential measurements. Malvern Zetasizer Nano Software was used to visualize the particle size distribution in terms of volume of each population.

The procedure used to carboxylate deagglomerated DNDs was according to Liu et al. (2007), with some modifications. Briefly, 15 mL acid mixture of H2SO4:HNO3 (3:1) were added into DNDs dispersion in water and heated at 75 °C on a stirrer plate for 24 h. Then 1 mL of 0.1 M NaOH was added at 90 °C for 2 h, and finally 0.1 M HCl at 90 °C for 2 h. Carboxylated nanodiamonds (cNDs) were washed with distilled water four times before collecting the sediment and dry. Dried cNDs were dispersed in distilled water and sonicated before use. Dried samples of DNDs before and after carboxylation were analyzed by FTIR spectroscopy, using a Spectrum GX System (Perkin Elmer).

TEM analysis was performed using a Jeol 2010F apparatus (JEOL Ltd., Akishima-shi, Japan) at 200 kV. Briefly, 10 μL of nanodiamonds suspension were deposited on a gold TEM grid and the sample was vacuum-dried for 24 h before observation.

To study DNDs-red blood cells interaction, whole blood from a healthy volunteer was drawn and transferred into EDTA-covered tubes. A dilution of blood in Phosphate-buffered saline (PBS), ratio 15 μL: 1000 μL, was prepared. One milliliter of red blood cells (RBC)/PBS dilution was mixed with DNDs 0.004% before and after deagglomeration. After 3 h of incubation at room temperature, samples were taken for viability, hemolysis and Raman assays. To test the viability of RBC after incubation with DNDs two aliquots from each condition: Positive Control (RBC + PBS), carboxylated DNDs and DNDs, were mixed with trypan blue (10 μL: 10 μL) and the unstained-viable cells were counted in a Neubauer chamber at the microscope. The presence of hemolysis in the supernatant after centrifugation was determined by measuring the absorbance at 540 nm with Perkin-Elmer Lambda 2 spectrophotometer (Perkin Elmer), using RBC + water as positive control. The Raman spectra were measured using a Raman micro-spectrometer Horiba LabRam-HR (Horiba) with 488 nm and 633 nm excitation wavelength. Raman and FTIR results were analyzed using OriginPro 9.0 software.

<table>
<thead>
<tr>
<th>Condition</th>
<th>DNDs/silica ratio</th>
<th>Total volume</th>
<th>Sonication DND sizes by DLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:0 silica</td>
<td>5 mL</td>
<td>4 × 30 s</td>
</tr>
<tr>
<td>2</td>
<td>1:1</td>
<td>5 mL</td>
<td>4 × 30 s</td>
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<tr>
<td>3</td>
<td>1:4</td>
<td>5 mL</td>
<td>4 × 30 s</td>
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<tr>
<td>4</td>
<td>1:4</td>
<td>10 mL</td>
<td>5 × 30 s</td>
</tr>
<tr>
<td>5</td>
<td>1:4</td>
<td>25 mL</td>
<td>5 × 30 s</td>
</tr>
</tbody>
</table>
Results and discussion

Silica beads are easy to obtain at laboratory and it has been reported that high power sonication up to 30 min, do not affect beads integrity (Russo et al., 2011). Based on these facts we decided to use silica instead of zirconium in BASD experiments. Table 1 shows that sonication without silica beads was not able to break the agglomerates; therefore, this condition was used only as a negative control. Under same sonication cycles, it was evident that the DNDs/silica ratio 1:4 was better than ratio 1:1, reducing agglomerate sizes from more than 1 μm to 167 nm. High quantities of silica particles in the mix facilitate the fragmentation of agglomerates by increasing the number of effective collisions. These impacts produce the needed energy to break the chemical bonds between particle surfaces that contribute to agglutination (Fig. 1).

Once the ratio was set at 1:4 for the rest of the experiments, some changes in sonication cycles and final volume were evaluated. These variations allowed to establish the best conditions to reduce DNDs aggregates to sizes of approximately 150 nm: 5 × 30 s sonication pulses, with 1 min inter-pulses, in ice (Table 1). Also, results suggest that the increase of total volume did not affect final DNDs sizes. Other experiment conditions, including a higher number of sonication cycles and different ratios DNDs/silica beads, could be studied in order to obtain lower sizes.

