

**APPLICATION OF
NANODIAMONDS FOR
BIOLOGICAL INVESTIGATION**

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PREFACE

Fluorescence imaging is a powerful tool for visualizing how biological molecules behave in cells. As a novel imaging probe, fluorescent nanodiamond has been received increasing attention. Its perfect photo-stability and optically detected magnetic resonance signal are expected to open up a new field of biology, especially magneto-, electro- and thermo-sensing.

This thesis contains authors works and discussions at Department of Molecular Engineering, Graduate School of Engineering, Kyoto University during the period from April 2010 to March 2015. The main purpose of this thesis is to prepare the bio-applicable nanodiamonds as optically detected magnetic resonance (ODMR) probe. Part II Chapter I provides the studies of ion irradiation for producing nanodiamonds containing negatively-charged nitrogen-vacancy centers. Chapter II provides charge state conversion for enhancing the ODMR intensity. Chapter III provides a comprehensive analysis for controlling chemical/physical states and properties of nanodiamonds subjected to annealing and oxidation processes. Chapter IV provides a selective targeting method of nanodiamonds to bio-molecules of interest. The summary of this thesis is provided in Part III.

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Part I

General Introduction

Fabrication of diamond crystal

Diamond is one of the crystal form of carbon. They are naturally formed at high-pressure (more than 30 kilobars) and high-temperature (more than 400 degrees) in the Earth mantle,[1, 2] which occurs over long period of more than one billion years. In recent years, artificial synthetic methods have developed such as High-Pressure High-Temperature (HPHT), Chemical Vapor Deposition (CVD) and Detonation. HPHT is the first developed synthesis of diamond by General Electric in 1954.[3] This method essentially mimics the natural process; diamonds are created deep in the earth where high-pressure high-temperature condition makes carbon crystals to the high density structure such as diamond (Figure. 1). This process makes it easy

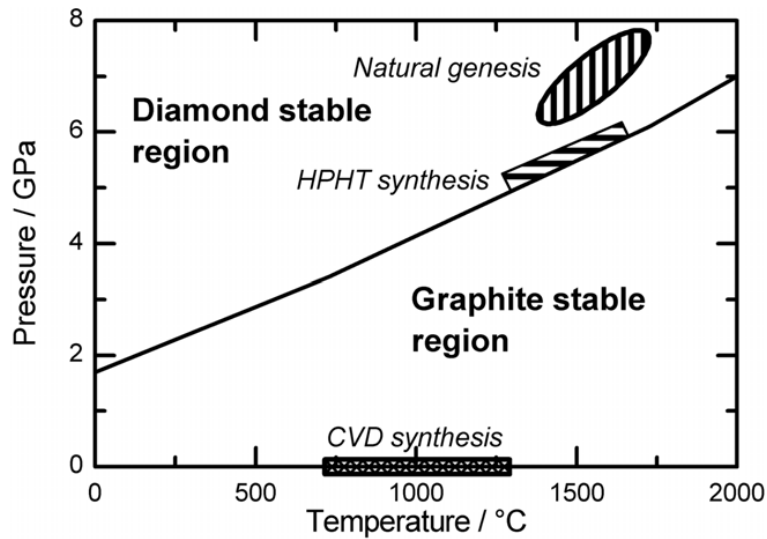


Figure 1. Phase diagram for carbon indicating main regions of pressure-temperature space in which diamond growth occurs.

to control the quality and geometry of diamond crystal, however, the experimental condition of pressure as 60,000 atmosphere is quite tricky and costly.

CVD is also the popular method for preparing synthetic diamond, which was

first performed in 1952 and work leading to industrialized processes was reported in the 1980s.[4, 5] The process generally takes place at below atmospheric pressure. The starting material, typically a mixture of methane and 1 to 5% of hydrogen gasses, is broke into fragments and reassemble into diamond on a surface of wafer by arc jets, microwaves, blowtorches of laser. The method enables the relatively large sized-diamond, but growth ratio is low (typically 1 μm per hour).

Detonation process is also famous method for the production of diamond. In 1963, Ukrainian physicist found that soot from the explosion of a 1:1 mixture of TNT-hexogen, which is military explosive, contained nano-sized diamonds in high concentration.[6] These diamonds are called detonation diamonds. They have been produced on industrial scale mainly in Russia and China since about 1990s. Their primary size is extremely small as 4-5 nm in diameter. Normally they form sub-micron-sized aggregation, so beads milling is required for deaggregation. Such detonation diamonds are commonly used as lubricant and abrading agent.

Color centers in diamond

Diamond has a lot of color centers in the crystal structure, for example, neutral vacancy (GR1), nitrogen-vacancy center (NVC), nitrogen-nitrogen-vacancy center (H_3) and nitrogen-nitrogen-nitrogen-vacancy center (N_3) (Figure 2). Each color center has been well characterized by optical.[7] In these centers, NVC is the well-studied one, which is formed by a substitutional nitrogen atom combining with an adjacent vacancy. NVCs absorb the photons in the range of green, and emit fluorescence in the near-infrared region (Figure 3).

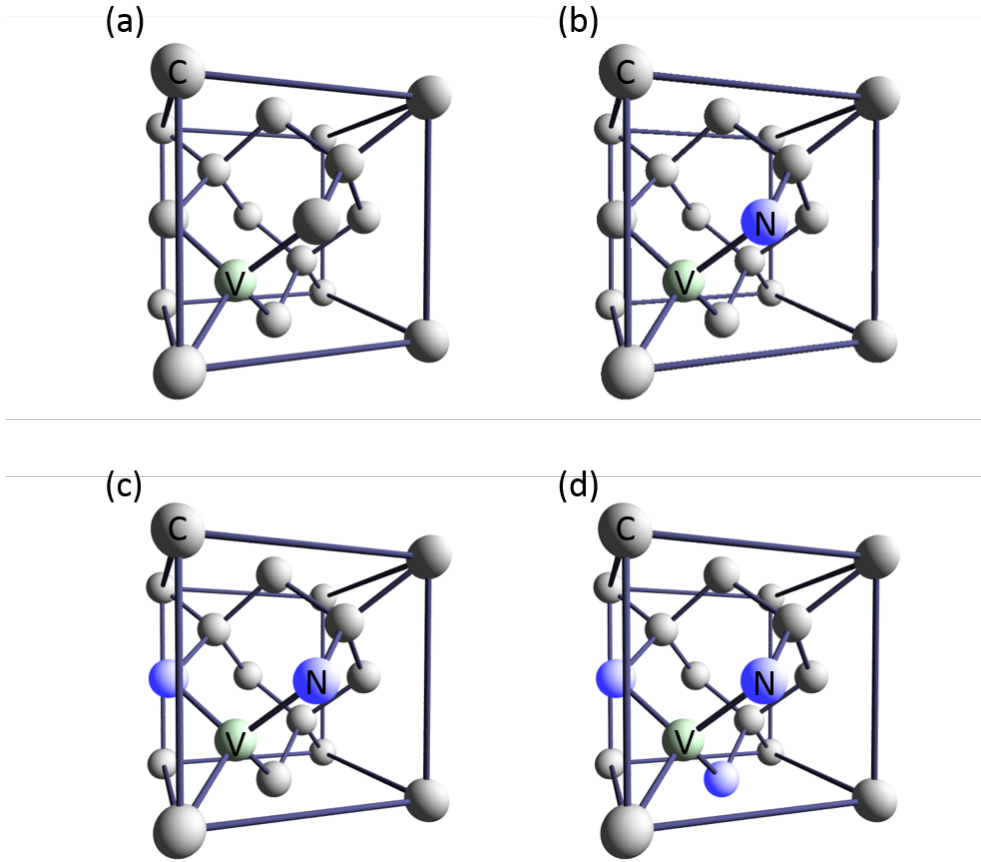


Figure 2. Vacancy related color centers in diamond: (a) GR1, (b) NVC, (c)H3, (d)N3.

Table 1. Properties of some vacancy-related defects.^a

Color centers	ZPL (nm)	λ_{em} (nm)	τ (ns)	Q.Y.
GR1	741.2	898	2.55	0.014
NV ⁻	637.6	685	11.6	0.99
NV ⁰	575.4	600	-	-
H3	503.5	531	16	0.95
N3	415.4	445	41	0.29

^a Listed are zero-phonon lines (ZPL), emission maxima (λ_{em}), emission lifetimes (τ), and quantum yields (Q.Y.)

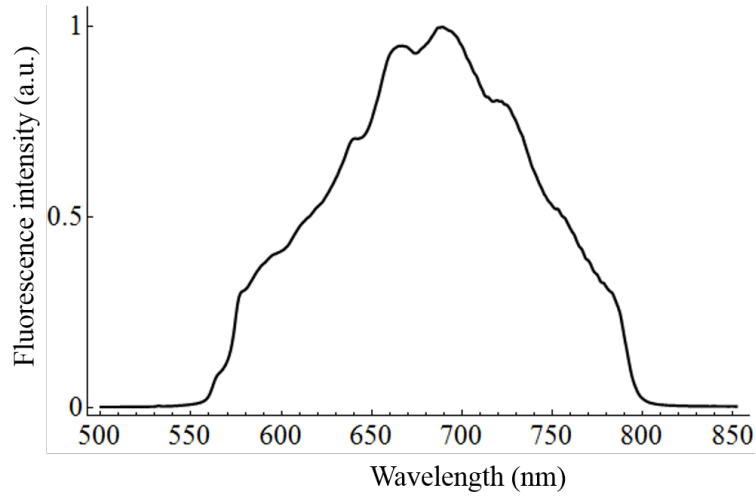


Figure 3. Fluorescence spectrum of NVCs.

In general, this color center rarely exists in natural diamond, so some additional process are required for the introduction of NVCs. In type Ib diamonds, nitrogen atoms are contained in the crystal as impurities for 1 to 100 ppm,[8] however, lattice vacancies are very few.[9] To improve the concentration of vacancies, irradiation by high energy particle such as electrons, protons, neutrons and ions is reported to be quite effective technique.[10, 11, 12, 13, 14] Then the diamond samples are thermally annealed at 800 °C in vacuum in order to pairing the each of nitrogen atom and vacancy, then forming an NVC.[14, 15] Subsequently, oxidation process such as acid treatment or thermal oxidation in air are applied for the elimination of surface contamination and graphite caused by annealing process, and therefore increase the brightness of NVCs.[16, 17]

Fluorescence properties of NVC

Nanodiamonds (NDs) emit visible fluorescence from intrinsic NVCs, but their

wavelength of emission is influenced by the particle size, excitation source, as well as how the diamond material is made.[18, 19, 20, 21, 22] There are two types of NVCs, each with a different charged state (Figure 4).[23, 24]

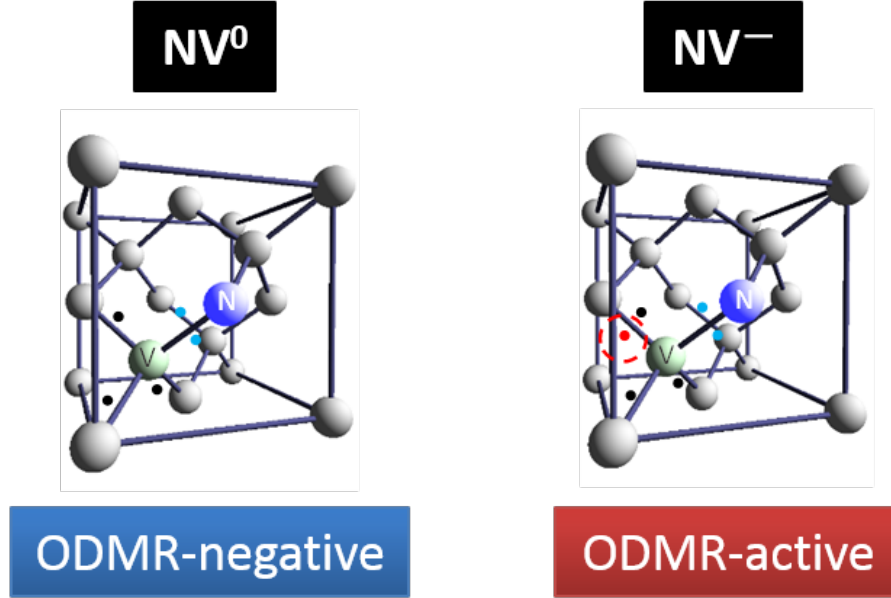


Figure 4. Two types of NVCs: Neutral NVC (NV^0) and negatively-charged NVC (NV^-).

One is a neutral NVC (NV^0 , electron spin: $S = 1/2$), while the other is a negatively-charged NVC (NV^- , electron spin: $S = 1$), represented by zero-phonon lines at 575 and 637 nm respectively.[25, 26] Both NVCs show excellent photo-stability. They emit fluorescence with a high quantum yield of 0.7, and it shows neither undesirable photo-properties such as photo-bleaching nor photo-blinking.[11, 27] Its excellent properties enable long-term *in vivo* and *in vitro* bio-imaging, which is not readily achieved by commonly used fluorescent agents such as organic dyes and fluorescent proteins.[13, 28, 29, 30, 31, 32] It has also been reported that the NDs are highly bio-compatible and non-toxic to various kinds of cell types, and are thus superior to quantum dots which often show cyto-toxicity.[33] Table 1 displays the comparison

of the properties of ND, quantum dot, fluorescence dye and fluorescent protein.

Table 2. Properties of various fluorescent agents.

	ND (NVC)	Quantum dot	Fluo. dye	Fluo. protein
Photo-bleaching	No	Yes	Yes	Yes
Photo-blinking	No	Yes	Yes	Yes
Quantum yield	0.7	0.1~0.8	0.1~0.3	0.6
Fluorescence lifetime	~20 ns	~10 ns	~2 ns	~4 ns
Toxity	No	Yes	-	No
Chemical stability	High	Low	-	Low

It is found that NDs are superior to another fluorescent agent in many aspects. These advantages of the NDs over conventional fluorophores could make them a potential candidate for optical bio-imaging probes.

Optically detected magnetic resonance

NVC has another unique optical property in addition to the the perfect photo-stability. Both NVCs (NV^0 and NV^-) emit fluorescence, but only NV^- allows for optical read out of electron spin (ODMR: optically detected magnetic resonance). NV^- has trigonal C_{3v} symmetry and ground state forms a spin triplet ($S = 1$). The spin dynamics is governed by the following Hamiltonian,

$$H = g\mu_B B S + D[S_z^2 - \frac{1}{3}S(S+1)] + E(S_x^2 - S_y^2), \quad (1)$$

Where g , μ_B , B , S , D and E are g -factor for electron, Bohr magneton, external magnetic field, electron spin vector ($S = 1$ for triplet state), fine-structure zero-field splitting and strain induced splitting coefficient, respectively. The working principle

of ODMR is following. Figure 5 shows the schematic energy diagram of NV^- .

