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Thermometric Characterization of Fluorescent Nanodiamonds Suitable for Biomedical Applications

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Abstract: Nanodiamonds have been studied for several biomedical applications due to their inherent biocompatibility and low cytotoxicity. Recent investigations have shown perspectives in using fluorescent nanodiamonds as nanothermometers because of their optical properties' dependence on temperature. Easy and accurate localized temperature sensing is essential in a wide variety of scientific fields. Our work demonstrated how the fluorescence spectrum of high-pressure high-temperature fluorescent nanodiamonds of three different sizes: 35 nm, 70 nm and 100 nm, changes with temperature within an important biological temperature range (25 °C to 60 °C). Taking advantage of this phenomenon, we obtained nanothermic scales (NS) from the zero phonon lines (ZPL) of the NV⁰ and NV[−] colour centres. In particular, the 100 nm-sized features the more intense fluorescence spectra whose linear dependence with temperature achieved 0.98 R² data representation values for both NV⁰ and NV[−]. This model predicts temperature for all used nanodiamonds with sensitivities ranging from 5.73% °C^{−1} to 6.994% °C^{−1} (NV⁰) and from 4.14% °C^{−1} to 6.475% °C^{−1} (NV[−]). Furthermore, the non-cytotoxic interaction with HeLa cells tested in our study enables the potential use of fluorescence nanodiamonds to measure temperatures in similar nano and microcellular aqueous environments with a simple spectroscopic setup.

Keywords: nanodiamond; nanothermometer; NV centres; fluorescence; bioimaging; HeLa

1. Introduction

A large number of nanoparticles are currently being considered for biomedical applications, such as imaging, diagnostics and drug delivery. However, many of these nanoparticles possess intrinsic limitations for their use in biological systems, such as control of surface properties, increased dispersion in physiological solutions, biocompatibility issues and effective targeting to intracellular locations [1]. Recently, carbon nanostructures attracted the scientific community's attention due to their potential applications in biomedicine and nanoscale device engineering [2–4].

Nanodiamonds receive special attention within the range of nanocarbon materials due to their outstanding physical and chemical features [5,6]. This versatile material exhibits low cytotoxicity at the cellular level [7,8] and is considered the most biocompatible of all carbon-containing derivatives [9,10]. Moreover, given their high surface-to-volume

ratio [11] and charged surface [12], nanodiamonds exhibit remarkable loading/binding capacities for a wide variety of therapeutic compounds [13–17]. With highly ordered aspect ratios close to unity, nanodiamonds have shown to be biologically stable [18], enabling them to prevent stress-inflammatory and apoptotic cellular reactions [19]. Some studies have supported nanodiamonds inherently susceptible biological performance in water suspension once incubated with cells [11,20].

Diamonds may contain luminescent defects or colour centres due to different impurities or defects embedded within the crystal lattice [21,22]. Amongst these diamond colour centres, the nitrogen-vacancy (NV) colour centres are the most notable as they possess a stable fluorescence spectrum spanning from the visible and near-infrared regions of the electromagnetic spectrum [22], which also broadens and quenches as the nanodiamond particle size decreases [23,24]. Characteristic emission lines are at 575 nm (NV^0) and 637 nm (NV^-) corresponding to the neutral or negatively charged centre, respectively. These colour centres are known to allocate 5 (NV^0) or 6 (NV^-) electrons and give rise to two possible electronic structures [25–27]. Each charge state population can be tuned to match a maximum of luminescent intensity given an excitation's wavelength and power [28]. Additionally, an excitation wavelength within 450–575 nm origins that the NV centres continuously switch between the two charge states [29]. Further studies show that under the typical 532 nm excitation wavelength, the probabilities of finding the NV centre in the NV^- and NV^0 configurations are 70% and 30%, respectively [28,30]. These optical properties make fluorescent nanodiamonds a suitable probe for bioimaging and thermic sensing applications in cellular environments [31,32]. Moreover, the NV^- centre is highly photostable, with no photobleaching signs, even under high power excitation at the single-molecule level [7,8].

