

8 nm nanodiamonds as markers for 2 photon excited luminescent microscopy

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Abstract. Structural and luminescent properties of stable suspensions of fluorescent nanodiamonds were investigated. Measurement of the effective hydrodynamic radius yields particles less than 30 nm diameter, while the TEM measurements made on the same particles shows average diameter about 8 nm. It was found that NDs have relatively low toxicity. Upon incubation, 3T3-L1 cells spontaneously take up nanodiamonds that uniformly distribute in cells cytoplasm. The possibility of fluorescent imaging using both single or two-photon excitation was shown.

1. Introduction

Carbon nanoparticles are ones of key materials for biomedical applications as they are biocompatible, biodegradable and are capable of providing novel imaging and therapeutic modalities, which are usually attributed to efficient photoluminescence either through quantum-confined states in small carbon particles or through the doping centers [1]. As it was shown before, [2, 3] nanodiamonds are one of the least toxic through all carbon nanomaterials. Nevertheless, the application of the luminescent nanodiamonds for bioimaging is limited by the low light penetration depth. Bulk diamond has bandgap 5.5 eV, so it should be excited with far-UV light. Nitrogen-doped nanodiamonds can be excited by the significantly lower energies in UV-visible range. It is widely known, that mammals' body has near-infrared window and the maximum penetration depth at the range 650-1350 nm while the UV and visible light is effectively absorbed by the body. One of the ways to increase the penetration depth is to excite the luminescence using the 2-photon absorption way [4]. It requires intensive laser irradiation, but the longer wavelength of the exciting irradiation can provide higher light penetration length. The up-to date studies usually use combustion-derived nanodiamonds with sizes from tenth to hundreds nanometers for single photon or multi-photon imaging [4]. Here, we by *in vitro* studies have showed that laser-ablation synthesized ultrapure nanodiamonds with sizes of 8-10 nm, could also be used as efficient non-linear optical labels with enhanced two-photon excited photoluminescence.



2. Synthesis and characterisation of nanodiamonds

Nanodiamonds with the sizes about 8 nm were ordered at Ray Techniques Ltd. The synthesis method of such nanodiamonds is Light Hydro-dynamic Pulse (LHDP) fabrication and described in details in [5]. High resolution TEM picture in figure 1a allows to clear visualize their crystalline nature. Atomic inter-plane distances estimated from the HRTEM pictures corresponds to the very well-known diamond crystalline structure.

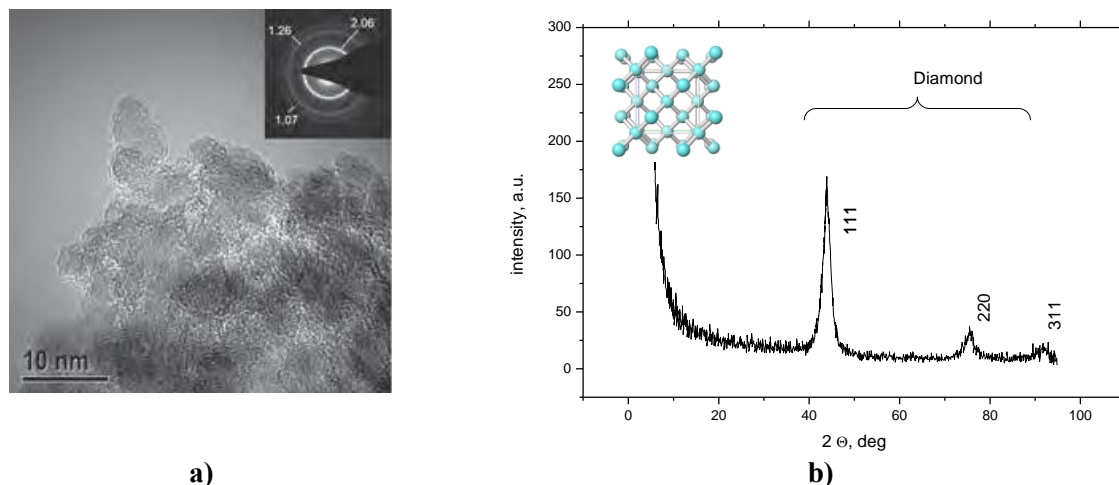


Figure 1. (a) TEM images of the nanodiamonds deposited from the solution. It is seen that near-10 nm nanodiamonds form the agglomerates inset shows the electron diffraction pattern (b) XRD pattern of the nanodiamonds powder. The XRD peak position corresponds to the bulk diamond structure (inset).

