Photoacoustic emission from fluorescent nanodiamonds enhanced with gold nanoparticles

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Abstract: Fluorescent nanodiamonds (FNDs) have drawn much attention in recent years for biomedical imaging applications due to their desired physical properties including excellent photostability, high biocompatibility, extended far-red fluorescence emission, and ease of surface functionalization. Here we explore a new feature of FNDs, i.e. their photoacoustic emission capability, which may lead to potential applications of using FNDs as a dual imaging contrast agent for combined fluorescence and photoacoustic imaging modalities. We observed significant enhancement of photoacoustic emission from FNDs when they were conjugated with gold nanoparticles (GNPs).

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1. Introduction

Live imaging based on laser-induced fluorescence techniques has played an especially pivotal role in biomedical research and applications, because it allows real-time observation of cell behavior in intact organisms using vital stains and fluorescent reporter proteins. However, conventional fluorescent biomarkers used in these observations are subject to several limitations. Optical absorption and scattering attenuate the signal, effectively reducing the ability to detect or image deep targets in tissue or other turbid media, as well as diffusing the recorded fluorescence, thus masking the location of the fluorophore source [1]. Another limitation is fluorophore photobleaching, which poses a challenge when imaging labeled samples for an extended period of time. Both of these limitations may be partially or completely overcome by the use of nanoparticles, which exhibit unique optical, electronic, magnetic, and material properties at the nanoscale, facilitating their use in commercial applications.

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industrial, military, and biomedical applications. The use of multi-component particles, combining different classes of materials with potentially complementary or synergistic properties, can be a productive approach for developing highly efficient contrast agents for imaging or sensing applications. The present work illustrates the use of a hybrid nanomaterial consisting of a nanodiamond combined with a gold nanoparticle and possessing photoacoustic response properties greatly enhanced over those of the component materials alone. Because of the ability of acoustic waves to propagate for long distances through tissue, the strong photoacoustic responses of these hybrid particles support their use as contrast agents in such applications as whole body imaging.

Fluorescent nanodiamonds (FNDs) have been demonstrated as an excellent alternative to conventional fluorescent biomarkers through a series of studies [2–7]. FNDs are carbon-based nanocrystallites containing nitrogen-vacancy sites as fluorescence-emission centers. FNDs are extremely photostable due to their ultrastable diamond structure, which ensures long-term monitoring of biological processes without fluorescence photobleaching problems [8–10]. FNDs emit extended far-red (600-800 nm) fluorescence with brightness over two orders of magnitudes higher than typical dye molecules, such as Alexa Fluor or Rhodamine 6G [2,11]. Moreover, FNDs are also non-toxic; this property allows them to be used as highly biocompatible, in vivo biomarkers [4,6,8,12,13]. At present, most of the studies on FNDs have been focused on their fluorescence imaging applications, but little focus has been on their potential photoacoustic applications.

Photoacoustic tomography (PAT) has gained popularity in recent years as a hybrid technology that combines rich optical contrast mechanisms and superior ultrasonic penetration depth and resolution in optically scattering biological tissues for three-dimensional in vivo imaging [14–17]. In PAT, a short-pulsed laser irradiates biological tissues, causing wideband ultrasonic waves to be created (referred to as photoacoustic emission) as a result of transient thermoelastic expansion if the tissue absorbs energy of the laser pulse. The magnitude of the photoacoustic emission is proportional to the local optical energy absorption by the tissue, creating contrasting signals dependent on the endogenous components of a given tissue type. For example, the contrast from hemoglobin in blood has been widely used to determine the blood oxygen saturation level, such as hypoxia, in photoacoustic imaging of suspicious tissues to diagnose tissue malignancy [18]. On the other hand, exogenous contrast agents with good photoacoustic emission properties can be used as extrinsic labels for molecular imaging of gene expressions, biological structures, or macromolecules such as pathogen-associated antigens [19–26]. In this letter, we report the study on photoacoustic features of FNDs to explore their potential applications as contrast agents for PA tomography and spectroscopy.

We conjugated gold nanoparticles (GNPs) with FNDs to further enhance the photoacoustic emission. In addition, the fluorescence properties of the conjugates were also studied.

2. Experiment

Fabrication of FNDs: The FND fabrication was based on the method described previously [3,27]. Briefly, FNDs were fabricated from type-Ib diamond nanocrystallites with a median size of ~100 nm under 40-keV He + ion beam irradiation from a lab-built ion beam facility, followed by vacuum annealing at 800 °C. Negatively charged nitrogen-vacancy centers (N-V−) were produced in the diamond nanocrystallites (Fig. 1(a)), which gave rise to red fluorescence emission (600-800 nm) when excited with green-yellow light (Fig. 1(b)).
Fig. 1. (a) Nitrogen-vacancy centers can be produced in type-Ib diamond nanocrystallites under irradiation of 40-keV He\(^+\) ion beam and followed by vacuum annealing at 800 °C. The nitrogen-vacancy centers of the FNDs are responsible for their fluorescence emission; (b) Absorption spectrum of type Ib diamond at 77 K due to nitrogen-vacancy centers (green) [28] and measured fluorescence emission spectrum of FNDs (red); and (c) Schematic diagram of conjugation of FNDs with GNPs.