Many cellular events are temperature dependent [33–35] and several studies have demonstrated how cancer cells exhibit a higher temperature than healthy cells due to increased metabolism [35,36]. At this point, accurate data on the local temperature in biological systems is necessary to understand their dynamical behaviour and general state [37]. In these systems, it is desirable to have a non-invasive method to perform local temperature measurements at micron and sub-micron scales. A solution to this problem is fluorescence thermometry, which makes use of fluorescent materials such as quantum dots, rare-earth-doped crystals [38–40], organic compounds [41,42] and nanodiamonds [43–45] to determine temperature without contact. However, doubts arise about the biocompatibility and toxicity of rare-earth-doped nanostructures [46]; for this reason, nanodiamonds are preferred due to their inherent biocompatibility [47]. Another nanodiamond advantage is NV centres are immobile at room temperature, conferring excellent stability and sensitivity [43]. Then, the thermometry based on nanodiamond fluorescence becomes an excellent alternative to assess the intracellular structures. Nevertheless, most works consider the changes in the electron spin resonances of NV^- centres when a magnetic field is applied [44,48,49]. The obtained optically detected magnetic resonances (ODMR) of NV^- centres under different temperatures have an excellent low-temperature uncertainty. Still, the implementation of those techniques demands high requirements of sophisticated experimental infrastructure [50–52]. In contrast, thermometry scales build from NV centres luminescence can be constructed with a simple experimental setup getting sensitivities around $\pm 1.3^\circ\text{C}$ for both NV centres. Examples are nano-thermometry using the Debye-Waller factor (DWF), that is the ratio of the area under the ZPL and the total emission band of the NV^- [44,53], the detection of small changes in the width, the area and the peak wavelength of the ZPL [54], recording of spectral shifts of ZPL [45] or the measurement of energy level shifts of NV centres by Raman and photoluminescence (PL) spectra [50].

In this work, we performed a thermometric characterization in a biological temperature range (25–60 °C) of commercial high-pressure high-temperature fluorescent nanodiamonds (HPHT FNDs) with three sizes: 35 nm, 70 nm and 100 nm. Our results showed that it is possible to establish nanothermic scales based on HPHT FNDs with affordable fluorescence microscope equipment. Additionally, the temperature measurement with NV^0 may apport an additional calibration tool. As an early biomedical application, the thermal

bioimage of HeLa cells at RT was obtained, opening a new perspective of using FNDs as nanothermometers in living cellular environments for thermal imaging and diagnosis aid.

2. Materials and Methods

2.1. High-Pressure High-Temperature Nanodiamonds

HPHT FNDs in aqueous solution were acquired from Sigma Aldrich with average particle sizes of 35 nm, 70 nm and 100 nm each (Table 1). HPHT FNDs of 100 nm of particle size were concentrated to 0.1%, 0.5% and 0.01% *w/v* with purified water (Milli-Q model C85358, Millipore Sigma) for the cytotoxicity assay and HeLa cells culture study.

Table 1. Sigma Aldrich's nanodiamonds specifications.

Part Number	Concentration	NV Centres/Particle	Size
900172-5 mL	1 mg/mL	≤4	35 nm
798169-5 mL	1 mg/mL	>300	70 nm
900174-5 mL	1 mg/mL	>900	100 nm

2.2. Fluorescent Thermometry

The fluorescence spectra of 35 nm and 70 nm-sized nanodiamonds were recorded at different temperatures using a laser scanning confocal microscope Olympus BX41 (Japan) equipped an InGaAs detector using a 10x long working distance microscope objective with a NA 0.25 and an OBIS 532 nm laser with 20 mW of continuous power as the optical excitation. The laser was directed through a series of mirrors into the microscope system. Then, the laser was focused on the sample, the detector collected the signal, and then processed with Horiba Scientifics LabSpec software. Once these colour centres were well-localised and the optimal microscope parameters established for a maximised output signal, fluorescence spectra were recorded at different temperatures within the physiological range of 25 °C to 60 °C with the help of a heating plate in steps of 1 °C and 20 min of thermal equilibrium time. Fluorescence spectra were recorded using a detection range from 550 nm to 850 nm in steps of 1 nm with a 2 nm wide slit and 0.1 s of integration time.