Size distribution of the used NDs has been performed from X-ray diffraction analysis. Typical X-ray diffraction pattern obtained on the NDs based powder is shown in figure 1b. Angle positions of the recorded peaks correspond to the cubic crystalline structure of diamond. According to Hall-Williamson's model, the NC size distribution was found to be centered at 8.3 ± 2.2 nm.

Since TEM shows the presence of agglomerates on the dried sample, size distribution of the nanoparticles' number was obtained using the dynamic light scattering (DLS) method (figure 2). Since it was shown in [6] 37 nm is the least detectable size for nanodiamonds, using DLS method, we can only conclude that we have no nanodiamonds agglomerates into the solution above 37 nm.

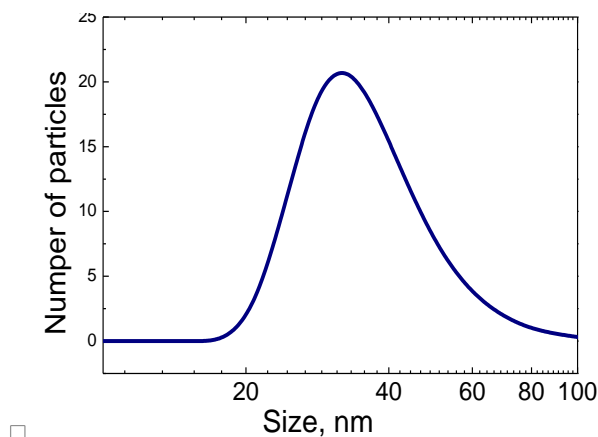


Figure 2. Size distribution of the nanoparticles' number obtained using the DLS of water suspensions of nanodiamonds.

Nanodiamonds are able to produce luminescence in green range under UV excitation. Figure 3 shows optical properties of the diamonds. Blue curve is an absorbance spectrum of the nanodiamonds, black one is a luminescence spectrum and the red is a PLE spectrum (intensity of PL at 490 nm under different excitation). The excited state lifetime is below 10 ns. The PL peak at 490 nm is attributed to the charge carriers recombination through the defects caused by nitrogen impurities, so the luminescence occurs predominantly due to recombination of charge carriers at the nitrogen-vacancy centers levels.

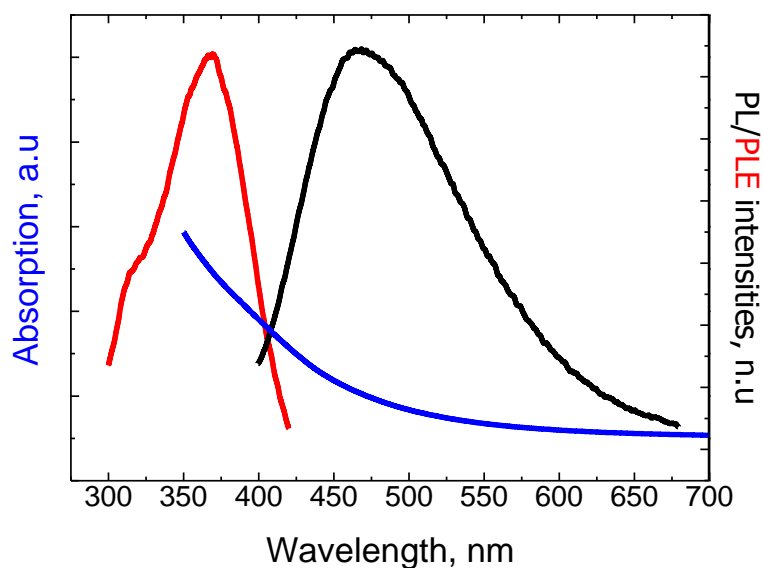


Figure 3. Photoluminescence and absorbance spectrum of the nanodiamonds (black and blue). PLE spectrum of the nanodiamonds (emission at 490 nm).

