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Original Research Article

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

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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 1
Different conditions studied to standardize BASD. DND sizes after silica separation by centrifugation can be seen in last column.

Condition	DNDs/silica ratio	Total volume	Sonication	DND sizes by DLS
1	1:no silica	5 mL	4 × 30 s	1325 nm
2	1:1	5 mL	4 × 30 s	204,3 nm
3	1:4	5 mL	4 × 30 s	167,1 nm
4	1:4	10 mL	5 × 30 s	154 nm
5	1:4	25 mL	5 × 30 s	140 nm

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