The fluorescence spectra of 100 nm-sized nanodiamonds were recorded at different temperatures using a Horiba Scientifics Fluorolog[®]-3 FL3-22 (Japan) equipped with a photomultiplier tube Hamamatsu R928P and an OBIS 532 nm laser with 20 mW of continuous power as optical excitation. The temperature control was achieved with the F-3030 temperature bath and the FL-1027 Single-Position Thermostatted Cell Holder accessories from Horiba Scientifics. The software FluorEssence (Horiba Scientifics, Japan) was set for an emission scan from 25 °C to 60 °C in steps of 1 °C with a tolerance of 0.1 °C and 20 min of thermal equilibrium time. The fluorescence spectra were recorded using a detection range from 550 nm to 850 nm in steps of 1 nm with a 2 nm wide slit and 0.1 s of integration time.

The software Origin (OriginLab, Northampton, MA, USA) was used to obtain peak information of the NV colour centres' zero phonon lines characteristics (NV⁰, 575 nm and NV⁻, 637 nm) in the form of intensity, full width at half maximum and position through a Gaussian fit. First, all the spectra were normalized from 0 to 1. Then, they had their baseline adjusted to include the phonon sidebands below ZPL, similarly to the background subtraction performed by Plathotnik et al. [44]. Finally, they had their NV centre peaks fit with a Gaussian curve. A linear fit was calculated for the thermal evolution of the NV centres' fluorescence, yielding very accurate results regarding their R² quality of fit value. Relative sensitivities were calculated according to the following equation:

$$S_Q = (1/Q)(dQ/dT) \quad (1)$$

where S_Q represents the sensibility, Q is the NV centre's fluorescence and T is the temperature.

2.3. HeLa Images and Cytotoxicity Assays

HeLa cells were seeded on a sterile 96 well plate and cultured in DMEM (Dulbecco's modified eagle medium), supplemented with FBS (10%), sodium pyruvate (1 mM), penicillin (10 µg/mL), streptomycin (0.1 mg/mL) and incubated at 37 °C, CO₂ (5%) and 80% humidity; until reaching 80% of a monolayer.

For nanodiamonds-cells interaction, the cells were washed with phosphate-buffered saline (PBS) solution to remove non-adherents, dead and rest of metabolized medium. The 100 nm nanodiamonds solution concentrated to 0.01% *w/v* was diluted in sterile DMEM at 0.01% *w/v* and 200 µL of the solution was added to each well, incubated for 1 h under the same conditions described previously. The cells were stirred in the dark, then the solution was removed, and they were subjected to 3 washing steps with PBS before observation at the microscope. The images of the interaction between nanodiamond and HeLa cells were obtained with a confocal microscope Nikon Eclipse Ti (Japan). The 532 nm laser beam of 5–6 mW was focused onto the samples by a 20x objective (air, NA 0.95) and fluorescence images (1024 × 1024 pixels) were processed with the imaging software NIS-Elements (Nikon, Japan).

The viability of HeLa cells exposed to nanodiamonds was measured using the well-known 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For this assay, serial dilutions of nanodiamonds were prepared in a sterile saline solution of DMEM (10^{−2}, 10^{−3}, 10^{−4}, 10^{−5}, 10^{−6}, 10^{−7} and 0% as a viability control). The culture medium was removed and replaced with each dispersion of nanodiamonds solutions (four replicates for each concentration per assay). The protocol was previously reported by Fernández-Quiroz in 2019 [55]. MTT was diluted in PBS (5 mg/mL) and filtered through a 0.22 µm sterile filter. After 24 h of incubation, the stimulus was removed. MTT diluted in DMEM (100 µL, 12 mM) was added to the wells and incubated at 37 °C (5% CO₂) for 2 h. MTT reactive was carefully removed, and a 100 µL of Dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals formed. Then, the absorbance was measured at 570 nm in a UV spectrophotometer (Thermo Scientific Multiskan GO), and the results were compiled using SkanIt software (Thermo Fisher Scientific, Waltham, MA, USA). Unstimulated-negative control cells were incubated with the respective complete medium. Positive control cells were treated with 10% DMSO to cause cell death. Cell viability is expressed as a percentage of the negative control, given by Equation (2).

$$\text{Cell Viability (\%)} = (A_{\text{Sample}} / A_{\text{Control}})(100\%) \quad (2)$$

where A_{Sample} represents the measurement from wells treated with nanodiamonds solutions, and A_{Control} represents the value obtained from the wells treated with complete medium.