3. Toxicity estimation

In order to determine a lowest toxic concentration of the NDs they were added to the culture of 3T3-L1 living cells, and the in-time evolution of cell index (proportional to alive cell number) was recorded using non-destructive impedance-based method by means of an xCELLigence set-up (see protocols and methods section). The result of toxicity experiment can be seen on figure 4.

During first 48 h we can observe the 3T3-L1 proliferation, than at the pointed time, nanodiamonds with different concentrations were added to different cell wells. There is a dose-response of the cells' behavior after NPs addition. Highest concentration of NDs (2 g/L) decrease number of alive cells, intermediate ones slow down the proliferation rate and concentrations below 0.5 g/l have no effect on cells proliferation.

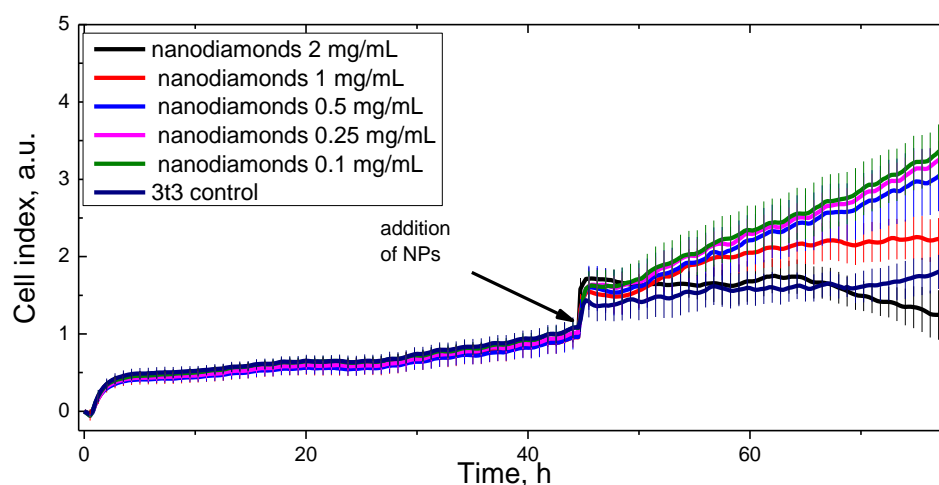


Figure 4. Cell index evolution curves for the 3T3-L1 cells. Arrow shows the time of the NDs addition.

4. One and two-photon excited imaging using NDs

Figure 6 shows bi-modal images of 3T3-L1 fibroblasts cells labelled with NDs. The labelling procedure is the 24h exposure of proliferating 3T3-L1 cells with 0.5 g/L of NDs. It is worth to notice the general healthy look of the cells, corroborating the non-toxic impact of the chosen NDs concentration. Figure 5a shows the PL microscopy image of the fixed 3T3-L1 cells. Inset shows the PL of the control cells under the same conditions. We can observe the uniform distribution of the nanodiamonds inside the cells' cytoplasm, while the nuclei remain unlabelled.

In figure 5 b yellow colour is related to the two-photon excited luminescence. Since auto-fluorescence intensity of the non-labelled cells is too weak under these excitation/detection conditions (see inset in figure 5-b), the observed TPEL of the labelled cells can be attributed to the NDs accumulated inside the cellular cytoplasm and ensuring its quite bright staining.