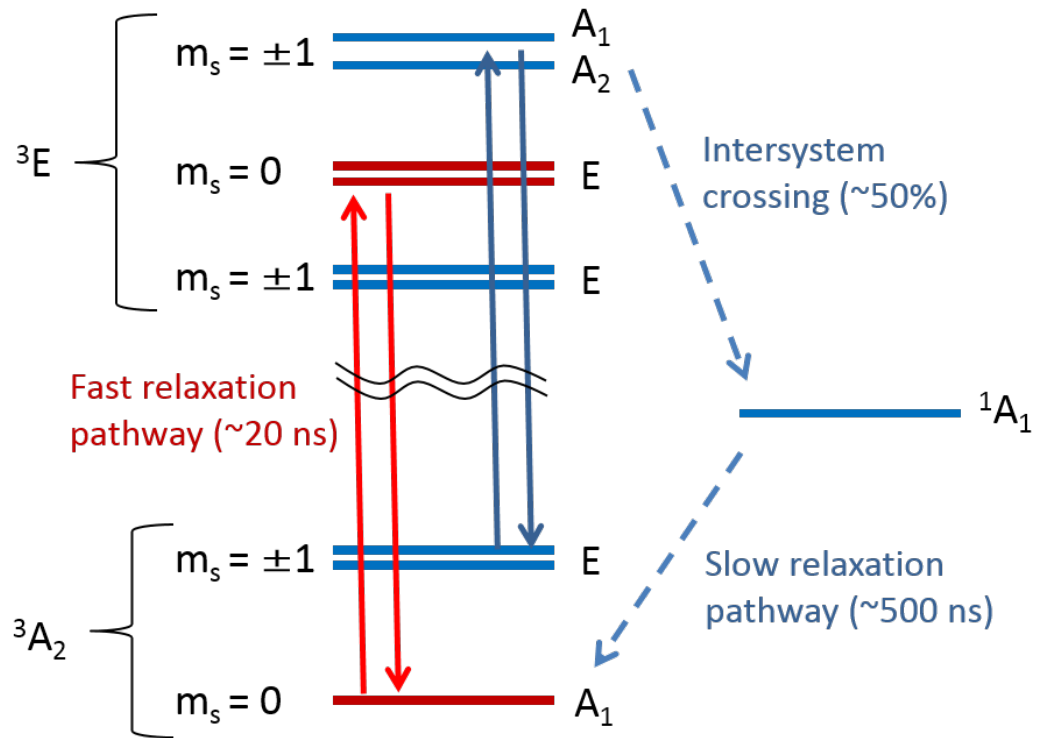


Figure 5. Schematic diagram of the principal of ODMR.

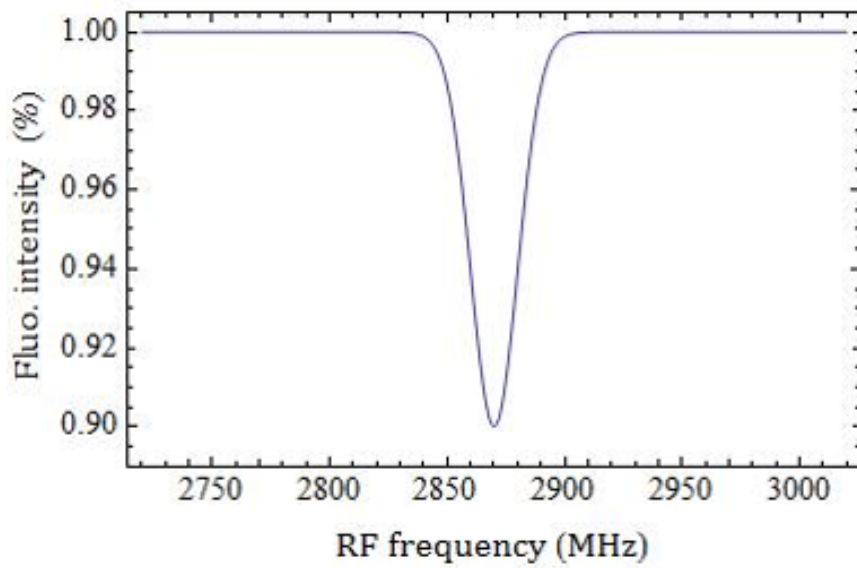


Figure 6. ODMR spectrum.

The crystal fields together with the on-site magnetic interaction separate three degenerated spin state into two energy levels of $m_s = 0$ and degenerated $m_s = \pm 1$, which is split by 2.87 GHz without external magnetic fields. Microwave (MW) irradiation at this frequency induces electron spin magnetic resonance between $m_s = 0$ and degenerated $m_s = \pm 1$ spin levels, which results in a reduction of the fluorescence intensity from NV^- due to spin-dependent intersystem crossing.[34] Thus $m_s = \pm 1$ fluorescence less than the $m_s = 0$ state, with a difference in initial fluorescence of ~ 20 to 40% . Namely, MW-assisted electron spin resonance can be detected by the fluorescence intensity. Figure 6 shows the typical ODMR spectrum.

Recent studies of biological investigation through nanodiamonds

A lot of papers have reported the biological investigation using ND as fluorescence or ODMR probes. Chang *et al.* demonstrated three-dimensional tracking of a single ND particle within the cell over 200 s.[13] Wu *et al.* showed fluorescent ND can identify transplanted CD45 $^-$ CD54 $^+$ CD157 $^+$ lung stem/progenitor cells in vivo, and track their engraftment and regenerative capabilities with single-cell resolution.[35] Kusco *et al.* demonstrated that NV^- in diamond can be used as robust temperature sensors that combine the virtue of submicrometre spatial resolution, subdegree thermal sensitivity and biocompatibility.[36] McGuinness *et al.* measured the location, orientation, spin levels and spin coherence times with nanoscale precision in living cells.[37] Geiselmann *et al.* showed the three dimensional spatial manipulation of individual NDs hosting a single NV spin.[38] Igarashi *et al.* demonstrated real-time back-ground free selective imaging of NDs in cell and *C.elegan*.[31] In spite of these

excellent studies, single molecule measurement has not been demonstrated yet. As NDs can determine the orientation of nitrogen-vacancy axis towards external magnetic fields with 1 degree precision, molecular dynamics of ND-labeled protein can be precisely measured in real-time and *in vivo*.

Preparation of bio-applicable nanodiamonds

Application of NDs to biological investigation is restricted by following problems. Concentration of NDs containing NV⁻s is too low to perform ODMR experiments. For improving the concentration of NV⁻s in NDs, ion irradiation is the potential method. In my study, I elucidated the optimal condition of ion irradiation for producing the NDs containing NV⁻s (Chapter I, Part II). Particle size of NDs are also important for bio-imaging, because protein labeling of too much large sized NDs hampers the protein dynamics. Ideally, it is preferable that the size of NDs is single-digit nanometer, however, magneto-sensing from such small NDs have not been achieved yet. Here I report the magneto sensing by detonation synthesized nanodiamonds (DNDs) (Chapter II, Part II). Particle properties and physical/chemical states of NDs through annealing and oxidation process, which are indispensable steps for producing fluorescent NDs, are still remains unknown. Specifically, the weight loss, particle size, crystal quality, chemical bonding states of carbon and oxygen, zeta potential, dispersibility, and fluorescent and optically detected magnetic resonance (ODMR) properties were determined using x-ray diffraction (XRD) analysis, transmission electron microscopy (TEM), elemental analysis, dynamic light scattering, Raman analysis, x-ray photoelectron spectroscopy (XPS), IR spectroscopy,

and a home-made fluorescence and ODMR microscope (Chapter III, Part II). Dispersibility of NDs in physiological media is quite low, and non-specific adsorption of biomolecules on the surface through hydrophobic interaction lead to decreased of reliability for target specificity. Here, I quantitatively elucidated that a hyper-branched polyglycerol modified NDs realize both excellent dispersibility and marked suppression of non-specific adsorption to proteins. Moreover, selective targeting to the proteins of interest, and a measurement of optically detected magnetic resonance from the NDs on living cells are demonstrated (Chapter IV, Part II).

Experimental set-up

In my study, I try to apply those excellent optical properties of NDs for biological investigation. Figure 7 is a schematic diagrams of the experimental set-up. The field of view was illuminated with a 532-nm Nd:YAG laser, and fluorescence images were measured by an electron multiplying charge coupled device (EMCCD) camera. The emitted light was collected by an oil immersion 60 or 100 $\times$ objective (NA=1.49), passed through a dichroic mirror centered at 575 nm and a long-wave (short-wave) pass filter longer than 590 nm (shorter than 845 nm) to detect the fluorescence signal from NVCs. The microscope was equipped with a MW coil and triaxial electron magnet above sample stage for irradiating the resonant frequency and external magnetic fields.

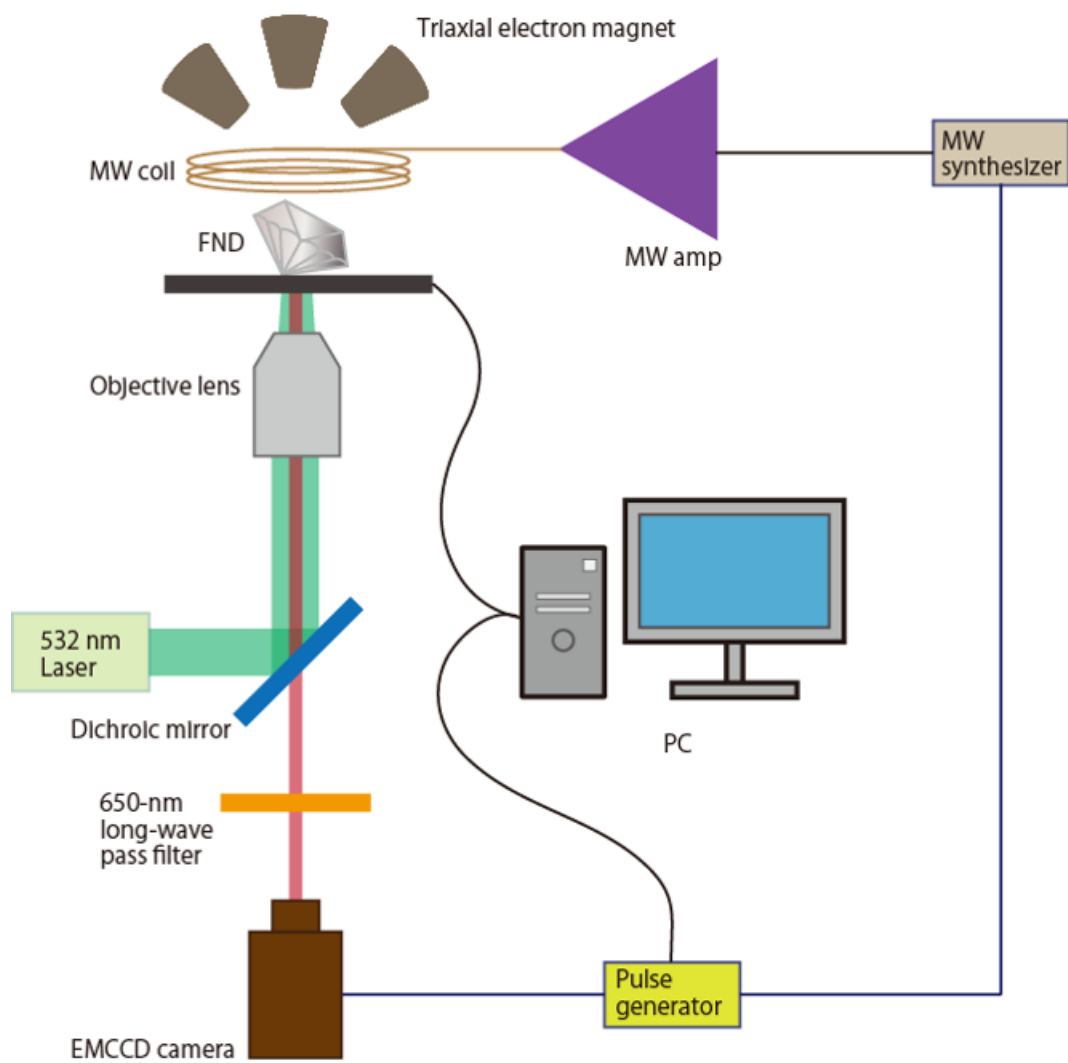


Figure 7. Schematic diagram of the experimental set-up.

References

- [1] H. Bratt, R. Tracy, and B. Qwens. Petrology. *Petrology: igneous, sedimentary, and metamorphic*, p. 212, 1996.
- [2] A. Finnerty and F. Boyd. Evaluation of thermobarometers for garnet peridotites. *Geochimica et Cosmochimica Acta*, Vol. 48, No. 1, pp. 15–27, January 1984.
- [3] F. Bundy, H. Hall, H. Strong, and R. Wnetorf. Man-made diamonds. *Nature*, Vol. 176, p. 51, 1955.
- [4] R. Balmer, J. Brandon, S. Clewes, H. Dhillon, J. Dodson, P. Madgwick T. Markham M. Mollart T. Perkins N. Scarsbrook G. Twitchen D. Whitehead J. Wilman J. Friel, I. Inglis, and S. Woollard. Chemical vapour deposition synthetic diamond: materials, technology and applications. *Journal of physics. Condensed matter : an Institute of Physics journal*, Vol. 21, No. 36, p. 364221, September 2009.
- [5] P. Martineau, M. Gaukroger, K. Guy, S. Lawson, D. Twitchen, I. Friel, J. Hansen, G. Summerton, T. Addison, and R. Burns. High crystalline quality single crystal chemical vapour deposition diamond. *Journal of physics. Condensed matter : an Institute of Physics journal*, Vol. 21, No. 36, p. 364205, September 2009.
- [6] L. Tang, C. Tsai, W. Gerberich, L. Kruckebeu, and D. Kania. Biocompatibility of chemical-vapour- deposited diamond. Vol. 16, No. 6, pp. 483–488, 1995.

- [7] Y. Hui and H. Chang. Recent Developments and Applications of Nanodiamonds as Versatile Bioimaging Agents. *Journal of the Chinese Chemical Society*, Vol. 61, No. 1, pp. 67–76, January 2014.
- [8] A. Schrand, S. Hens, and O. Shenderova. Nanodiamond Particles: Properties and Perspectives for Bioapplications. *Critical Reviews in Solid State and Materials Sciences*, Vol. 34, No. 1, pp. 18–74, January 2009.
- [9] V. Acosta, E. Bauch, and M. Ledbetter. Temperature dependence of the nitrogen-vacancy magnetic resonance in diamond. *Physical review ...*, Vol. 5, pp. 397–405, 2010.
- [10] G. Dantelle, A. Slablab, L. Rondin, F. Lainé, F. Carrel, P. Bergonzo, S. Peruchas, T. Gacoin, F. Treussart, and J. Roch. Efficient production of NV colour centres in nanodiamonds using high-energy electron irradiation. *Journal of Luminescence*, Vol. 130, No. 9, pp. 1655–1658, September 2010.
- [11] S. Yu, M. Kang, H. Chang, K. Chen, and Y. Yu. Bright fluorescent nanodiamonds: no photobleaching and low cytotoxicity. *Journal of the American Chemical Society*, Vol. 127, No. 50, pp. 17604–5, December 2005.
- [12] Y. Tzeng, O. Faklaris, B. Chang, Y. Kuo, J. Hsu, and H. Chang. Superresolution imaging of albumin-conjugated fluorescent nanodiamonds in cells by stimulated emission depletion. *Angewandte Chemie (International ed. in English)*, Vol. 50, No. 10, pp. 2262–5, March 2011.

- [13] Y. Chang, H. Lee, K. Chen, C. Chang, D. Tsai, C. Fu, T. Lim, Y. Tzeng, C. Fang, C. Han, H. Chang, and W. Fann. Mass production and dynamic imaging of fluorescent nanodiamonds. *Nature nanotechnology*, Vol. 3, No. 5, pp. 284–8, May 2008.
- [14] C. Santori, P. Barclay, K. Fu, and R. Beausoleil. Vertical distribution of nitrogen-vacancy centers in diamond formed by ion implantation and annealing. *Physical Review B*, pp. 1–8, 2009.
- [15] X. Song, G. Wang, X. Liu, F. Feng, and J. Wang. Generation of nitrogen-vacancy color center in nanodiamonds by high temperature annealing. *Applied Physics Letters*, Vol. 102, p. 133109, 2013.
- [16] S. Osswald, G. Yushin, V. Mochalin, S. Kucheyev, and Y. Gogotsi. Control of sp²/sp³ carbon ratio and surface chemistry of nanodiamond powders by selective oxidation in air. *Journal of the American Chemical Society*, Vol. 128, No. 35, pp. 11635–42, September 2006.
- [17] J. Havlik, V. Petrakova, I. Rehor, V. Petrak, M. Gulka, J. Stursa, J. Kucka, J. Ralis, T. Rendler, S. Lee, R. Reuter, J. Wrachtrup, M. Ledvina, M. Nesladek, and P. Cigler. Boosting nanodiamond fluorescence: towards development of brighter probes. *Nanoscale*, Vol. 5, No. 8, pp. 3208–11, April 2013.
- [18] P. Chung, E. Perevedentseva, and C. Cheng. The particle size-dependent photoluminescence of nanodiamonds. *Surface Science*, Vol. 601, No. 18, pp. 3866–3870, September 2007.