Synthesis of GNP-FND conjugates: FNDs are composed of sp\(^3\) carbon. Its surface can be readily grafted with carboxyl groups. To facilitate this grafting process, freshly prepared FNDs were oxidized in air at 450 °C to remove graphitic surface structures and subsequently treated in strong oxidative acids to functionalize their surfaces with rich carboxyl groups [29–31]. GNPs with amine groups were covalently linked with FNDs through dehydration reactions (Fig. 1(c)). For that, the carboxyl groups on the FNDs (200 µg) surface were first activated with the aid of 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) molecules for about 15 minutes (pH at 4.5), with the molar ratios of –COOH: EDC: NHS = 1: 250: 100. The GNPs were then added into the FND solution (with pH value of 8.0) and the particle ratio between the GNPs and FNDs was chosen to be 5:1. The mixture was then vortexed for 4 hours. The non-reacted molecules were removed by centrifugation at a speed of 15000 rpm. We selected amine terminated GNPs (Nanopartz) with a medium size of 20 nm, because their surface plasmon resonant (SPR) wavelength matches with the absorption spectrum of FNDs that has a strong absorption in the spectral range of 500-620 nm.

Fluorescence measurement: The fluorescence measurement setup is shown in Fig. 2, where a supercontinuum fiber laser (Fianium, SC-400-pp) was used as the excitation source with a wide range of tunability (400 – 2500 nm). Excitation band pass filters were used to select desired excitation wavelengths. In this study, we selected two excitation wavelengths using a filter (Chroma, HQ565/30) centered at 565 nm with a bandwidth of 30 nm and another filter (Chroma, HQ530/30) centered at 530 nm with a bandwidth of 30 nm, respectively. The laser beam passing through the bandpass filter was reflected by a dichroic beamsplitter with a separation wavelength around 610 nm. The beam was then focused by a microscope objective lens (10 ×, NA = 0.25, Leica) into a cuvette containing FNDs or FND-5GNP conjugates in deionized water. The FND solution has a concentration of 250 µg/mL, and the FND-5GNP solution has the same concentration in terms of the FND contents. Fluorescence emission from each sample was collected with the same objective lens and passed through the dichroic beamsplitter. It was further filtered using a long-pass filter with a cut-off wavelength at 600
nm (Chroma, LP600) and focused into a fiber-coupled spectrometer (Photon Control, Spectrometer-SPM-002) and then detected with a Hamamatsu S9840 back-thinned CCD array.

**Photoacoustic measurements:** Fig. 3 shows a schematic diagram of the system we built for the photoacoustic signal measurements [25,26]. The excitation source was from a tunable optical parametric oscillator (OPO) (Opolette HR 355 II), which has a pulse duration of ~5 ns, a wavelength tunable from 410 to 2200 nm, a repetition rate of 1 - 20 Hz, and a maximum pulse energy of 3 mJ.
For this study, we selected two excitation wavelengths at 530 and 565 nm. The former is at the absorption peak of the pure GNPs and the latter is close to the absorption maximum of the (N-V)$^-$ center in FNDs. The pulse energy from the OPO system was adjusted to 1.5 mJ at the sample well. The diameter of each sample well in a microtiter plate was 6 mm. The excitation laser beam passed through the sample well from its bottom with a beam diameter of 3 mm, resulting in an energy density of 21 mJ/cm$^2$.

A row of sample wells containing FNDs, GNPs, FND-5GNP, and pure deionized water, respectively, were placed in a water tank and their positions were controlled with a programmable translational stage. Photoacoustic emissions occurred when the sample absorbed the energy from the excitation laser due to thermoelastic effects. Different from a conventional method of using a piezoelectric ultrasound transducer for the detection of photoacoustic signals, we used a probe beam deflection technique (PBDT) [32,33]. The principle of this technique is to detect the change in the direction of a probe laser beam affected by the refractive index gradient produced when the photoacoustic signal passing through the water tank. The sensitivity of the PBDT is linear with the rate of change of pressure with time, and is governed by such factors as the intensity and wavelength of the laser probe beam, the medium properties such as change (dn/dp) of refractive index with transient pressure, and photodiode diode sensitivity and amplifications. Recent work demonstrated that a typical PBDT implementation provided competitive signal to noise ratio compared to a conventional Lithium Niobate ultrasound transducer with a 5-mm aperture [34]. A compact diode laser emitting 3 mW at 670 nm was used as the probe beam. The elliptical beam of the diode laser was focused to a beam waist of 150 µm for the long axis of the beam, which was 3 mm under the sample well. The long axis of the beam was aligned to be parallel to the bottom surface of the well for enhanced detection sensitivity. A position sensitive detector containing a pair of photodiodes was used to monitor the deflection of the probe beam due to propagation of photoacoustic signals in the water tank. This PBDT approach is sensitive to photoacoustic signals, while insensitive to background electronic noise, minimizing the need for acoustic isolation or shielding as in a conventional piezoelectric ultrasound transducer [25,26].