After DNDs were partially disaggregated to 140 nm we performed the carboxylation reaction, which triggered the re-aggregation of the resulting dried carboxylated nanodiamonds (cNDs) to 1 μm. This result was mentioned by Mochalin et al. (2012) when stated that the drying process promotes re-aggregation due to capillary forces, as well as attractive van der Waals forces have an effect that leads to aggregation. Although cNDs were sonicated again (using an ultrasonic bath), the size was immovable over 1 μm until a pH adjustment was achieved.

In aqueous media, the pH of the sample is one of the most important factors that affects its zeta potential, which can be a measure of suspensions stability (Kirby and Hasselbrink, 2004). The zeta potential values of DNDs in suspension, measured in a previous experiment under different pH ranges, showed that higher zeta potential value was obtained at pH = 10–11 (Data not shown), but it was not reflected in a considerable size reduction. Using these previous results, we increased the pH of the cND suspension to pH = 10 and the DLS spectra showed a size reduction to approximately 100 nm and the emergence of a population of cNDs with sizes around 30 nm (Fig. 2). The incidence of pH in DNDs dispersion is related with the isoelectric point (pI) of the surface functional groups. Changing from acid to an alkaline pH allows that the carboxyl groups on DNDs surfaces become electrically charged, surpassing its pi. Similar and homogenous electrical charge on every particle surface promotes repulsion and facilitates dispersion. Same results were found for an additional sample disaggregated using parameters from condition 5. Starting at 159 nm after BASD, sizes around 90 nm were obtained by DLS after carboxylation (Supplementary information).

At this point we had DNDs agglomerates that decreased its sizes from more than 1 μm to 150 nm when BASD was applied. After subsequent carboxylation reaction and pH adjustment we obtained DNDs with sizes around, or less than, 100 nm. This last decrease of the size could be related with a reduction of some of the causes of agglomeration, becoming carboxylation reaction an indirect second step in the deagglomeration process. According to Panich and Alekseenkii (2012), disaggregation by carboxylation becomes possible after carboxyl groups interact with water and the interaction drives the formation of a double electric layer around DND particles. This layer is responsible of the electrostatic repulsion between DNDs, reducing the inter-particle coulombic interaction. That effect could be lost at the drying step but re-dispersion in water and pH change provided the environment to make it possible again. Another complementary explanation could be that the strong acid oxidation led to a reduction of the graphitic phase that is surrounding DND cores. This phase could be increased during BASD because of the high local temperatures that can be generated by sonication and high-speed collisions between DNDs and beads (Chao et al., 2007).

Results obtained by DLS were corroborated using TEM. As Fig. 3 shows, we were able to find nanodiamond agglomerates of sizes around 100 nm and others with less than 50 nm in diameter.

Analyzing the FTIR spectra of original DNDs and carboxylated particles (Fig. 4A) we observed the absence of typical Si-O and Si-O-Si bond stretching peaks around 1080 cm⁻¹ and 800 cm⁻¹ (Russo et al., 2011), but the possibility of an overlapping with diamond bands could be suspected. XPS technique was able to confirm the presence of a small peak of silica in carboxylated DNDs spectra, showing that silica beads used in BASD are a source of contamination. Nevertheless, this silica peak was completely eliminated when we executed a later hydroxylation reaction (Supplementary material), due to strong oxidative conditions. Another finding from FTIR spectra analysis is related with the corroboration of the results obtained by Tu et al. (2006) in their experiments with carboxylated nanodiamonds: the chemical structure and interaction of the surface functional groups depend on the size of nanodiamond and can be associated to the C=O stretching frequency.

Fig. 4B shows that the spectrum of non carboxylated (original) DNDs was similar to re-aggregated cNDs in the range of 1550–1850 cm⁻¹ with a size for both particles over 1 μm. The FTIR
spectra differed only in the proportion of C=O peak being higher for cNDs than original DNDs. Additionally, the hydroxyl bending mode adsorption band at 1635 cm$^{-1}$ remained invariable for the three analyzed particles; as it was previously reported by Tu et al. (2006): this peak position does not depend on particle size. These authors published a shift in the position of C=O peak on the zone of 1650–1850 cm$^{-1}$ and a reduction in the absorbance, when the size decrease. In Fig. 4, we can see changes in C=O peak, between different size carboxylated particles (cNDs over 1 μm and cNDs at pH = 10 under 100 nm in size). Tu attributed the observed shift to the interaction of C=O with the environment, led by the formation of hydrogen bonds between the COOH groups in the carboxylated nanodiamond surfaces. In our case, the pH modification introduced a new environment that allowed carboxylated nanodiamonds to disaggregate to size less than 100 nm after BASD, exhibiting differences in the FTIR spectra when compared with original DNDs.