- [19] V. Mochalin and Y. Gogotsi. Wet chemistry route to hydrophobic blue fluorescent nanodiamond. *Journal of the American Chemical Society*, Vol. 131, No. 13, pp. 4594–5, April 2009.
- [20] S. Hu, F. Tian, P. Bai, S. Cao, J. Sun, and J. Yang. Synthesis and luminescence of nanodiamonds from carbon black. *Materials Science and Engineering: B*, Vol. 157, No. 1-3, pp. 11–14, February 2009.
- [21] I. Aharonovich, S. Castelletto, D. Simpson, A. Stacey, J. McCallum, A. Green-tree, and S. Prawer. Two-level ultrabright single photon emission from diamond nanocrystals. *Nano letters*, Vol. 9, No. 9, pp. 3191–5, September 2009.
- [22] I. Vlasov, O. Shenderova, S. Turner, O. Lebedev, A. Basov, I. Sildos, M. Rähn, A. Shiryaev, and G. Tendeloo. Nitrogen and luminescent nitrogen-vacancy defects in detonation nanodiamond. *Small (Weinheim an der Bergstrasse, Germany)*, Vol. 6, No. 5, pp. 687–94, March 2010.
- [23] Y. Doi, T. Makino, H. Kato, D. Takeuchi, M. Ogura, H. Okushi, H. Morishita, T. Tashima, S. Miwa, S. Yamasaki, P. Neumann, J. Wrachtrup, Y. Suzuki, and N. Mizuochi. Deterministic Electrical Charge-State Initialization of Single Nitrogen-Vacancy Center in Diamond. *Physical Review X*, Vol. 4, No. 1, p. 011057, March 2014.
- [24] K. Fu, C. Santori, P. Barclay, and R. Beausoleil. Conversion of neutral nitrogen-vacancy centers to negatively charged nitrogen-vacancy centers through selective oxidation. *Applied Physics Letters*, Vol. 96, No. 12, p. 121907, 2010.

- [25] B. Grotz, M. Hauf, M. Dankerl, B. Naydenov, S. Pezzagna, J. Meijer, F. Jelezko, J. Wrachtrup, M. Stutzmann, F. Reinhard, and J. Garrido. Charge state manipulation of qubits in diamond. *Nature communications*, Vol. 3, p. 729, January 2012.
- [26] P. Siyushev, H. Vörös Pinto, M. Gali, F. Jelezko, and J. Wrachtrup. Optically Controlled Switching of the Charge State of a Single Nitrogen-Vacancy Center in Diamond at Cryogenic Temperatures. *Physical Review Letters*, Vol. 110, No. 16, p. 167402, April 2013.
- [27] T. Gaebel, I. Popa, A. Gruber, M. Domhan, F. Jelezko, and J. Wrachtrup. Stable single-photon source in the near infrared. *New Journal of Physics*, Vol. 6, pp. 98–98, July 2004.
- [28] C. Fu, H. Lee, T. Wu H. Lin P. Chen, K. Lim, P. Chang H. Wei, P. Tsao, and W. Fann. Characterization and application of single fluorescent nanodiamonds as cellular biomarkers. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 104, No. 3, pp. 727–32, January 2007.
- [29] C. Fang, V. Vaijayanthimala, C. Cheng, S. Yeh, C. Chang, C. Li, and H. Chang. The exocytosis of fluorescent nanodiamond and its use as a long-term cell tracker. *Small (Weinheim an der Bergstrasse, Germany)*, Vol. 7, No. 23, pp. 3363–70, December 2011.
- [30] K. Liu, C. Wang, C. Cheng, and J. Chao. Endocytic carboxylated nanodiamond for the labeling and tracking of cell division and differentiation in cancer and

- stem cells. *Biomaterials*, Vol. 30, No. 26, pp. 4249–59, September 2009.
- [31] R. Igarashi, Y. Yoshinari, H. Yokota, T. Sugi, F. Sugihara, K. Ikeda, H. Sumiya, S. Tsuji, I. Mori, H. Tochio, Y. Harada, and M. Shirakawa. Real-time background-free selective imaging of fluorescent nanodiamonds in vivo. *Nano letters*, Vol. 12, No. 11, pp. 5726–32, November 2012.
- [32] Y. Kuo, T. Hsu, Y. Wu, and H. Chang. Fluorescent nanodiamond as a probe for the intercellular transport of proteins in vivo. *Biomaterials*, Vol. 34, No. 33, pp. 8352–60, November 2013.
- [33] R. Hardman. A Toxicologic Review of Quantum Dots: Toxicity Depends on Physicochemical and Environmental Factors. *Environmental Health Perspectives*, Vol. 114, No. 2, pp. 165–172, February 2006.
- [34] A. Gruber, C. Dräbenstedt, L. Tietz, J. Fleury, J. Wrachtrup, and C. Borczyskowski. Scanning Confocal Optical Microscopy and Magnetic Resonance on Single Defect Centers. *Science*, Vol. 276, No. 5321, pp. 2012–2014, June 1997.
- [35] T. Wu, W. Cheng C. Kuo Y. Chien C. Chang H. Tzeng, Y. Chang, and J. Yu. Tracking the engraftment and regenerative capabilities of transplanted lung stem cells using fluorescent nanodiamonds., September 2013.
- [36] G. Kucsko, P. Maurer, N. Yao, M. Kubo, P. Park H. Noh, H. Lo, and M. Lukin. Nanometre-scale thermometry in a living cell. *Nature*, Vol. 500, No. 7460, pp. 54–8, August 2013.

- [37] L. McGuinness, Y. Yan, A. Stacey, D. Simpson, L. Hall, D. Maclaurin, S. Prawer, P. Mulvaney, J. Wrachtrup, F. Caruso, R. Scholten, and L. Hollenberg. Quantum measurement and orientation tracking of fluorescent nanodiamonds inside living cells. *Nature nanotechnology*, Vol. 6, No. 6, pp. 358–63, January 2011.
- [38] M. Geiselmann, M. Juan, J. Renger, J. Say, L. Brown, F. Abajo, Javier G., F. Koppens, and R. Quidant. Three-dimensional optical manipulation of a single electron spin., March 2013.

Part II

Preparation, Characterization and Application of Nanodiamonds

Chapter I

Effective Production of Fluorescent Nanodiamonds Containing Negatively-Charged Nitrogen-Vacancy Centers by Ion Irradiation

Abstract

Fluorescence from negatively-charged nitrogen-vacancy centers (NV⁻s) in diamonds has unique optical properties with none of the undesirable effects such as photo-bleaching and photo-blinking. In addition, the spin-dependent fluorescence intensity of NV⁻ allows us to perform optically detected magnetic resonance (ODMR) investigation for evaluating the presence of NV⁻s and for the electronic local environment. In this work, we irradiated H⁺, He⁺, Li⁺ and N⁺ ions to nanodiamonds with a median size of 26 nm at various irradiation energies and doses for improving the NV⁻s concentration. ODMR observations of the nanodiamonds showed that ion irradiation increased the number of nanodiamonds containing NV⁻s up to 200 ppm, whereas without ion irradiation, only few NV⁻s were found. The number of nanodiamonds containing NV⁻s at various ion irradiation doses was not monotonous, but had maxima at certain irradiation doses. These results suggest a competition in two opponent roles of vacancies, effective for pairing with nitrogen atoms and as defects for developing damage in crystalline. We also found that sharp and strong ODMR signals were obtained from nanodiamonds irradiated at the optimal condition for the highest yield of NV⁻s. We concluded that He⁺ ion irradiations with 60 or 80 keV at a dose of 1×10^{13} ions per cm² are the conditions required for the most efficient production of a high quantity of nanodiamonds containing NV⁻s.

1. Introduction

Fluorescent nanodiamonds (FNDs) have been attracting much attention as new bio-imaging probes due to their unique optical properties. The negatively-charged nitrogen-vacancy color center (NV^-) in FNDs, which is formed by a substitutional nitrogen atom combining with an adjacent vacancy, is responsible for fluorescence that is emitted in the near-infrared region (600-800 nm) with a high quantum yield of 0.7.[1, 2] Its well-known photo-stability of displaying neither photo-blinking nor photo-bleaching enables long-term in vivo and in vitro imaging, which is not readily achieved by commonly used fluorescent agents such as organic dyes and fluorescent proteins.[3–5] It has also been reported that FNDs are highly bio-compatible and non-toxic to various kinds of cell types, and are thus superior to quantum dots which often show cyto-toxicity.[6, 7] These advantages of FNDs over conventional fluorescent dyes could make them a potential candidate for optical bio-imaging probes.[5, 8, 9]

Optically detected magnetic resonance (ODMR) has been demonstrated for detection of a single NV^- at ambient conditions[10], and significant attention has been paid in the field of quantum information[11], magnetic sensing[12, 13] and bio-application[14, 15]. In particular, advanced applications in the nano-scale sensing of magnetic[16, 17] and electric fields[18] and temperature[19] require a long-time coherence of spin state, which strongly depends on the quality of the diamond matrix. For this reason, methods for synthesizing high quality nanodiamonds and the efficient production of NV^- s are highly desired. Here, we report the effects of ion irradiation on the production of NV^- s in nanodiamonds.

There are two types of nitrogen-vacancy centers (NVCs), each with a different charged

state.[20, 21] One is a neutral NVC (NV^0 , electron spin: $S = 1/2$), while the other is a negatively-charged NVC (NV^- , electron spin: $S = 1$), represented by zero-phonon lines at 575 nm and 637 nm respectively.[22, 23] Both NVCs emit fluorescence, but only the NV^- allows for measurement by ODMR. The ground state of NV^- is a spin-triplet, and the spin sublevels, $m_s = 0$ and $m_s = \pm 1$ are split by 2.87 GHz. The spin sublevels $m_s = \pm 1$ are degenerated in the absence of an external magnetic field. Microwave (MW) irradiation at this frequency induces electron spin magnetic resonance between $m_s = 0$ and degenerated $m_s = \pm 1$ spin levels, which results in a reduction of the fluorescence intensity from NV^- due to spin-dependent intersystem crossing. On the other hand, NV^0 is insensitive to MW irradiation. By comparing the fluorescence intensity upon MW irradiation, we can distinguish the two types of NVCs, as shown later.

In general, the concentration of NVCs in diamond is on the order of sub ppm.[16] The existence probability of NVCs contained in nanodiamonds is lower than that of in bulk diamonds, because vacancies are annihilated at the surface.[24] In order to increase the concentration of NVCs in nanodiamonds, the method of ion irradiation is commonly used for creating carbon vacancies.[3, 25] In this technique, ionized atoms are accelerated and are allowed to penetrate into nanodiamonds, where collisions of the ions with atoms force the carbon atoms to be expelled, resulting in the creation of vacancies. Subsequent thermal annealing at 800 °C leads to trapping of moving vacancies by nitrogen atoms which pre-exist in the diamond lattice, thereby forming an NVC.[26] Although this method is quite effective, optimal conditions for producing a high quantity of NV^- s in nanodiamonds are still unknown. In this study, we also investigated how ion irradiation affects the production of nanodiamonds containing NV^- s, and their ODMR spectra when

irradiated by H^+ , He^+ , Li^+ and N^+ ions. Furthermore, we elucidated the optimal irradiation condition.

2. Experimental section

2.1 Sample preparation

In our experiments, we used synthetic type Ib nanodiamonds which contain typically 100 ppm nitrogen atoms (Micron+ MDA Element Six). A suspension of nanodiamonds in Milli-Q water was centrifuged at 15,000 rpm for 20 min, and the supernatant was freeze-dried. The median size of the nanodiamond particles was determined by dynamic light scattering (DLS) to be 25.9 nm (Figure 1).

2 μ l of a nanodiamond suspension in Milli-Q water at a concentration of 2 mg/ml was dropped on the surface of a silicon wafer measuring one centimeter square, and subsequently spin-coated at 7,000 rpm for 20 s. To estimate the number of nanodiamonds on the silicon wafer, we carried out atomic force microscope (AFM) measurements and obtained the images of prepared samples (Figure 2), from which we directly counted the number of nanodiamonds which cover an area of 1 mm² on a wafer to be 1.6×10^6 particles.

2.2 Ion irradiation

The samples were irradiated by H^+ , He^+ , Li^+ and N^+ ions in vacuum at 10^{-5} Pa under various energy and dose conditions at Takasaki Advanced Radiation Research Institute JAEA. The ion penetration depth and the number of vacancies created per incident ion at a given irradiation energy were evaluated in advance by SRIM (<http://www.srim.org/>)

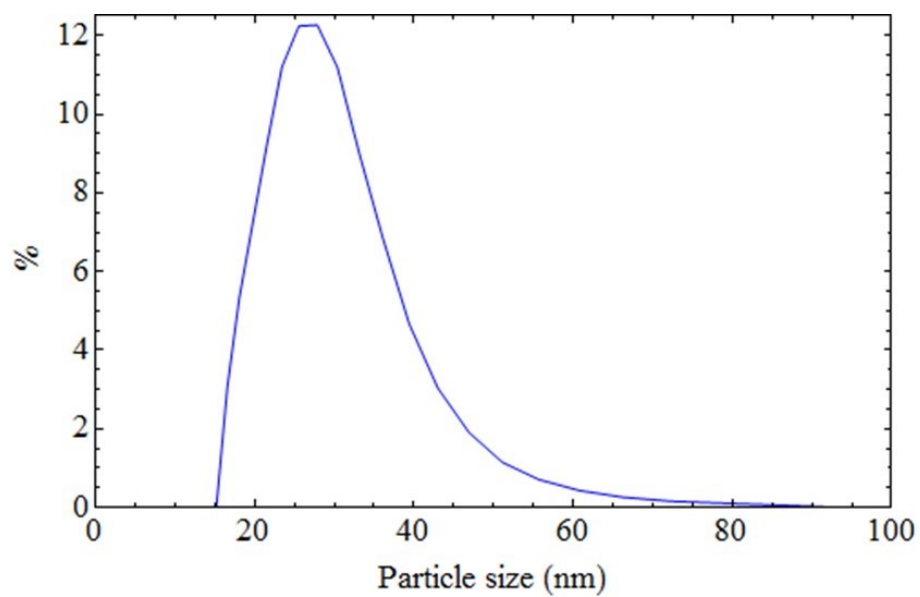


Figure 1. Particle-size distribution of nanodiamonds as measured by dynamic light scattering (DLS).

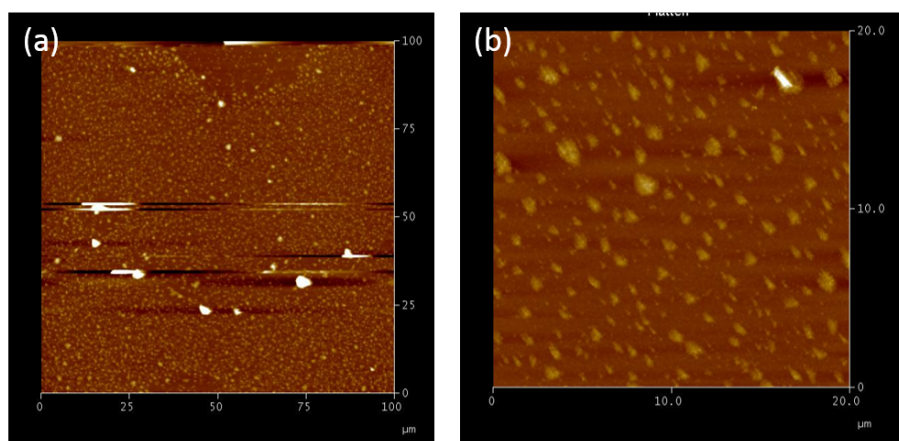


Figure 2. (a) AFM image of spin-coated nanodiamonds on silicon wafer. (b) Close-up image of (a).

(Table 1). For obtaining desired dosages, ion beam currents in the range of 0.1 nA to 300 nA and the exposure times were adjusted. Under the conditions employed in this experiment, most ions passed through the nanodiamond arrays. After the irradiation, the samples were thermally annealed at 800 °C under a reduced pressure of ~1 Pa for two hours, for the purpose of pairing nitrogen atoms and vacancies. Since the graphite layer on the surfaces of nanodiamonds interferes with fluorescence emission from NV⁻s, the layer was removed by oxidizing at 500 °C under atmospheric conditions for two hours.