3. Results and discussion

**Photoacoustic signal enhancement of FNDs:** Fig. 4(a), 4(b) and 4(c) show the raw data of the photoacoustic signals emitted from FNDs, GNPs, and FND-5GNP conjugates, respectively, when these samples are excited by the OPO system tuned to 530 nm.
Each curve is from the recording of the signal by the position-sensitive detector that monitors the change in the position of the probe laser beam due to the modulation of the photoacoustic signal generated by a single laser pulse on the sample. The photoacoustic signal from FNDs is much weaker compared with the signals from the pure GNPs and the FND-5GNP conjugates. Figure 4(d) shows the accumulated signals for 10 laser shots for FNDs, GNPs, and FND-5GNP conjugates under the excitation at 530 nm or 565 nm, respectively. The photoacoustic signals are enhanced by a factor of 30, when the FNDs are conjugated with GNPs at a ratio of 5 GNPs for each FND.

In the experiments the molar concentration of the GNPs is the same as that of FND-5GNP in terms of its GNP contents, while the molar concentration of the FNDs is the same as that of the FND-5GNP in terms of its FND contents. Therefore, the enhancement of the photoacoustic signals of FNDs is not due to variation of sample concentrations. We attribute the enhancement effect to the energy transfer between the FNDs and the GNPs within the conjugates. As the photoacoustic signal arises from thermoeelastic expansion after a sample absorbs the energy from a nanosecond laser pulse and converts the energy to heat, it is desirable for the sample to not dissipate the absorbed energy other than converting it into heat to optimize the photoacoustic emission. The high fluorescence quantum yield of the (N-V) center in FNDs limits the heat conversion and therefore results in low efficiency in photoacoustic signal generation. By conjugating GNPs with FNDs, it not only increases the energy absorption of FNDs due to the enhanced local field around the GNPs, but also efficiently suppresses the fluorescence emission from FNDs by fluorescence quenching effects via the GNPs. Thus, the observed photoacoustic signals from the FND-5GNP conjugates were significantly enhanced compared with pure FNDs. The fluorescence quenching effect of the conjugates has been verified through the experiments described below.

**Fluorescence quenching of FND-5GNP conjugates:** The fluorescence spectra of FNDs and FND-5GNP conjugates when excited at 565 and 530 nm are illustrated in Fig. 5(a) and 5(b), respectively.

The fluorescence of FNDs was dramatically quenched by about 9-fold and 4-fold for the excitation at 565 nm and 530 nm, respectively. Because the FNDs are about 100 nm in diameter, the strong quenching effects suggest that the (N-V) centers responsible for the FND fluorescence emission are located on or close to the surface of the FNDs. Thus, efficient energy transfer may occur within the conjugates between the surface plasmon resonance of the GNPs and the (N-V) centers of the FNDs. This leads to significantly increased non-radiative decay of FNDs, thus reducing the fluorescence emission and enhancing the photoacoustic emission.
4. Summary

An investigation of the photoacoustic property of FNDs was performed. Although the photoacoustic signal of pure FNDs was weak, the signal can be significantly enhanced by a factor of 30 when the FNDs were conjugated with GNPs. The large increase in photoacoustic signal was attributed to the local field enhancement of the GNPs and the energy transfer between FNDs and GNPs, which increased the non-radiative decay processes of FNDs and therefore the conversion efficiency of the absorbed laser energy into heat for enhanced thermoelastic effects. This conclusion was further confirmed by the fluorescence measurements which showed efficient fluorescence quenching for the FND-5GNP conjugates. This study suggests that the fluorescence and photoacoustic properties of FNDs may be modulated with conjugated GNPs. Potential applications of using FNDs as a dual imaging contrast agent for combined fluorescence and photoacoustic imaging modalities may become possible in future if photocleavable linkers are used for synthesizing the FND-GNP conjugates. In addition, FND-GNP hybrid particles may be ideal contrast agents for use in photoacoustic imaging modalities.

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