3. Results and Discussion

3.1. NV Centre Electronic Structure

In general, the electronic structure of the NV centre has six electrons. Two coming from the nitrogen atom, and another three are dangling bonds from the three carbon atoms surrounding the vacancy. The sixth electron is captured from the lattice, resulting in NV[−] charge state [56]. During the FNDs crystal growing others NV charge states may occur, such as NV⁰ and NV⁺. Among the created centres, NV⁰ and NV[−] are optically active but only NV[−] is both magnetically and optically active. The electron density of those centres is distributed in a plane vertical to the central NV axis, with the highest density on the three carbon atoms and the vacant site [57,58]. In this scheme, the electronic structure of NV⁰ has a ²E ground state, ²A₂ optical excited level, and an intermediate metastable ⁴A₂ level. The ²A₂ has no orbital degeneracy and exhibits no zero-field fine structure, but the ground state ²E has orbital degeneracy causing a spin-orbit fine structure [59]. For NV[−] centre, the ground base (³A₂) is a spin-triplet that at zero field state split in three sub-spin levels: a doublet ($m_s = \pm 1$) and a singlet ($m_s = 0$). Moreover, the NV[−] centre presents an additional

1A_1 metastable state [60]. In contrast, the excited electronic state 3E exhibits a complex fine structure composed mainly by spin-spin and spin-orbit interactions [61]. Figure 1a. Thermal energy enhances all the luminescent relaxation processes but also activates surface or defects states. These nonradiative relaxation channels are activated as temperature rises. The thermal relaxations may compete directly with the radiative response from ZPL in FNDs, diminishing their fluorescence signal [62].

Figure 1. (a) Energy level diagram of the NV^0 and NV^- centres in the diamond crystal lattice. The solid arrows denote the energy separation from the energy level to the valence or conduction band, and E_g is the diamond's bandgap. The dashed arrows characterize the intersystem crossing between two energy levels. The wavy coloured arrows are the observed radiative transitions. **(b)** Fluorescence spectra of HPHT nanodiamonds of different sizes recorded at 25 °C. The top-coloured lines are normalized fluorescence spectra, the top dashed black lines are the baselines, the bottom-coloured lines are the filtered spectra, the bottom solid black lines are the Gaussian fits, and the bottom-coloured areas correspond to fluorescence peaks of the NV centres.

3.2. Nanothermic Scale of High-Pressure High-Temperature Nanodiamonds

The fluorescence spectrum of 35 nm, 70 nm and 100 nm-sized nanodiamonds are shown in Figure 1b. The two fluorescence peaks detected at 575 nm and 637 nm correspond to the NV⁰ and NV⁻ colour centres defects' fluorescence, respectively. An energy level diagram for both charge states is shown in Figure 1a, illustrating which energy levels are found in either charge state configuration symmetry. The NV⁰ zero-phonon line transition ²A₂-²E has an energy of 2.15 eV corresponding to 575 nm. The NV⁻ zero-phonon line transition ³E-³A₂ has an energy of 1.94 eV corresponding to 637 nm. The two peaks detected at 659 nm and 680 nm are NV⁻ phonon sidebands ascribed to an electron-phonon coupling [63,64]. Optical excitation and emission are separated into zero-phonon lines and phonon sidebands. Vibrational properties determine the shape and extent of the phonon sidebands. In a radiative transition, the initial state is a vibrational ground state of an excited energy level, while the final state can be any vibrational state of the electronic ground state; hence, the phonon sideband transitions are usually Stokes-shifted [59,64,65]. The radiative transition ¹A₁-¹E of energy 1.19 eV corresponds to an infrared photon of a wavelength of 1042 nm and lies within the NV⁻ electronic structure.

Our results demonstrated how the NV centres' fluorescence from nanodiamonds is sensitive to temperature changes and can be accurately described by a linear model within the 25 °C to 60 °C range. Figure 2 shows how both charge states' fluorescence transforms concerning to temperature changes and their nanothermic scales adjusted employing a linear fit.

Figure 2. Fluorescence spectra of NV^0 and NV^- centres in HPHT nanodiamonds of 35 nm (a,b), 70 nm (d,e) and 100 nm (g,h) for temperatures from 25 °C to 60 °C. The dependence of the maximum fluorescence intensity on temperature (c,f,i) is obtained through normalization and using a Gaussian model. A linear fit gives R^2 values are 0.97825, 0.82308 and 0.98147 for the NV^0 centre and 0.90851, 0.93936 and 0.98033 for the NV^- centre, for nanodiamonds of 35 nm, 70 nm and 100 nm, respectively.