2.3 Count of nanodiamonds containing NV⁻s and measurement of its ODMR

The number of nanodiamonds containing NV⁻s generated by ion irradiation was counted by the following method using a home-built fluorescence microscope. In our experimental set-up, the field of view was illuminated with a 532-nm Nd:YAG laser at 7 mW, and fluorescence images (40 μm × 40 μm) were measured by an electron multiplying charge coupled device (EMCCD) camera. The emitted light was collected by an oil immersion 60 × objective lens (NA=1.49), and passed through a dichroic mirror centered at 575 nm and a long (short)-wave pass filter longer than 590 nm (shorter than 842 nm) to detect the intrinsic signal. The microscope was equipped with a computer-controlled moving stage. Firstly, the sample was roughly scanned in a broad range of areas in steps of 50 μm. At each position, fluorescence images were measured while MW was swept point-by-point across the resonant frequency from 2.865 to 2.875 GHz. The intrinsic fluorescence from NV⁻s was deduced by the selective imaging protocol reported by Igarashi et al.[15] This procedure provides a conventional fluorescence image as well as a selective one, the latter displaying only a signal arising from NV⁻ fluorescence. Typical

Table 1. Mean penetration depths and the distribution widths of stopped ions (H^+ , He^+ , Li^+ and N^+) implanted into a diamond crystal of sufficient thickness. The calculation was made by SRIM.

Ion	Energy (eV)	Range ($\text{\AA}$)	Straggle ($\text{\AA}$)	Vacancy (/ion)
H^+	20	1281	229	4.7
H^+	40	2135	248	5.4
H^+	60	2903	258	5.7
H^+	80	3647	298	6.2
He^+	20	936	220	37
He^+	40	1594	287	44
He^+	60	2157	321	49
He^+	80	2671	357	51
Li^+	25	879	222	59
Li^+	50	2135	320	76
Li^+	75	2903	359	85
Li^+	100	3647	406	91
N^+	100	1150	218	190
N^+	150	1650	271	224
N^+	200	2126	293	245
N^+	250	2565	335	265

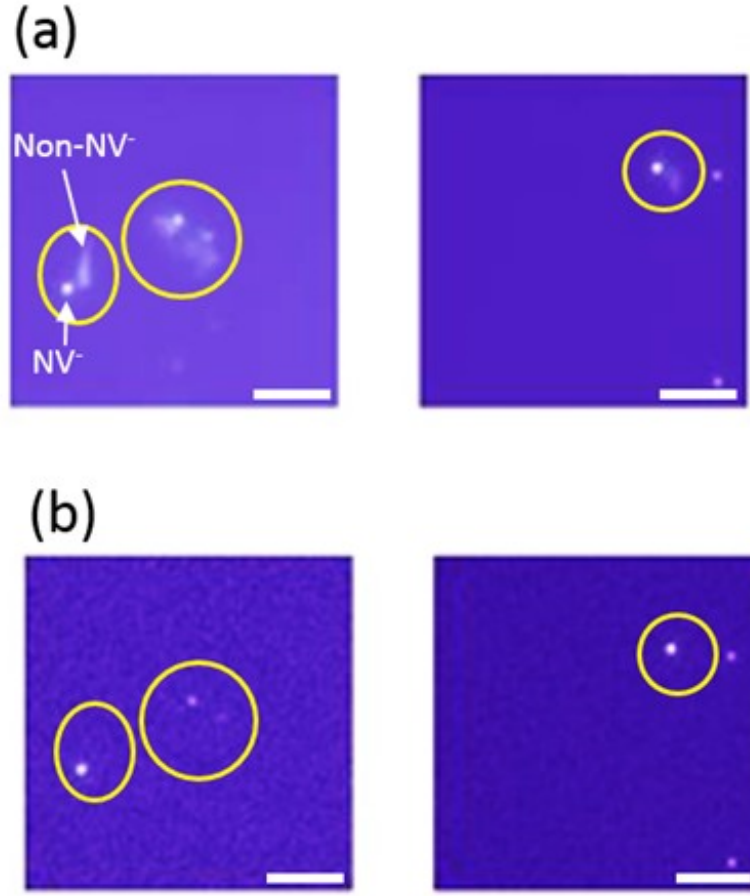


Figure 3. (a) Conventional fluorescence and (b) selective images of NV^- s at the same area in the sample [He^+ , 60 keV, 1×10^{13}]. Yellow circles indicate an area of co-existence of fluorescence signals from NV^- s and non- NV^- s. The selective imaging as shown in (b) clearly eliminates extrinsic fluorescence. Scale bar shows 10 μm .

obtained images are shown in Figure 3. After the pre-scanning, the allocation numbers of images which contained the fluorescence from NV^- s were listed and stored. In order to precisely measure the entire ODMR spectrum for each nanodiamond containing NV^- s in the allocated field, a second run was performed with the MW swept widely from 2.820 to 2.920 GHz point-by-point. After acquiring hundreds of images in the latter process, the number of nanodiamonds containing NV^- s was counted. The extraction of intrinsic signals was done by setting a threshold for the signal intensity, so as to pick up the nanodi-

amonds which contain roughly more than two NV⁻s. Below, we abbreviate a particular condition of ion irradiation by the notation [ion species, energy, dose per cm²], such as [He⁺, 20 keV, 1×10^{13}].

3. Results and Discussion

The numbers of nanodiamonds containing NV⁻s produced per mm² as a function of energy and ion dose for H⁺, He⁺, Li⁺ and N⁺ irradiation are listed in Table 2. Figure 4 shows a bar chart representation of the values given in Table 2. The results of H⁺ irradiation show no or very few instances of nanodiamonds containing NV⁻s after irradiation with less than or equal to 1×10^{12} ions per cm². Production of nanodiamonds containing NV⁻s increased as ion dose increased, but doses higher than 1×10^{14} ions per cm² caused a decline in NV⁻ production. This turning point indicates the optimal condition for NV⁻ production by H⁺ ion irradiation. The results for He⁺ ion irradiation were found to be similar to those observed for H⁺ irradiation, with the dose being one order lower in magnitude. Li⁺ ion irradiation showed a significantly lower efficiency of NV⁻ production, and N⁺ ion irradiation was observed to produce no NV⁻s (not shown). The results indicate that the ion doses exert significant influence on NV⁻ production, and that the optimal conditions appear to be shifted to lower doses as the irradiation energy decreases. Considering our irradiation energies and the corresponding depth profiles (Table 1), some of the incident ions may be trapped in the nanodiamonds at a finite probability and contribute effectively to create vacancies. As a consequence, we found that the optimal conditions for producing nanodiamonds containing NV⁻s are 1×10^{14} ions per cm² for H⁺ and 1×10^{13} ions per cm² for He⁺.

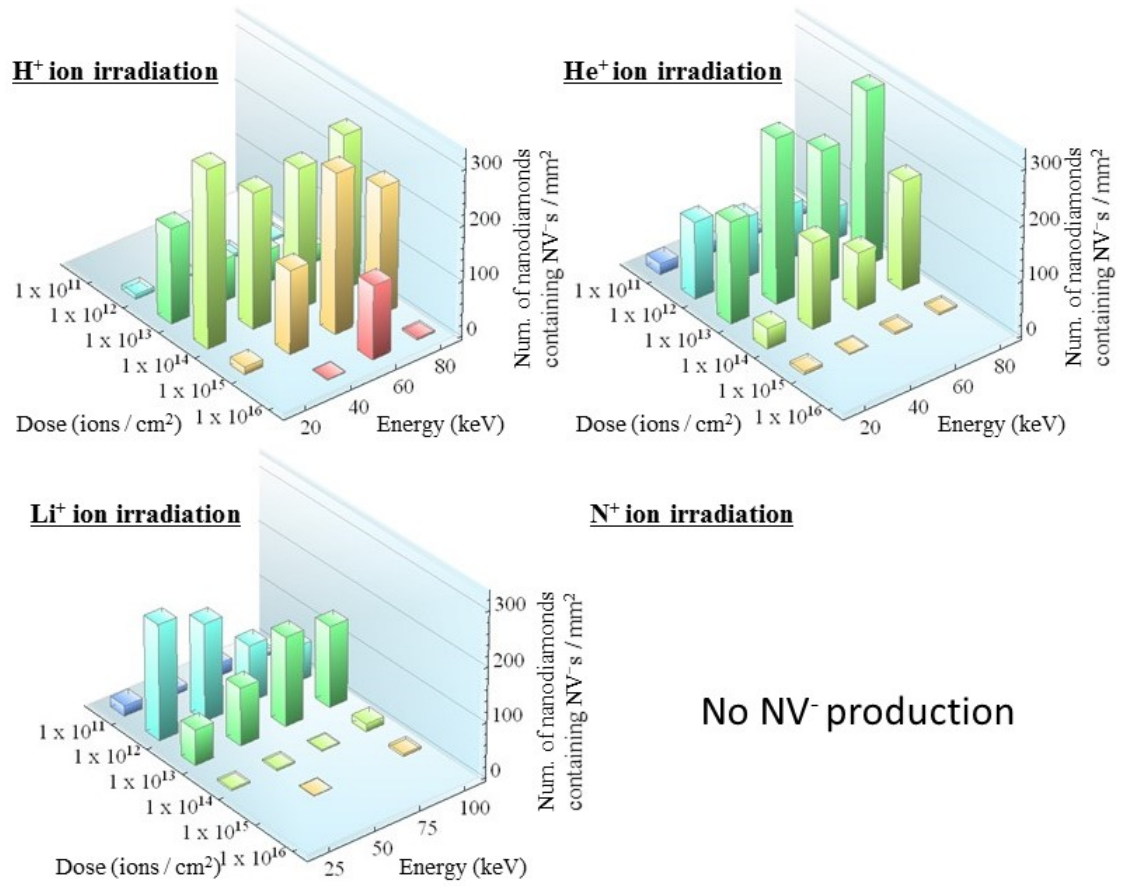


Figure 4. Numbers of nanodiamonds containing NV⁻s created by ion irradiation of H⁺, He⁺ and Li⁺ ions as a function of irradiation energy and dose. The bar height indicates the number per 1 mm². The Dose axis is a log scale.

Table 2. Numbers of nanodiamonds containing NV⁻s per 1 mm² versus irradiation energy and dose of H⁺, He⁺ and Li⁺ ions.

Ion	Energy (eV)	Dose (ions / cm ²)					
		1×10^{11}	1×10^{12}	1×10^{13}	1×10^{14}	1×10^{15}	1×10^{16}
H ⁺	20	-	9	173	322	14	0
H ⁺	40	-	4	77	246	152	2
H ⁺	60	-	7	60	255	290	134
H ⁺	80	-	5	31	281	231	4
He ⁺	20	20	140	184	37	6	-
He ⁺	40	20	110	300	157	3	-
He ⁺	60	8	99	244	103	5	-
He ⁺	80	9	59	318	198	4	-
Li ⁺	25	19	211	64	4	0	-
Li ⁺	50	11	178	105	4	1	-
Li ⁺	75	24	104	163	3	0	-
Li ⁺	100	6	65	148	14	5	-

The number of nanodiamonds containing NV⁻s produced by ion irradiation was estimated as follows. In our experimental set-up, approximately 1.6×10^6 nanodiamond particles cover an area of 1 mm^2 on a silicon wafer. As 322 nanodiamonds containing NV⁻s were observed for [H⁺, 20 keV, 1×10^{14}], one nanodiamond containing NV⁻s is created per 5×10^3 particles, yielding a density of 200 ppm. This number is a marked improvement as compared to the case of non-irradiated nanodiamonds, in which no NV⁻s were observed.

In order to investigate the damage in the diamond crystal due to ion irradiations, the ODMR spectra of the nanodiamonds containing NV⁻s were evaluated, since the line shape of the magnetic resonance spectra are sensitive to distortions in the crystal lattice around NV⁻s. We analyzed the spectra taken for NV⁻s created by H⁺, He⁺ and Li⁺ irradiation at various conditions. Each ODMR spectrum was then fitted to a Gaussian function (Figure 5a):

$$f(\nu) = \text{const} - \alpha \exp\left\{-\frac{1}{2}\left(\frac{\nu - \nu_0}{\Delta\nu}\right)^2\right\}, \quad (1)$$

where ν , ν_0 , α and $\Delta\nu$ are the MW frequency, resonance frequency, depth and frequency width, respectively. We focused on the depth α and width $\Delta\nu$ because they are good indicators for the evaluation of inhomogeneity around NV⁻s. In particular, electrical inhomogeneity caused by ion irradiation induces a distribution of the local crystal field around NV⁻s, thus causing broad line width and weak signal depth in the spectrum. As shown in Figures 5b and 5c, the histograms of the parameters α and $\Delta\nu$ of the spectra of ion irradiated nanodiamonds have maxima around 3% and 12 MHz, and spread asymmet-

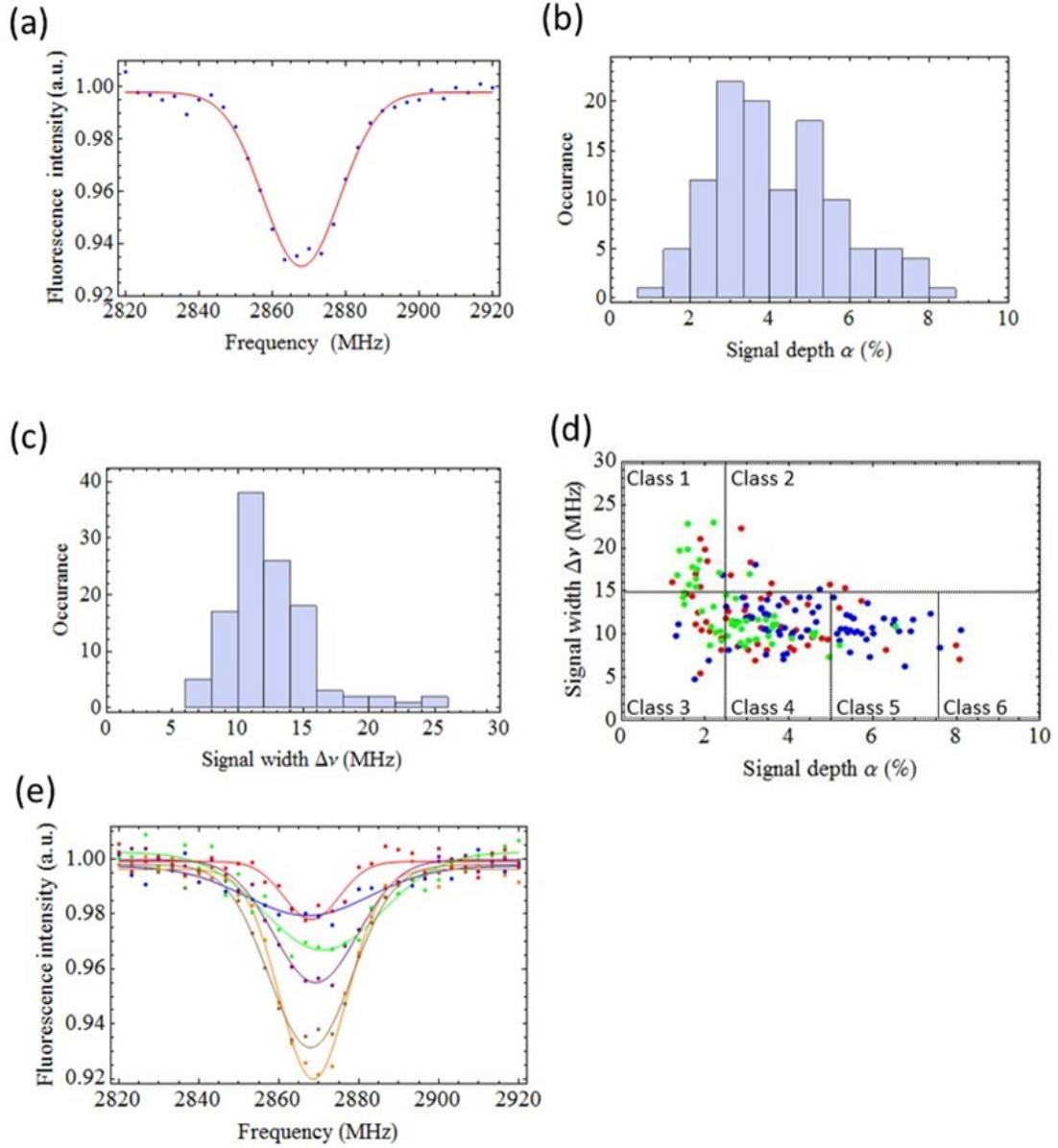


Figure 5. Numbers of nanodiamonds containing NV^- s created by ion irradiation of H^+ , He^+ and Li^+ ions as a function of irradiation energy and dose. The bar height indicates the number per 1 mm^2 . The Dose axis is a log scale.