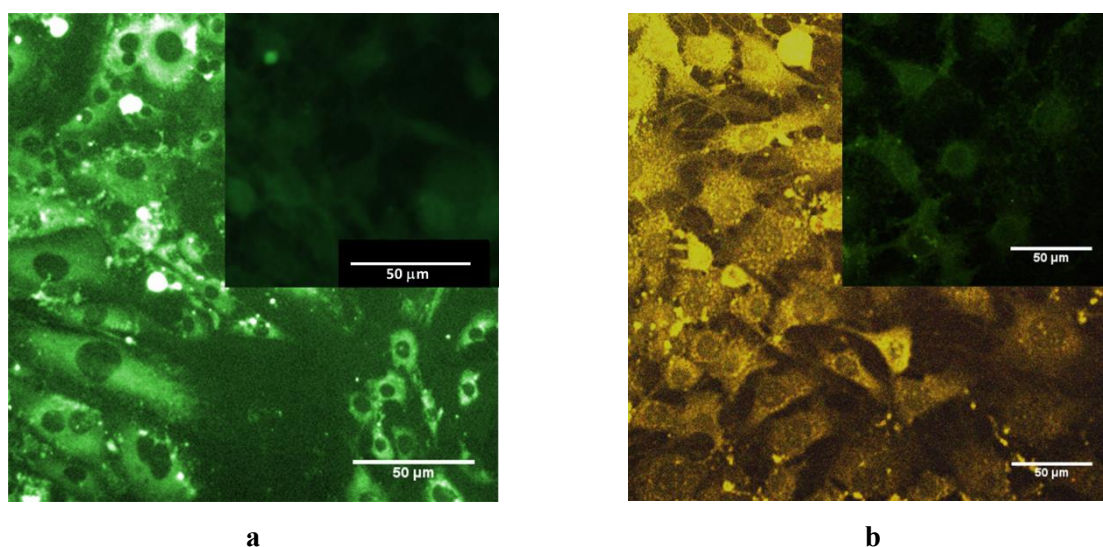


Figure 5. PL microscopy image of the 3T3-L1 cells labeled with 0.25 mg/mL of nanodiamonds for 24h.

5. Protocols and methods

TEM pictures were obtained by EM-002B (Topcon, Japan) high-resolution transmission electron microscope operating at 200 kV.

The x-ray diffraction experiments were performed using Cu k alpha x-ray source (0.15418 nm), SmartLab diffractometer at the nanoparticles dried powders.

For DLS size distribution measurements Zetasizer Nano Z zeta potential analyzer had been used. All the measurements were applied at 22⁰ C in water solution.

3T3-L1 (fibroblasts) cells used for this work were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum 100 IU penicillin, 0.1mg streptomycin, and 0.25 mg/L amphotericin B at 37°C in a water saturated atmosphere with 5% CO₂ in air, in a Heraeus incubator. The cells were trypsinized and about 2500 cells (estimated by millipore Scepter™ 2.0 cell counter) were added to each well in a 96-well plate for cell proliferation rate measurements and onto the glass coverslip for cell imaging experiments. Then the cells were incubated for 48 h. NDs with concentrations 2, 1, 0.5, 0.25 and 0.1 mg/ml were added to the cell cultures which were additionally incubated for 24 h. After NDs were washed out from the extracellular environment with phosphate-buffered saline and the cells were washed with PBS twice. Then cells were fixed with 60% ethanol solution for the cell images acquisition.

Cell number measurements were performed using non-destructive impedance-based method (xCELLigence). The cells were grown on special plate with electrodes on its bottom. The system measures electrical impedance across interdigitated microelectrodes situated at the bottom of culture wells. The measurements are done by applying an alternative excitation signal (20 mV control voltage amplitude) at three different frequencies (10, 25 and 50 kHz) through the microelectrodes in the E-plates while monitoring the voltage drop across the electrodes where the quotient voltage/current yields the impedance. Software shows cell index as a result of processing impedance data. Cell index is proportional to the cell number, single cell surface area and adhesion factor. For a given cell line under basal conditions, cell number is the main factor affecting cell index. Each curve is the mean of cell index measured on 8 wells.

The luminescent microscopy images were obtained by means of Leica DMI 4000B microscope with the following filter combination: UV/violet excitation band: 354 – 424 nm and observation spectral range: >470 nm.

For optical nonlinear imaging we employed a Nikon A1R multiphoton upright microscope (NIE-Nikon) coupled with an Insight Deepsee tunable laser oscillator (Spectra-Physics, 120 fs, 80 MHz, 680 - 1300 nm). The nonlinear signals were epi-collected by a Nikon 25 water immersion objective (CFI75 APO, N.A.1.1) spectrally filtered by tailored pairs of dichroic mirrors and interference filters and acquired in parallel either by a normal photomultiplier (600 - 655 nm) or a GaAsP photomultiplier (385 - 492 nm).

6. Conclusion

Fluorescent nanodiamond is a new valuable tool for long-term labeling, imaging, tracking. In this work we have successfully show that 8 nm NDs produced by the LHDP method were used as biocompatible solid-state labels for bi-modal non-linear optical imaging *in vitro*.

Acknowledgments

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