The 35 nm nanodiamonds yielded a very accurate linear data representation and sensitivities of 97%, 5.37% °C⁻¹ (NV^0) and 90%, 5.618% °C⁻¹ (NV^-), demonstrating outstanding thermometric performance. The 70 nm nanodiamonds yielded linear data representations of 82%, 6.365% °C⁻¹ (NV^0) and 93%, 6.475% °C⁻¹ (NV^-), performing slightly worse than the 35 nm-sized ones. The 100 nm nanodiamonds performed best among all the samples. They exhibited superior linear data representations of 98%, 6.994% °C⁻¹ (NV^0) and 98%, 4.14% °C⁻¹ (NV^-) than the other samples, mainly attributed to their high nitrogen-vacancy centre concentration [66–68] and their enhanced fluorescence signal of the NV^- dominant charge state [23]. Remarkably, the reported sensitivities represent uncertainties around ± 1.3 °C for both NV centres marginally bigger than those reported in ODMR experiments (≈ 0.5 – 1 °C) [69,70].

We observed how the 70 nm-sized nanodiamonds' NV^0 intensity exhibited an anomalous feature in the analysis, where a linear curve could only represent 82% of the data, whereas the 35 nm (93%) and 100 nm (98%) sized ones reached higher R^2 values. This

fact may be attributed to sp^2 carbon contamination of the surface during the growth process [71] and the proximity of NV centres to her [72]. Furthermore, the nanothermic scales for 35 nm-sized nanodiamonds performed nearly as good as the 100 nm-sized nanodiamonds, differing in the overall spectrum broadening due to a smaller particle size [23].

As an example of thermal bioimaging at room temperature, a fluorescence image of the HeLa cells incubated with 100 nm nanodiamonds is shown in Figure 3a. Moreover, their original shape remains unaltered because of a non-cytotoxic interaction. The HPHT FNDs rendered biocompatible ($>90\%$) for concentrations up to 0.1%, as depicted in Figure 3b. We observe that most nanodiamonds are accumulated preferentially on the protuberance generated by the nucleus' volume (Figure 3a). This feature has been used to indirectly measure the temperature on the malignant cell's nucleus, which presents a higher gene expression rate and protein production and demands higher energy levels of ATP consumption [73]. The thermal bioimaging for measuring cell temperature of protuberances may be an efficient technique to differentiate between healthy and infected cells [74]. Improvements to thermal bioimage could be achieved with the implantation of selected ions to the surface of FNDs to slow NV^- to NV^0 conversion energy. Being H, an efficient fluorescent termination as demonstrated by Kratochvílová et al. [75].

Figure 3. (a) Fluorescence of 100 nm HPHT FNDs in HeLa cells. The fluorescence image was recorded around the membrane after 24 h of incubation at 37 °C. (b) MTT Cytotoxicity assay of different concentrations of 100 nm HPHT FNDs in HeLa cells, the red bar corresponds to the concentration used in (a).

4. Conclusions

In general, the overall fluorescence intensity of the fluorescent nanodiamonds used for this work decreases as temperature rises within the range of biologically relevant temperatures (25 °C to 60 °C). We demonstrated how this fluorescence intensity mostly follows a linear dependence with the local nanodiamond temperature. Furthermore, there is a size dependency on the processes involved in the changes in the overall fluorescence spectrum of nanodiamonds which relates the smaller nanodiamonds with a broader and less intense signal, while larger nanodiamonds with a sharper and brighter spectrum. However, the ZPL position remains unaltered in all cases, and then the fitting process can discriminate and account for them successfully.

The NV^0 and NV^- centres' fluorescence allowed the construction of nanothermic scales. Additionally, this study unveils the possibility of using nanodiamonds as nanothermometers to distinguish delimitation of tumoral margins and several types of pathologies through the point measurement of the membrane's temperature (70 nm or 100 nm size nanodiamonds) or inner structures of malignant cells (with 35 nm or smaller nanodiamonds).

Here, biomedical applications such as, the imaging and diagnosing instrumentation based on fluorescence became simple and could be implemented easily in biomedical facilities inside hospitals and health clinics.

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