rically from 1% to 10% and from 5 MHz to 30 MHz, respectively. We further made in Figure 5d a correlation plot between these two parameters to investigate the effects of ion irradiation on the ODMR spectra, and divided the domain into six segments (classes 1 to 6). This classification made it easy to compare the spectral profiles belonging to these categories. For example, the spectra of samples at $[\text{He}^+, 80 \text{ keV}, 1 \times 10^{13}]$ (blue dots in Figure 5d) shown mainly in the lower-right area (classes 5 or 6) exhibit strong signal depth and narrow widths. These features indicate that the irradiation condition produces NV^- s without large perturbation of the crystalline lattice. In contrast, the spectra of samples at $[\text{He}^+, 80 \text{ keV}, 1 \times 10^{14}]$ (green dots in Figure Fig. 5d) shown in the upper-left area (classes 1 or 3) show shallow and broad signals. Figure 5e displays examples of ODMR spectra picked from each class for comparison. The spectra from class 5 (brown) or 6 (orange) show strong and narrow signals, which are suitable for application using ODMR. In contrast, those from class 1 (blue) yielded shallow and broad signals, thus indicating that such NV^- is not suitable. Consequently, a search for optimal conditions for irradiation was conducted by calculating the ratio of the number of NV^- s classified in class 5 or 6 to the total number of NV^- s. The results are listed in Table 3. The most suitable condition for generating nanodiamonds giving high-quality ODMR was found to be $[\text{He}^+, 60 \text{ or } 80 \text{ keV}, 1 \times 10^{13}]$, where more than 40% of the generated NV^- s belonged to class 5 or 6. $[\text{H}^+, 40, 60 \text{ or } 80 \text{ keV}, 1 \times 10^{14}]$ and $[\text{He}^+, 40 \text{ keV}, 1 \times 10^{13}]$ are in second place, with approximately 30% of the generated NV^- s located in class 5 or 6. These conditions coincide with those producing the largest number of NV^- s, as seen in Figure Li^+ irradiation was not effective both for producing a large number of NV^- s and for the creation of NV^- s yielding high quality ODMR signals.

Table 3. Ratios of the number of nanodiamonds containing NV⁻s classified in class 5 or 6 to the total numbers of nanodiamonds containing NV⁻s with H⁺, He⁺ and Li⁺ ions irradiation.

Ion	Energy (eV)	Dose (ions / cm ²)			
		1×10^{12}	1×10^{13}	1×10^{14}	1×10^{15}
H ⁺	20	-	0.06	0.11	-
H ⁺	40	-	0.10	0.30	0.00
H ⁺	60	-	0.13	0.28	0.15
H ⁺	80	-	0.00	0.32	0.00
He ⁺	20	0.05	0.18	0.00	-
He ⁺	40	0.12	0.29	0.00	-
He ⁺	60	0.15	0.44	0.05	-
He ⁺	80	0.11	0.40	0.04	-
Li ⁺	25	0.01	0.00	0.00	-
Li ⁺	50	0.03	0.00	0.00	-
Li ⁺	75	0.01	0.00	0.00	-
Li ⁺	100	0.00	0.01	0.00	-

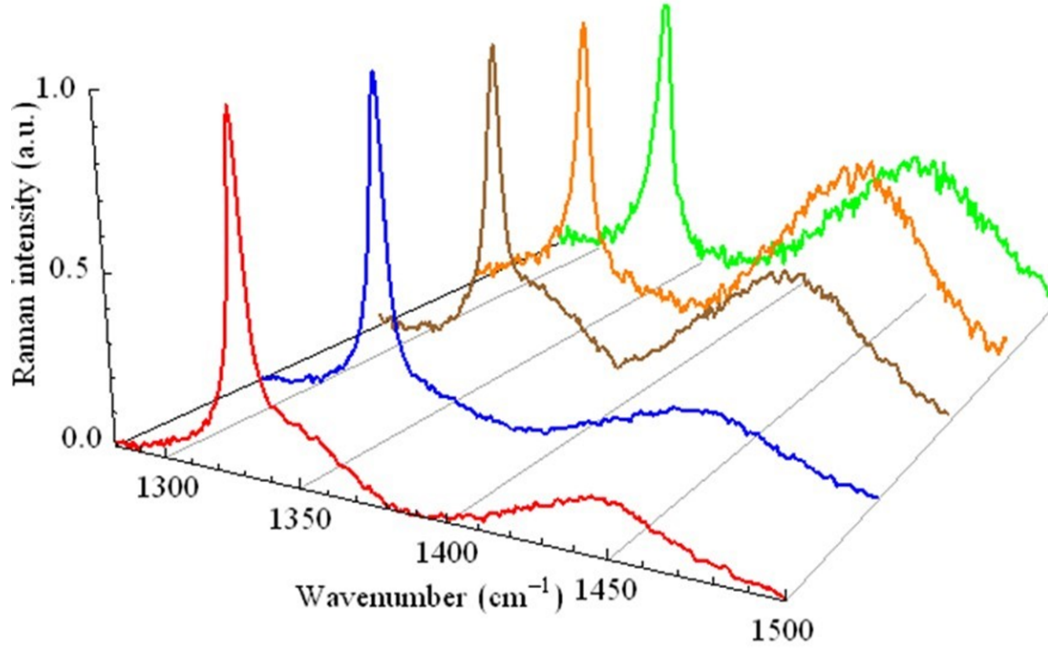


Figure 6. Examples of Raman spectra of nanodiamonds irradiated with He^+ ions at 20 keV and dose 1×10^{11} (red), 1×10^{12} (blue), 1×10^{13} (brown), 1×10^{14} (orange) and 1×10^{15} (green) ions per cm^2 . The signal intensities are normalized for comparison at 1332 cm^{-1} .

We also carried out Raman spectroscopic measurements to elucidate why the irradiation with heavy ion species or an excess dose of lighter ions leads to a decrease in the generation of NV^- s and the low quality of ODMR. Raman spectroscopy enables us to evaluate the crystalline quality of diamonds.[27, 28] Diamond has a strong and sharp Raman signal at 1332 cm^{-1} attributed to sp^3 bonding carbon, while disorders of the crystal lattice cause the appearance of a broad signal at 1500 cm^{-1} .

Figure 6 shows examples of Raman spectra of the nanodiamonds containing NV^- s created in this study. At all irradiation energies, the 1332 cm^{-1} sp^3 peak decreased with an increase in ion irradiation dose, while the band around 1450 cm^{-1} , which is attributed to disorders of crystal lattices, increased. We evaluated the quality of the crystal structure of the nanodiamonds containing NV^- s in terms of the intensity of the sp^3 diamond peak divided by that of the disorder-originating band peak (Table 4). The ratio decreased as the

Table 4. Crystal quality of nanodiamonds irradiated by H^+ or He^+ ion. The number in each column is a ratio of Raman signal intensity of sp^3 bonding carbon ($\sim 1332\text{ cm}^{-1}$) divided by that of disorder induced band peak ($\sim 1500\text{ cm}^{-1}$).

Ion	Energy (eV)	Dose (ions / cm^2)			
		1×10^{12}	1×10^{13}	1×10^{14}	1×10^{15}
H^+	20	-	1.34	0.75	-
H^+	40	-	2.95	1.58	1.04
H^+	60	-	3.49	1.11	0.44
H^+	80	-	2.38	2.36	1.30
He^+	20	2.61	1.06	-	-
He^+	40	4.46	1.22	0.61	-
He^+	60	3.78	1.65	0.88	-
He^+	80	5.89	1.24	0.70	-

irradiation dose increased, indicating that the high dose perturbed the crystal structures. These results suggest that the generation of vacancies and the disruption of the crystalline structure are competitive events. Generation of vacancies contributes to the formation of NV^- s until ion irradiation reaches the optimal dose, after which disruption of the crystal structure is dominant, preventing the formation of NV^- s and enhancing the electrical inhomogeneity that causes weak and broad ODMR signals. For similar reasons, ion irradiation of heavy ion species not only creates excess vacancies in nanodiamonds, but also significantly disrupts the crystalline structure, resulting in no functional NV^- s.

4. Conclusion

We explored the optimal conditions of ion irradiation for creating nanodiamonds containing NV^- s with a median particle size of 26 nm. The obtained best conditions were He^+ ion irradiation at 60 keV or 80 keV with a dose of 1×10^{13} ions per cm^2 for the

production of high-quantity and high-quality nanodiamonds containing NV⁻s. The consequent NV⁻s exhibited strong intensities and narrow widths in ODMR spectra, which is generally an indication of good crystalline. We also demonstrated that irradiation with heavy ion species or an excessive ion dose leads to the destruction of nanodiamond crystals, resulting in a decrease in the quantity and quality of NV⁻s. In particular, Raman spectroscopy clearly revealed the gradual deterioration of crystalline upon those irradiations, evidenced by the observation of the intensity shifted gradually from the diamond sp³ peak to the band characteristic of crystal disorder. The optimal irradiation condition seems to imply a subtle competition of the role of vacancies not only effective for pairing with nitrogen atoms but also as defects for developing damage in crystalline. Our experimental data supplies a guideline for optimal conditions of ion irradiation and facilitates a wide range of studies involving NV⁻s.

References

- [1] S. Yu, M. Kang, H. Chang, K. Chen, and Y. Yu. Bright fluorescent nanodiamonds: no photobleaching and low cytotoxicity. *Journal of the American Chemical Society*, Vol. 127, No. 50, pp. 17604–5, December 2005.
- [2] T. Gaebel, I. Popa, A. Gruber, M. Domhan, F. Jelezko, and J. Wrachtrup. Stable single-photon source in the near infrared. *New Journal of Physics*, Vol. 6, pp. 98–98, July 2004.
- [3] Y. Chang, H. Lee, K. Chen, C. Chang, D. Tsai, C. Fu, T. Lim, Y. Tzeng, C. Fang, C. Han, H. Chang, and W. Fann. Mass production and dynamic imaging of fluorescent nanodiamonds. *Nature nanotechnology*, Vol. 3, No. 5, pp. 284–8, May 2008.
- [4] C. Fu, H. Lee, K. Chen, T. Lim, H. Wu, P. Lin, P. Wei, P. Tsao, H. Chang, and W. Fann. Characterization and application of single fluorescent nanodiamonds as cellular biomarkers. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 104, No. 3, pp. 727–32, January 2007.
- [5] J. Chao, E. Perevedentseva, P. Chung, K. Liu, C. Cheng, C. Chang, and C. Cheng. Nanometer-sized diamond particle as a probe for biolabeling. *Biophysical journal*, Vol. 93, No. 6, pp. 2199–208, September 2007.
- [6] A. Schrand, H. Huang, C. Carlson, J. Schlager, Eiji. Sawa, S. Hussain, and L. Dai. Are diamond nanoparticles cytotoxic? *The journal of physical chemistry. B*, Vol. 111, No. 1, pp. 2–7, January 2007.
- [7] H. Huang, E. Pierstorff, E. Osawa, and D. Ho. Active nanodiamond hydrogels for

chemotherapeutic delivery. *Nano letters*, Vol. 7, No. 11, pp. 3305–14, November 2007.

[8] P. Sharma, S. Brown, G. Walter, S. Santra, and B. Moudgil. Nanoparticles for bioimaging. *Advances in colloid and interface science*, Vol. 123-126, pp. 471–85, November 2006.

[9] Y. Lin, E. Perevedentseva, L. Tsai, K. Wu, and C. Cheng. Nanodiamond for intracellular imaging in the microorganisms in vivo. *Journal of biophotonics*, Vol. 5, No. 11-12, pp. 838–47, November 2012.

[10] A. Gruber, C. Dräbenstedt, L. Tietz, J. Fleury, J. Wrachtrup, and C. Borczyskowski. Scanning Confocal Optical Microscopy and Magnetic Resonance on Single Defect Centers. *Science*, Vol. 276, No. 5321, pp. 2012–2014, June 1997.

[11] H. Bernien, B. Hensen, W. Pfaff, G. Koolstra, M. Blok, L. Robledo, T. Taminiau, M. Markham, D. Twitchen, L. Childress, and R. Hanson. Heralded entanglement between solid-state qubits separated by three metres. *Nature*, Vol. 497, No. 7447, pp. 86–90, May 2013.

[12] T. Staudacher, F. Shi, S. Pezzagna, J.. Meijer, J Du, C. Meriles, F. Reinhard, and J. Wrachtrup. Nuclear magnetic resonance spectroscopy on a (5-nanometer)³ sample volume., February 2013.

[13] M. Geiselmann, M. Juan, J. Renger, J. Say, L. Brown, F. Abajo, F. Koppens, and R. Quidant. Three-dimensional optical manipulation of a single electron spin., March 2013.

- [14] L. McGuinness, Y. Yan, A. Stacey, D. Simpson, L. Hall, D. Maclaurin, S. Prawer, P. Mulvaney, J. Wrachtrup, F. Caruso, R. Scholten, and L. Hollenberg. Quantum measurement and orientation tracking of fluorescent nanodiamonds inside living cells. *Nature nanotechnology*, Vol. 6, No. 6, pp. 358–63, January 2011.
- [15] R. Igarashi, Y. Yoshinari, H. Yokota, T. Sugi, F. Sugihara, K. Ikeda, H. Sumiya, S. Tsuji, I. Mori, H. Tochio, Y. Harada, and M. Shirakawa. Real-time background-free selective imaging of fluorescent nanodiamonds in vivo. *Nano letters*, Vol. 12, No. 11, pp. 5726–32, November 2012.
- [16] Gopalakrishnan Balasubramanian, I Y Chan, Roman Kolesov, Mohannad Al-Hmoud, Julia Tisler, Chang Shin, Changdong Kim, Aleksander Wojcik, Philip R Hemmer, Anke Krueger, Tobias Hanke, Alfred Leitenstorfer, Rudolf Bratschitsch, Fedor Jelezko, and Jörg Wrachtrup. Nanoscale imaging magnetometry with diamond spins under ambient conditions. *Nature*, Vol. 455, No. 7213, pp. 648–51, October 2008.
- [17] J. Maze, P. Stanwix, J. Hodges, S. Hong, J. Taylor, P. Cappellaro, L Jiang, M. Dutt, E. Togan, S. Zibrov, A. Yacoby, R. Walsworth, and M. Lukin. Nanoscale magnetic sensing with an individual electronic spin in diamond. *Nature*, Vol. 455, No. 7213, pp. 644–7, October 2008.
- [18] F. Dolde, H. Fedder, M. Doherty, T. Nöbauer, F. Rempp, G. Balasubramanian, T. Wolf, F. Reinhard, L. Hollenberg, F. Jelezko, and J. Wrachtrup. Electric-field sensing using single diamond spins. *Nature Physics*, Vol. 7, No. 6, pp. 459–463, April 2011.