The use of this fast and affordable method to disaggregate and obtain cNDs of 100 nm and less, allowed us to have nanodiamonds in sizes that, as was mentioned before, can be incorporated by tumor cell lines and could be used in biomedicine. However, to be useful for biomedical applications, this cNDs require also a proven safety for living cells, and that is why we performed a study of the interaction between cNDs and RBC.

As it was previously demonstrated (Lin et al., 2012; Liu et al., 2007), a concentration of cND less than 100 μg/mL is safe to cells and effective to study diamond-cell interaction. Using this information we decided to evaluate the behavior of RBC incubated

![Volume based DND size distribution obtained by DLS. A: Sample before BASD. B: Sample after BASD. C: Sample of cND at pH = 10.](image)
Fig. 3. Transmission Electron Microscopy images of Detonation Nanodiamonds. A: DNDs before disaggregation. B, C, D: DND agglomerates after BASD and Carboxylation, with sizes around 100 nm and less.

Fig. 4. FTIR analysis of different diamond particles. A: Complete spectra of two samples: DNDs before BASD (non carboxylated) and DNDs after BASD, carboxylation and pH variation to 10. B: Region of 1500–2000 cm⁻¹ of A with the addition of the cND sample without pH variation.
with nanodiamonds in PBS at different concentrations: cND 0.04% (400 μg/mL), cND 0.01% (100 μg/mL), cND 0.004% (40 μg/mL), and non carboxylated DND (ncND) 0.004% (40 μg/mL). This range of concentrations allowed to study the effect of a previously reported safe concentration as well as the consequences of using a ten times higher value. The results of viability test and hemolytic effect analysis are shown in Fig. 5.

Trypan blue exclusion test of cell viability is a widely used tool that allows to estimate the number of viable cells in suspension, by counting at microscope the intact clear cells that have not been stained with trypan dye (Strober, 2001). In our experiment, the number of living cells in each condition was not lower than positive control. This result is in accordance with the absence of a high hemolytic effect in all tested concentrations after 3–5 h, having values under 10% in all cases. In an extensive report, Lin et al. (2012), found that carboxylated nanodiamonds of 5 and 100 nm interacting with RBC, had a benignant behavior at similar concentrations. Now, we can state that DNDs agglomerates, under BASD and carboxylation procedures (which includes aggressive physical and chemical conditions) were reduced to steady particles with low sizes and modified surfaces that do not affect RBC viability at tested concentrations during 3 h.

To study the impact of our cNDs on cells at molecular level, we proceed to analyze the Raman spectra of RBC under two conditions: control (RBC + PBS) and RBC + cND 0.004%. After 3 h of incubation, the spectra were acquired at both 488 and 633 nm excitation wavelength. Positive signals were averaged and normalized, and the origin graphics are showed in Fig. 6.

Both spectra on each laser wavelength have same peaks in same positions; which means that no differences between control condition and presence of cNDs were detected in terms of Hb structural transformations. Main bands are in accordance with the results obtained by Wood and McNaughton (2002) for RBC spectra at different excitation wavelengths. Silica peak at 972 cm⁻¹ from Si substrate can be observed. Other characteristics of RBC-cND interactions as deformability of the cell membrane and incidence in oxygenation/deoxygenation process, are being studied by our group, showing that cNDs could have a positive impact in RBC recovery after irradiation (Acosta-Elías et al., 2015).
Conclusions

We applied a previously described method for deagglomeration of detonation nanodiamonds: BASD, which was changed to be more accessible and less-time consuming, including the use of silica beads instead of zirconia. The procedure proved its effectiveness to disaggregate DNDs from sizes around 1 μm to less than 100 nm, in a non-toxic way and did not introduce harmful contamination to RBC at the concentrations of DNDs tested, during 3 h. Raman studies with 488 and 633 nm laser wavelengths showed that hemoglobin spectra were unaltered in both cases, as a signal of the benign character of DNDs at a molecular level.

Although other methods have been used to solve the same problem: deagglomeration of DNDs in sizes of 1 μm and more, the intended use of nanodiamonds and the sizes to be achieved have not been always the same and can not be compared. This work represents our contribution in the way to find an affordable and easy method to disintegrate agglutinated DNDs, where BASD with silica beads followed by carboxylation is showed as one of the possible alternatives. In particular, this procedure could be elected when the final intention is the use of nanodiamonds in biomedicine, because of the sizes achieved in the range of 50 to 100 nm and the absence of harmful effects when interacting with cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jab.2016.09.003.

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