- [19] D. Toyli, F. Charles, David. Christle, V. Dobrovitski, and D. Awschalom. Fluorescence thermometry enhanced by the quantum coherence of single spins in diamond. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 110, No. 21, pp. 8417–21, May 2013.
- [20] Y. Doi, T. Makino, H. Kato, D. Takeuchi, M. Ogura, H. Okushi, H. Morishita, T. Tashima, S. Miwa, S. Yamasaki, P. Neumann, J. Wrachtrup, Y. Suzuki, and N. Mizuochi. Deterministic Electrical Charge-State Initialization of Single Nitrogen-Vacancy Center in Diamond. *Physical Review X*, Vol. 4, No. 1, p. 011057, March 2014.
- [21] K. Fu, C. Santori, P. Barclay, and R. Beausoleil. Conversion of neutral nitrogen-vacancy centers to negatively charged nitrogen-vacancy centers through selective oxidation. *Applied Physics Letters*, Vol. 96, No. 12, p. 121907, 2010.
- [22] B. Grotz, M. Hauf, M. Dankerl, B. Naydenov, S. Pezzagna, J. Meijer, F. Jelezko, J. Wrachtrup, M. Stutzmann, F. Reinhard, and J. Garrido. Charge state manipulation of qubits in diamond. *Nature communications*, Vol. 3, p. 729, January 2012.
- [23] P. Siyushev, H. Pinto, M. Vörös, a. Gali, F. Jelezko, and J. Wrachtrup. Optically Controlled Switching of the Charge State of a Single Nitrogen-Vacancy Center in Diamond at Cryogenic Temperatures. *Physical Review Letters*, Vol. 110, No. 16, p. 167402, April 2013.
- [24] C. Bradac, T. Gaebel, N. Naidoo, J. Rabeau, and A. Barnard. Prediction and mea-

surement of the size-dependent stability of fluorescence in diamond over the entire nanoscale. *Nano letters*, Vol. 9, No. 10, pp. 3555–64, October 2009.

- [25] J. Cui, X. Chen, L. Fan, Z. Gong, C. Zou, F. Sun, Z. Han, and G. Guo. Generation of Nitrogen-Vacancy Centers in Diamond with Ion Implantation. *Chinese Physics Letters*, Vol. 29, No. 3, p. 036103, March 2012.
- [26] B. Smith, D. Inglis, B. Sandnes, J. Rabeau, A. Zvyagin, C. Gruber, D. and Noble, R. Vogel, E. Osawa, and T. Plakhotnik. Five-nanometer diamond with luminescent nitrogen-vacancy defect centers. *Small (Weinheim an der Bergstrasse, Germany)*, Vol. 5, No. 14, pp. 1649–53, July 2009.
- [27] S. Osswald, G. Yushin, V. Mochalin, S. Kucheyev, and Y. Gogotsi. Control of sp²/sp³ carbon ratio and surface chemistry of nanodiamond powders by selective oxidation in air. *Journal of the American Chemical Society*, Vol. 128, No. 35, pp. 11635–42, September 2006.
- [28] J. Cebik, J. McDonough, F. Peerally, R. Medrano, I. Neitzel, Y. Gogotsi, and S. Osswald. Raman spectroscopy study of the nanodiamond-to-carbon onion transformation. *Nanotechnology*, Vol. 24, No. 20, p. 205703, May 2013.

Chapter II

Optically Detected Magnetic Resonance Signals Arising from Nitrogen-Vacancy Centers in Nanodiamonds as Small as 5-nm

Abstract

Negatively-charged nitrogen-vacancy center (NV^-) in nanodiamond is one of the prominent candidate for magneto-sensing probe, however such sensing have not achieved by detonation nanodiamond (DND, size 5 nm). Here we report the magneto-sensing by a single NV^- contained in a DND through optically detected magnetic resonance (ODMR) signal. However, the signal is too small to conduct such experiment, so we first demonstrated the ODMR signal can be enhanced by the surface chemical termination of nanodiamonds (size 25 nm). We then elucidated that this enhancement is effective for DNDs. Furthermore we demonstrated the determination of orientation towards external magnetic field of ODMR enhanced single DND, indicating such small nanodiamond can be used for single spin probe for nanomagnetometry in biological system.

1. Introduction

NVCs are well-studied chromophore centers in diamond crystal, each of which consists of a substitutional nitrogen atom and adjacent vacancy in the crystal lattice of a diamond. NVCs form in two different types of charge state, neutral-charged NVCs (NV^0) and negatively-charged NVCs (NV^-), characterized by their zero-phonon line at 575 nm and 638 nm, respectively.[1] Among these two states, optical read out of the electron spin, which is perfectly polarized in the triplet ground state $m_s = 0$ sublevel, is possible only for NV^- s.[2, 3] Owing to this property, single electron spin resonance can be measured by optically detected magnetic resonance (ODMR) with high sensitivity even at room temperature.[4, 5] Recently, many challenging studies such as ultrasensitive magnetometry in a nanoscale region,[6, 7] temperature sensing with sub-micrometer spatial resolution and sub-degree thermal sensitivity in a single cell,[8] and long-term tracking of cells in vivo have been reported[9]. However, most of these studies used diamond nanoparticles with the size of larger than 40 nm, and have not achieved with NV^- s contained in detonation synthesized nanodiamonds (DNDs).

DNDs are currently produced in bulk scale as colloidal dispersion with primary particle size of 5 nm.[10, 11] Their mechanically rigid core and easiness of the surface chemical modification is attractive for various fields of science.[12] Owing to their small size, they are attracted in the application for bio-medical application.[13, 14] DNDs with NVCs has reported by Smith et al[15], and Bradac et al and Baranov et al have reported the ODMR signal arise from NV^- s contained in DNDs[16, 17], but single NV^- detection has not been experimented. Generally, knowing the position of an NV^- in diamond crystal is quite difficult, so the spatial resolution of those sensing is determined by the size of a nan-

odiamond particle. Hence, detection and control of the spin in such NV^- give promising applications for the sensing with ultra-high resolution.

In the natural state, DNDs are supposed to contain the NV^- s for effective concentration, so we are tried to the charge state conversion by surface termination. Recently, charge state conversion from NV^0 to NV^- has attracted a great deal of attention owing to growing interest in ODMR experiments. Because ODMR intensity is determined by the ratio $NV^- / (NV^0 + NV^-)$, charge state conversion from NV^0 to NV^- enables more sensitive ODMR detection. It has been reported that the charge state of NVCs located close to the surface are largely influenced by chemical terminations on the surface.[18, 19] Surface termination by hydrogen, which has lower electron negativity than carbon, exhibits negative electron affinity on the diamond's surface. Water layers adsorbed on the surface of the diamond accept electrons and lead to the surface band bending upwards, resulting in an accumulation of hole and enhancement of the population of NV^0 s.[20, 21] By contrast, surface termination by atoms such as oxygen that have higher electronegativity than carbon results in a higher density of NV^- s. From this point of view, fluorine termination is reported to be more effective for charge state conversion from NV^0 to NV^- as compared with oxygen termination.[22] Nevertheless, previous studies have provided only limited information such as the ratio $NV^- / (NV^0 + NV^-)$ by comparing individual fluorescence intensity of zero-phonon lines. Direct measurement of how much the ODMR signal is enhanced by surface termination would be informative; however, studies that report the intensity level have not been reported so far. At first, we investigated the effect of surface chemical terminations in nanodiamonds (NDs, size 25 nm) on ODMR intensity.

2. Results and Discussion

We prepared bare-, COOH- and OH-terminated NDs (ND-bare, ND-COOH and ND-OH, respectively) (see experimental section). ND-COOH and ND-OH were expected to enhance ODMR intensity because the charge state of NVCs is converted from ODMR-inactive NV^0 s to ODMR-active NV^- s. In addition, these groups are easily activated through carbodiimide chemistry or silanization unlike fluorine termination[23] So they are favorable for chemical, medical and biological applications of NDs.

The ODMR signal is detected as a decrease in fluorescence intensity when microwave (MW) irradiation is applied at a frequency of 2870 MHz. The ground state of NV^- is a spin-triplet, and the spin sublevels, $m_s = 0$ and degenerated $m_s = \pm 1$, are split by 2870 MHz.[24] MW irradiation at this frequency induces electron spin magnetic resonance between the $m_s = 0$ and $m_s = \pm 1$ spin levels, which results in a reduction in the fluorescence intensity from NV^- due to spin-dependent intersystem crossing. Here, we measured ODMR signals arising from NDs with a chemically modified surface. To detect the ODMR signals, we used a custom-built microscope established by Igarashi et.al.,[25] comprising an inverted fluorescence microscope equipped with a MW irradiation system to manipulate the electron spin of the NVCs. We used this microscope to monitor the fluorescence intensity of NVCs while sweeping the MW frequency.

First, we observed the fluorescence of NDs scattered on glass plates. Figure 1a shows an example of the time course of the fluorescence intensity of an ND without MW irradiation and the corresponding histogram of fluorescence intensities. It can be observed that the fluorescence intensity is constant without photo-blinking or photo-bleaching, and the histogram shows a single peak in distribution without MW irradiation. Figure 1b shows

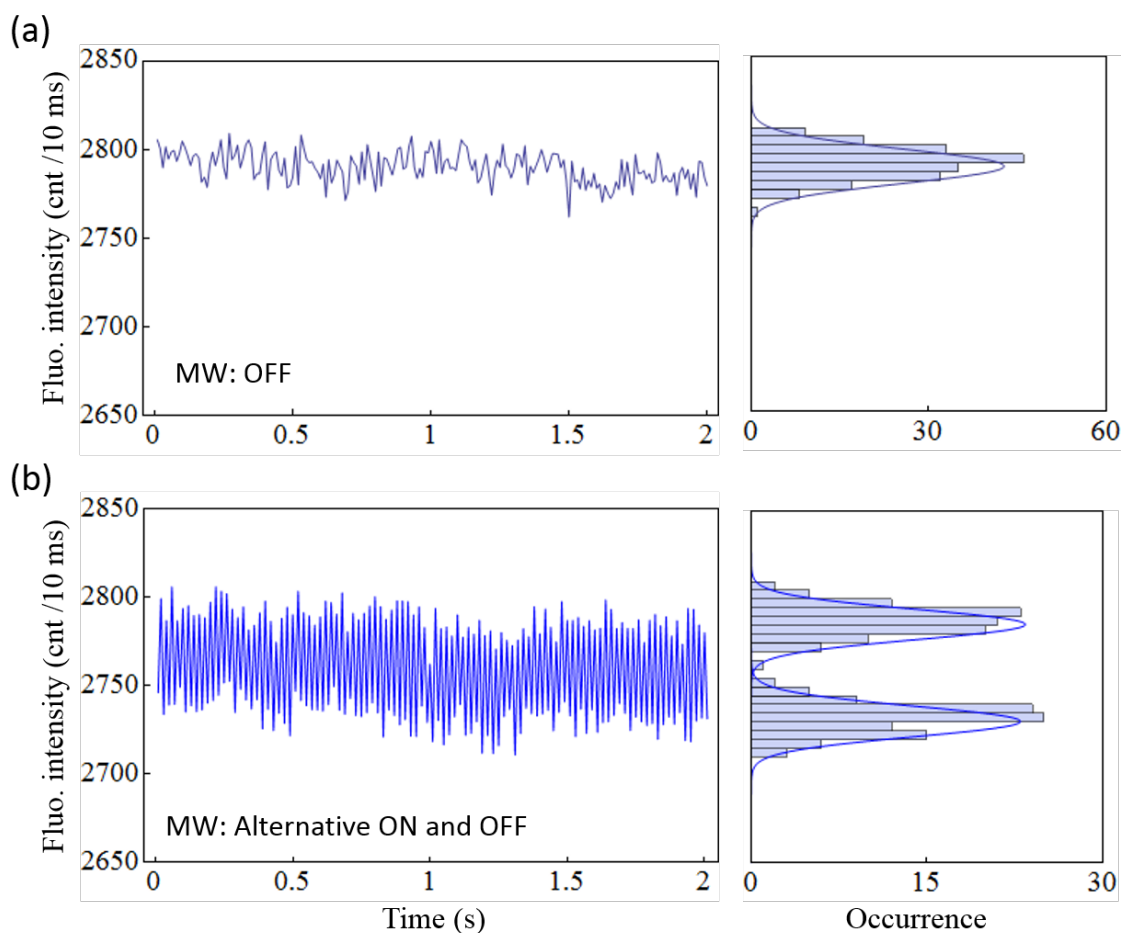


Figure 1. Example time course of the fluorescence intensity of an ND and its corresponding histogram. (a) Without microwave irradiation. The histogram is fitted with a single Gaussian. (b) With MW irradiation alternatively OFF and ON at 2870 MHz. The histograms are fitted with Gaussians.

the time course of fluorescence intensity and the corresponding histogram of the same ND, with alternately on-and-off irradiation at 2870 MHz. Synchronization of the fluorescence intensity modulation with the MW alternation can be observed, in contrast to Figure 1a. The histogram of Figure 1b shows two peaks in distribution corresponding to intensities with and without MW irradiation. This newly emerged distribution exhibits a reduction in fluorescence intensity due to MW-assisted ESR. These data verify that it is possible to detect ODMR signals by our system. We determined the ODMR intensity of

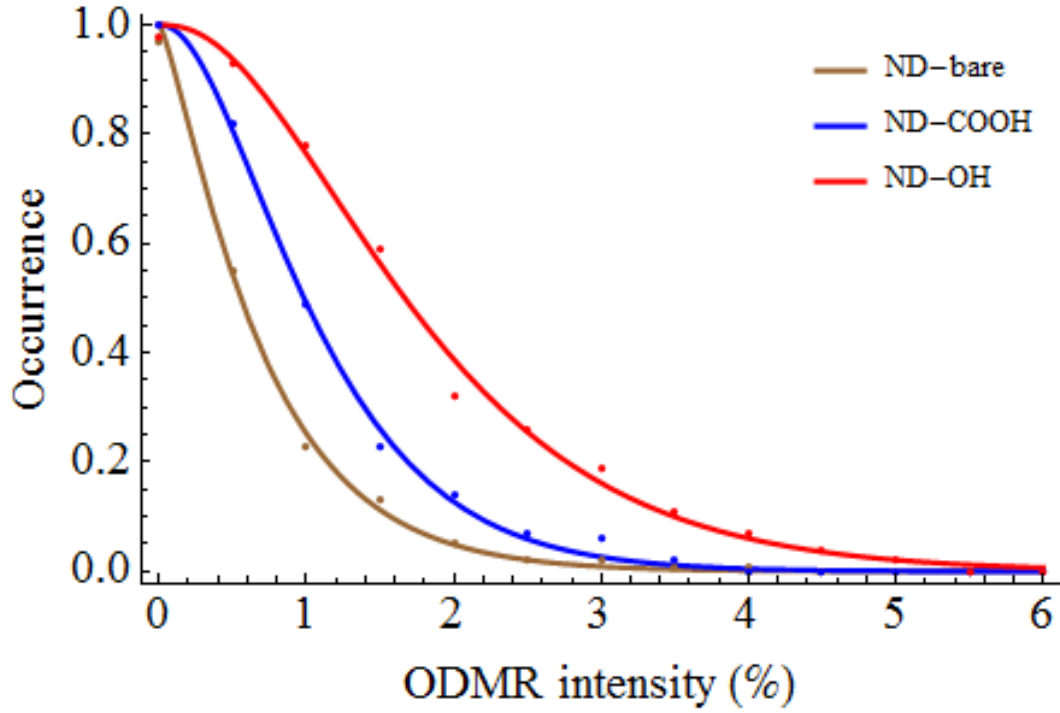


Figure 2. Probability of the occurrence of NDs emitting ODMR intensities larger than the values indicated on the horizontal axis. Data were fitted to Gamma cumulative distribution functions. For each surface-terminated ND, 100 samples were measured.

one ND by the following equation:

$$I_{ODMR} = (I_{OFF} - I_{ON}) / I_{OFF} \quad (1)$$

where I_{ODMR} , I_{OFF} , and I_{ON} indicate the intensity of the ODMR, and the peak intensities of the distribution when the external magnetic fields are off and on, respectively. Next, we measured the ODMR signal of surface-terminated NDs.

Figure 2 shows the relative frequency of the occurrence of NDs giving an ODMR intensity larger than the values indicated on the x axis. Data were fitted to Gamma cumulative distribution functions. The particle shapes of NDs are not perfectly spherical but have an irregular shape, so the effect of surface termination on NVCs is likely to be

highly random. The Gamma function was thought to be optimal for describing these data because it is often used for the representation of random variables. In fact, the Gamma distribution was in good agreement with the data. The results indicated that ODMR intensity is enhanced by surface modification with COOH- or OH- termination. The average ODMR intensity calculated by the fitting was 0.7%, 1.2% and 1.9% for ND-bare, ND-COOH and ND-OH, respectively. This indicates that the ODMR intensity increased 1.7- and 2.7-fold for COOH- and OH-termination, respectively, as compared with non-surface modification. Thus, as predicted by previous studies, COOH-termination is effective for enhancing ODMR intensity. Moreover, we revealed that OH-termination is more effective than COOH-termination by means of directly measuring ODMR signals.

We next investigated whether the effect of ODMR enhancement by surface modification would be applicable to DNDs. Owing to their small size, DNDs are the leading candidate for novel ODMR probes for analyzing nanometer-sized bio-molecules. However, there has not been reported about the magneto-sensing of NV^- contained in a DND despite recent increasing attention in ODMR. An easy method for the preparation of ODMR-detectable DNDs and its sensing would open up novel biological applications. Because DNDs are so small, almost all NVCs are thought to exist close to the surface. Hence, modifying the surface termination of DNDs is expected to have a strong effect on ODMR signals. We therefore synthesized OH-terminated DNDs. Since DNDs are very small-sized nanodiamonds, there is a possibility that the diamond crystal structure is disturbed by the process of chemical treatment. We measured XRD spectra, and confirmed that the crystal structure is maintained for OH-terminated DNDs (Figure 3). We then measured the ODMR spectra of them.

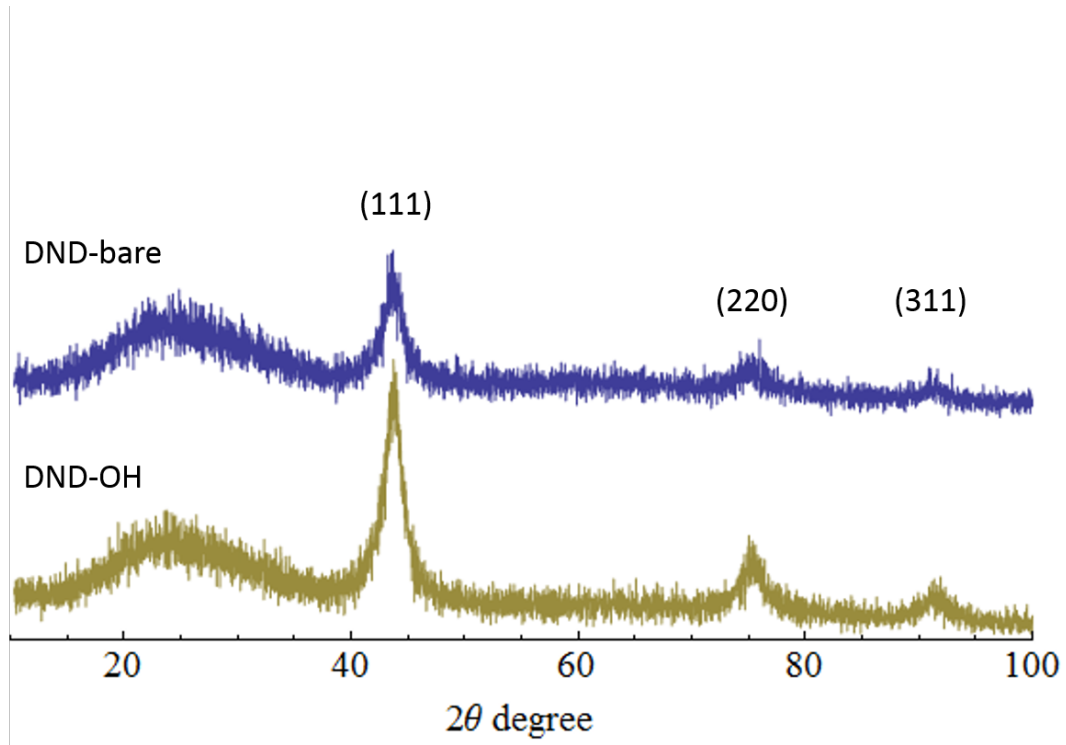


Figure 3. XRD spectra of DND-bare and DND-OH. Three peaks corresponding to (111), (220) and (311) diffraction of diamond are observed, indicating that each sample maintains a diamond crystal structure.

First, we measured the ODMR signals from bulk DNDs. Figure 4 exhibits the fluorescence images and ODMR spectra of bulk DND-OH and DND-bare (ODMR spectra correspond to the yellow square in the fluorescence image; 3 typical images of each DND are shown). From the spectra of DND-OH, a reduction in fluorescence intensity caused by MW-induced magnetic resonance of electron spin in the NV^- was clearly observed at around 2870 MHz. By contrast, ODMR-detectable DND-bare were not observed even in bulk measurements. As a result, bulk measurements indicate that ODMR intensity can be effectively enhanced by OH-termination also in DNDs.

Next, we demonstrated the detection of a single spin from a DND-OH and magnetosensing of them. Figure 5 shows the 3 samples of ODMR spectra from an OH-terminated

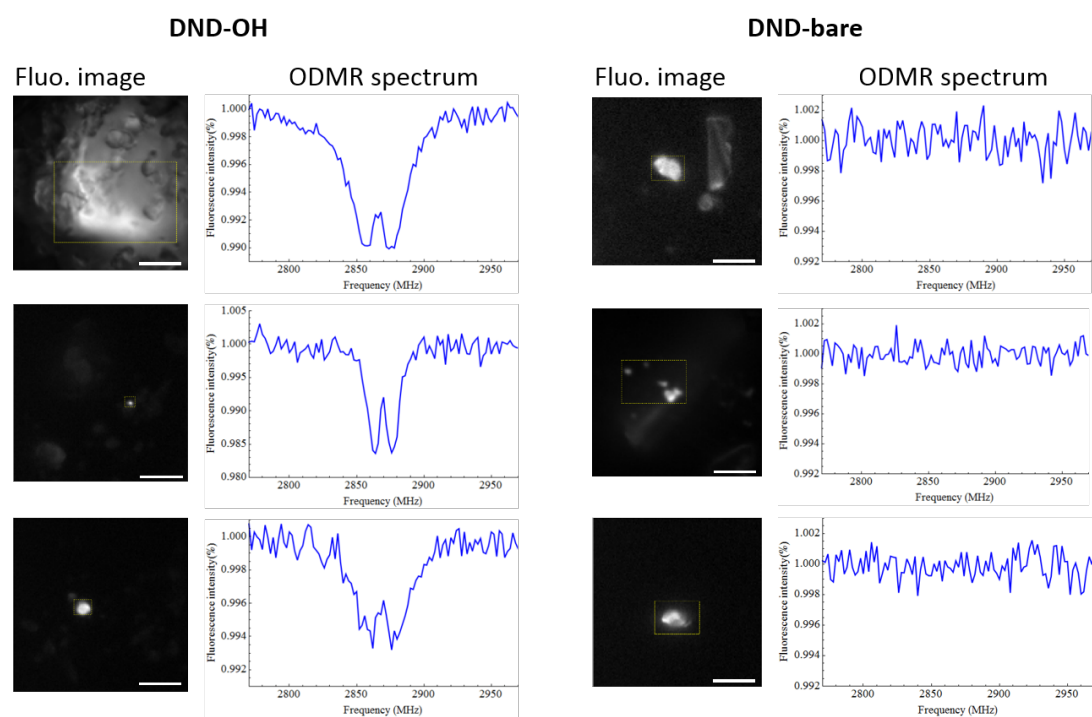


Figure 4. Typical fluorescence images (left column) and corresponding ODMR spectra for the area indicated in the yellow square (right column) for bulk DND-OH and DND-bare. Bulk DND-bare could not be detected by ODMR. Scale bar show a 10 μm .

single DND. Green and red plots show the ODMR spectrum without and with the external magnetic field, respectively. Each plot are fitted by two Lorentz function (solid line). A reduction in fluorescence intensity is clearly observed in the spectrum around 2870 MHz under zero magnetic field. The degenerate spin sublevels $m_s = \pm 1$ are lifted by the Zeeman effect in the applied external magnetic field; we then observed two dips corresponding to the electron spin resonance frequencies between $m_s = 0$ to $+1$ and $m_s = 0$ to -1 , indicating the detection of external magnetic fields by means of the single NV^- . Thus, the ODMR signal can be successfully detected from not only bulk DND-OH, but also a single DND-OH. In addition to this result, selective imaging protocol experiments[17] were performed, which suggested that the quantity of ODMR-detectable DNDs is increased by OH-termination (Figure 6).

Taken together, these results indicate that OH-termination might be an effective method for manipulating the electron spin of a single NV^- in a DND. Furthermore, we tried to determine the orientation of NV^- towards the external magnetic field. The spin Hamiltonian of NV^- s is given by:

$$H = g\mu_B B S + D[S_z^2 - \frac{1}{3}S(S+1)] + E(S_x^2 - S_y^2), \quad (2)$$

Where g , μ_B , B , S , D and E are g -factor for electron, Bohr magneton, external magnetic field, electron spin vector ($S = 1$ for triplet state), fine-structure zero-field splitting and strain induced splitting coefficient, respectively. So the strength of the external magnetic field and the orientation of nitrogen-vacancy axis towards the fields can be determined from the ODMR spectrum. These magnetic and angular parameters of each NV^- in a

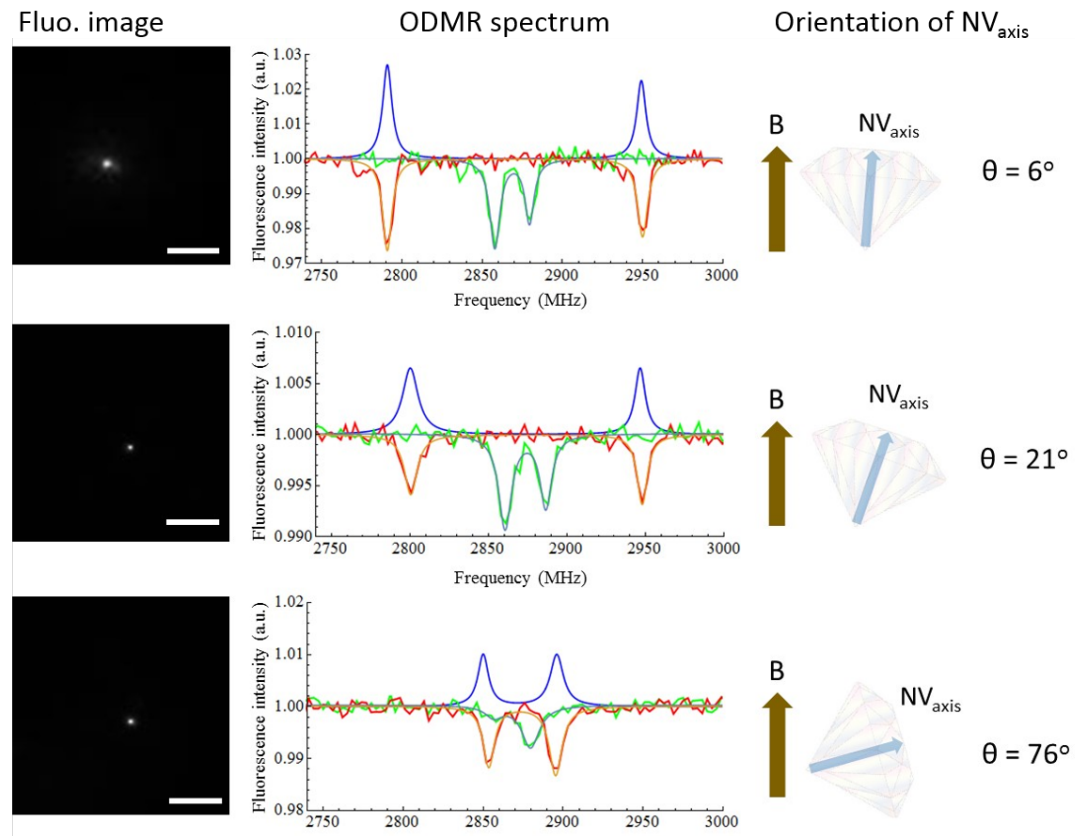


Figure 5. Fluorescence image, ODMR spectrum and orientation towards applied magnetic field of a NV axis estimated by the corresponding ODMR spectrum (3 samples). Scale bar in the fluorescence image shows 5 μm . Red and green plots represent the ODMR spectrum with or without the external magnetic field, respectively. Schematic illustration indicate the direction and strength of applied magnetic field and orientation of N-V axis. Blue plot in ODMR spectrum is the theoretical one calculated by these parameter, note that vertical axis is reversed.

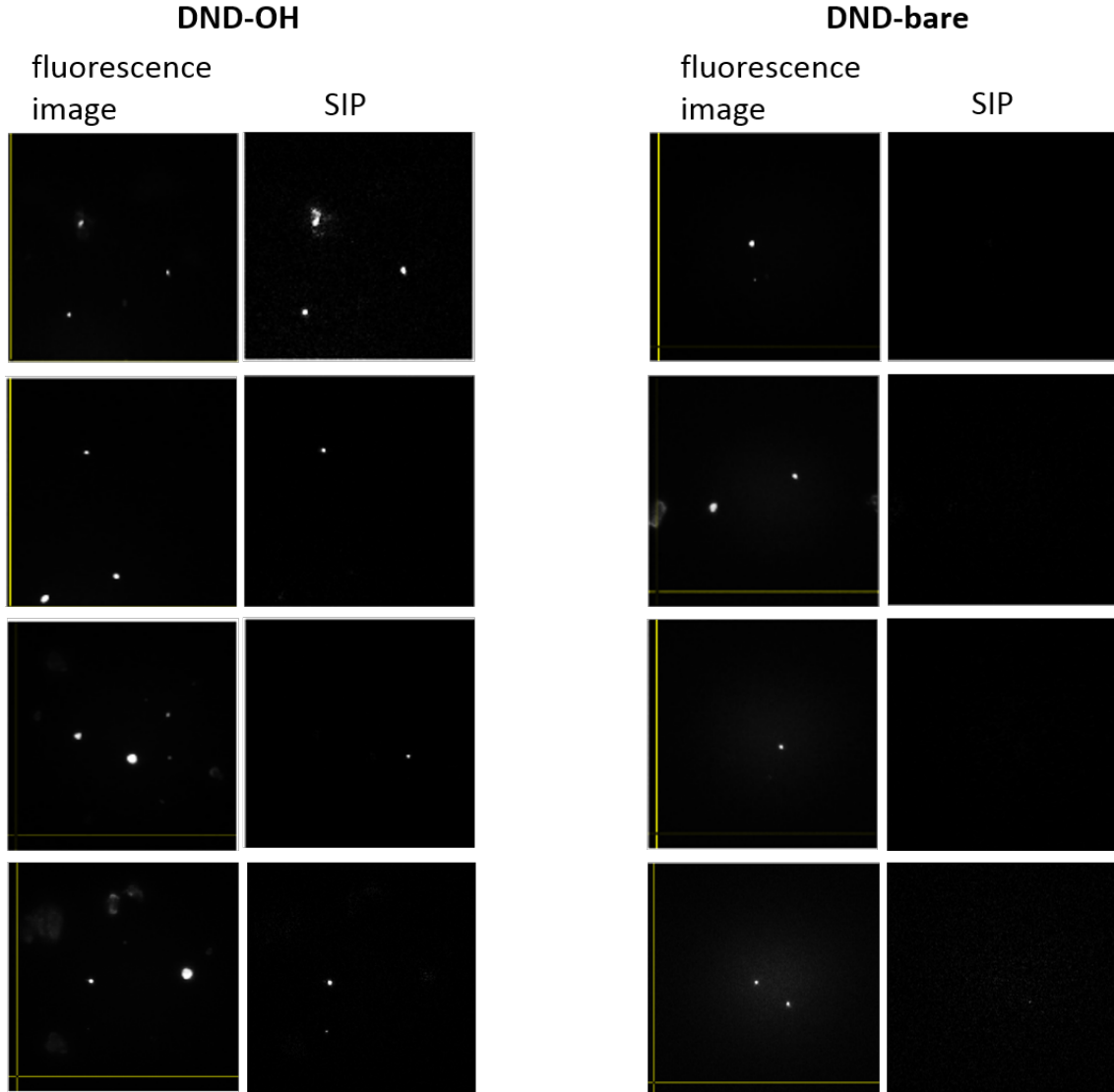


Figure 6. Fluorescence (left column) and selective imaging protocol images (right column) of DD-OH and DD. This protocol is a technique established by Igarashi et. al. (*Nano Lett.* 2012, 12, 5726), which enables the detection of intrinsic fluorescence from ODMR-active NV⁻s. DND-OH exhibits some ODMR-active nanodiamond particles; by contrast, DND rarely shows ODMR-active fluorescence.

DND are displayed in Figure 5. Blue plot in Figure 5 is the theoretical ODMR spectrum calculated from these parameters, note that the vertical axis is inverted for facilitating visualization. These results showed that the experimental and theoretical data were in good accordance, indicating that a single DND is applicable for magneto-sensing such as

the orientation tracking.

In summary, we have demonstrated that the enhancement of ODMR signals depends on the surface chemical termination. As expected from previous studies, COOH-termination of the surface of NDs is effective for augmenting the ODMR signal. In addition, we have shown that OH-termination is more effective than COOH-termination. This effect is likewise valid for DNDs: OH-terminated DNDs yield a strong ODMR signal, even under an externally applied magnetic field. This termination enables the detection of a single spin within a DND. Furthermore, we first demonstrated the orientation detection of nitrogen-vacancy axis towards applied external magnetic fields. Our results will contribute to the measurement of dynamics of proteins or organelles, which requires the small-sized probes for preventing interfere to the dynamics, and are an important step for controlling and utilizing ODMR spectroscopy from DNDs. We believe that the findings of this study will open up a high-sensitive and high-resolution magnetometry for biological research.

3. Experimental Section

3.1 Surface modification of NDs

NDs (Micron+ MDA Element Six) in water were centrifuged at 15,000 rpm for 20 minutes, and the supernatant was lyophilized. The median size of the NDs was determined to be 25.9 nm by dynamic light scattering (DLS). These samples were thermally annealed at high temperature (800 °C) for two hours under vacuum, yielding NVCs in NDs (ND-bare); the surface graphite layer and volatile impurities were then removed by oxidation at 550 °C for two hours under atmospheric conditions. The oxidized samples were treated with a mixture of H₂SO₄ : HNO₃ (9:1 v/v) at 70 °C for three days, and then centrifuged

for 30 minutes at 15000 rpm, and the supernatant was discarded. Next, 0.1 M NaOH was added and the suspension was stirred for two hours at 90 °C. After centrifugation, the NDs were stirred in 0.1 M HCl for two hours at 90 °C. Lastly, the NDs were centrifuged and washed three times with milli-Q water, yielding ND-COOH. In addition, the oxidized samples were treated with borane in THF (1 M) under nitrogen atmosphere with refluxing for 12 hours. The reaction was quenched with HCl aqueous solution. The samples were centrifuged and washed three times with milli-Q water, yielding ND-OH. Synthesis of these NDs was confirmed by IR spectroscopy (Figure 7a).

3.2 Surface modification of DNDs

DNDs (NanoAmando, size 5.3 ± 1 nm) were provided by Dr. Eiji Osawa. They were freeze-dried and thermally annealed at high temperature (800 °C) for two hours under vacuum, and then oxidized at 425 °C for five hours. COOH-termination and OH-termination were conducted by a method similar to that of surface modification of NDs. Synthesis of these DNDs was confirmed by IR spectroscopy (Figure 7b).

3.3 ODMR measurements

The field of view was illuminated with a 532-nm Nd:YAG laser, and fluorescence images were measured by an electron multiplying charge coupled device (EMCCD) camera. The emitted light was collected by an oil immersion 60 × objective (NA=1.49), passed through a dichroic mirror centered at 575 nm and a long-wave (short-wave) pass filter longer than 590 nm (shorter than 845 nm) to detect the fluorescence signal from NVCs. The microscope was equipped with a MW coil and detachable neodymium mag-

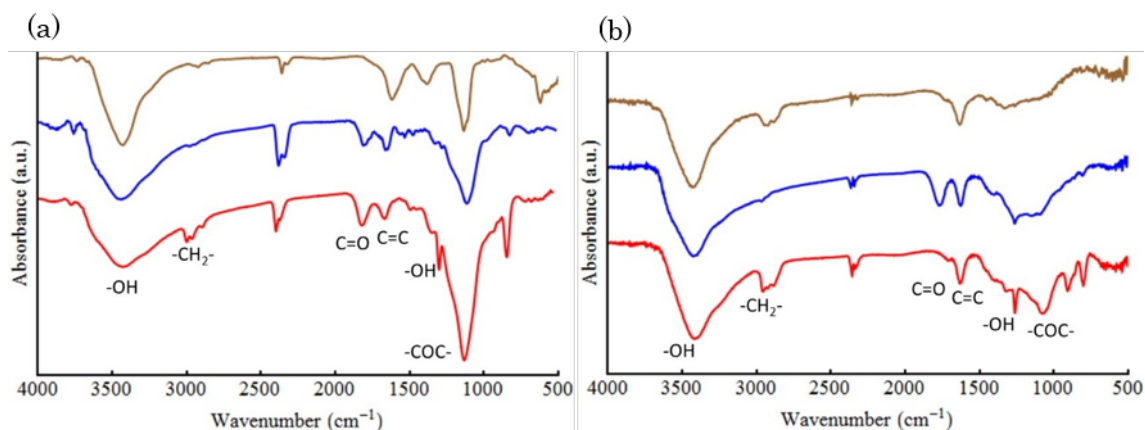


Figure 7. IR spectra of (a) ND-bare (brown), ND-COOH (blue) and ND-OH (red), and (b) DND (brown), DND-COOH (blue) and DND-OH (red). Typical adsorption bands in the area are labeled. COOH-termination was confirmed by the emergence of C=O vibration band of $1740\text{--}1760\text{ cm}^{-1}$, and OH-termination was confirmed by C-H stretching vibration band of $2850\text{--}2960\text{ cm}^{-1}$ and O-H deformational vibration band of $1250\text{--}1300\text{ cm}^{-1}$.

net above sample stage for irradiating the resonant frequency and external magnetic fields.

By means of this experimental set-up, ODMR intensity was calculated according to Equation (1), and ODMR spectrum was measured with the MW swept widely from 2670 to 3070 MHz point-by-point with or without external magnetic fields.

References

- [1] Y. Chang, H. Lee, K. Chen, C. Chang, D. Tsai, C. Fu, T. Lim, Y. Tzeng, C. Fang, C. Han, H. Chang, and W. Fann. Mass production and dynamic imaging of fluorescent nanodiamonds. *Nature nanotechnology*, Vol. 3, No. 5, pp. 284–8, May 2008.
- [2] A. Gruber, C. Dräbenstedt, L. Tietz, J. Fleury, J. Wrachtrup, and C. Borczyskowski. Scanning Confocal Optical Microscopy and Magnetic Resonance on Single Defect Centers. *Science*, Vol. 276, No. 5321, pp. 2012–2014, June 1997.
- [3] P. Nizovtsev, S.Ya. Kilin, F. Jelezko, I. Popa, A. Gruber, and J. Wrachtrup. NV centers in diamond: spin-selective photokinetics, optical ground-state spin alignment and hole burning. *Physica B: Condensed Matter*, Vol. 340-342, pp. 106–110, December 2003.
- [4] P. Maletinsky, S. Hong, M. Grinolds, B. Hausmann, M. Lukin, R. Walsworth, M. Loncar, and A. Yacoby. A robust scanning diamond sensor for nanoscale imaging with single nitrogen-vacancy centres. *Nature nanotechnology*, Vol. 7, No. 5, pp. 320–4, May 2012.
- [5] V. Horowitz, B. Alemán, D. Christle, A. Cleland, and D. Awschalom. Electron spin resonance of nitrogen-vacancy centers in optically trapped nanodiamonds. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 109, No. 34, pp. 13493–7, August 2012.
- [6] G. Balasubramanian, I. Chan, R. Kolesov, M. Al-Hmoud, J. Tisler, C. Shin, C. Kim, A. Wojcik, P. R Hemmer, A. Krueger, T. Hanke, A. Leitenstorfer, R. Bratschitsch,

- F. Jelezko, and J. Wrachtrup. Nanoscale imaging magnetometry with diamond spins under ambient conditions. *Nature*, Vol. 455, No. 7213, pp. 648–51, October 2008.
- [7] L. McGuinness, Y. Yan, A. Stacey, D. Simpson, L. Hall, D. Maclaurin, S. Prawer, P. Mulvaney, J. Wrachtrup, F. Caruso, R. Scholten, and L. Hollenberg. Quantum measurement and orientation tracking of fluorescent nanodiamonds inside living cells. *Nature nanotechnology*, Vol. 6, No. 6, pp. 358–63, January 2011.
- [8] G. Kucsko, P. Maurer, N. Yao, M. Kubo, H. Noh, P. Lo, H. Park, and M. Lukin. Nanometre-scale thermometry in a living cell. *Nature*, Vol. 500, No. 7460, pp. 54–8, August 2013.
- [9] T. Wu, Y. Tzeng, W. Chang, C. Cheng, Y. Kuo, C. Chien, H. Chang, and J. Yu. Tracking the engraftment and regenerative capabilities of transplanted lung stem cells using fluorescent nanodiamonds., September 2013.
- [10] A. Krüger, F. Kataoka, M. Ozawa, T. Fujino, Y. Suzuki, A.E. Aleksenskii, Ya. Vul, and E. Osawa. Unusually tight aggregation in detonation nanodiamond: Identification and disintegration. *Carbon*, Vol. 43, No. 8, pp. 1722–1730, July 2005.
- [11] O. A. Shenderova, V. V. Zhirnov, and D. W. Brenner. Carbon Nanostructures. *Solid State and Material Science*, Vol. 27, pp. 227–356, 2002.
- [12] Amanda S. Barnard and E. Osawa. The impact of structural polydispersivity on the surface electrostatic potential of nanodiamond. *Nanoscale*, Vol. 6, No. 2, pp. 1188–94, January 2014.

- [13] A. Krüger, Y. Liang, G. Jarre, and J. Stegk. Surface functionalisation of detonation diamond suitable for biological applications. *Journal of Materials Chemistry*, Vol. 16, No. 24, p. 2322, 2006.
- [14] E. Chow, X. Zhang, M. Chen, R. Lam, E. Robinson, H. Huang, D. Schaffer, E. Osawa, A. Goga, and D. Ho. Nanodiamond therapeutic delivery agents mediate enhanced chemoresistant tumor treatment. *Science translational medicine*, Vol. 3, No. 73, p. 73ra21, March 2011.
- [15] B. Smith, D. Inglis, B. Sandnes, J. Rabeau, A. Zvyagin, D. Gruber, C. Noble, R. Vogel, E. Osawa, and T. Plakhotnik. Five-nanometer diamond with luminescent nitrogen-vacancy defect centers. *Small (Weinheim an der Bergstrasse, Germany)*, Vol. 5, No. 14, pp. 1649–53, July 2009.
- [16] C. Bradac, T. Gaebel, N. Naidoo, M. Sellars, J. Twamley, L. Brown, S. Barnard, T. Plakhotnik, A. Zvyagin, and J. Rabeau. Observation and control of blinking nitrogen-vacancy centres in discrete nanodiamonds. *Nature nanotechnology*, Vol. 5, No. 5, pp. 345–9, May 2010.
- [17] P. Baranov, A. Soltamova, D. Tolmachev, N. Romanov, R. Babunts, F. Shakhov, S. Kidalov, A. Vul', G. Mamin, S. Orlinskii, and N. Silkin. Enormously high concentrations of fluorescent nitrogen-vacancy centers fabricated by sintering of detonation nanodiamonds. *Small (Weinheim an der Bergstrasse, Germany)*, Vol. 7, No. 11, pp. 1533–7, June 2011.
- [18] L. Rondin, G. Dantelle, A. Slablab, F. Grosshans, F. Treussart, P. Bergonzo, S. Per-

- ruchas, T. Gacoin, M. Chaigneau, H. Chang, V. Jacques, and J.-F. Roch. Surface-induced charge state conversion of nitrogen-vacancy defects in nanodiamonds. *Physical Review B*, Vol. 82, No. 11, p. 115449, September 2010.
- [19] K.-M. C. Fu, C. Santori, P. E. Barclay, and R. G. Beausoleil. Conversion of neutral nitrogen-vacancy centers to negatively charged nitrogen-vacancy centers through selective oxidation. *Applied Physics Letters*, Vol. 96, No. 12, p. 121907, 2010.
- [20] M. Hauf, B. Grotz, B. Naydenov, M. Dankerl, S. Pezzagna, J. Meijer, F. Jelezko, J. Wrachtrup, M. Stutzmann, F. Reinhard, and J. Garrido. Chemical control of the charge state of nitrogen-vacancy centers in diamond. *Physical Review B*, Vol. 83, No. 8, pp. 1–4, February 2011.
- [21] V. Petráková, A. Taylor, I. Kratochvílová, F. Fendrych, J. Vacík, J. Kučka, J. Štursa, P. Cígler, M. Ledvina, A. Fišerová, P. Kneppo, and M. Nesládek. Luminescence of Nanodiamond Driven by Atomic Functionalization: Towards Novel Detection Principles. *Advanced Functional Materials*, Vol. 22, No. 4, pp. 812–819, February 2012.
- [22] S. Cui and E. Hu. Effect of fluorinated diamond surface on charge state of nitrogen-vacancy centers. *arXiv preprint arXiv:1304.1407*, 2013.
- [23] A. Krueger and D. Lang. Functionality is Key: Recent Progress in the Surface Modification of Nanodiamond. *Advanced Functional Materials*, Vol. 22, No. 5, pp. 890–906, March 2012.
- [24] J. Maze, P. Stanwix, J. Hodges, S. Hong, J.. Taylor, P Cappellaro, L. Jiang, G. Dutt,

- E. Togan, S. Zibrov, A. Yacoby, R. Walsworth, and M. Lukin. Nanoscale magnetic sensing with an individual electronic spin in diamond. *Nature*, Vol. 455, No. 7213, pp. 644–7, October 2008.
- [25] R. Igarashi, Y. Yoshinari, H. Yokota, T. Sugi, F. Sugihara, K. Ikeda, H. Sumiya, S. Tsuji, I. Mori, H. Tochio, Y. Harada, and M. Shirakawa. Real-time background-free selective imaging of fluorescent nanodiamonds in vivo. *Nano letters*, Vol. 12, No. 11, pp. 5726–32, November 2012.

Chapter III

**Comprehensive and Quantitative
Analysis for Controlling the
Physical/Chemical States and Particle
Properties of Nanodiamonds for
Biological Applications**

Abstract

The physical/chemical states and properties of nanodiamonds subjected to thermal annealing and air oxidation, which are indispensable processes for the preparation of fluorescent nanodiamonds, were investigated. Specifically, the weight loss, particle size, crystal quality, chemical bonding states of carbon and oxygen, zeta potential, dispersibility, and fluorescent and optically detected magnetic resonance (ODMR) properties were determined using transmission electron microscopy (TEM), elemental analysis, dynamic light scattering, Raman analysis, x-ray diffraction (XRD) analysis, x-ray photoelectron spectroscopy (XPS), IR spectroscopy, and a home-made fluorescence and ODMR microscope. The study focused on small-sized nanodiamonds (~50 nm), which are applicable for biological research. The obtained results should be useful for controlling the mutually-related physical/chemical states and properties of diamond nanoparticles.

1. Introduction

In recent years, numerous reports have appeared in the literature on the properties of nitrogen-vacancy centers (NVCs) as lattice defects in nanodiamonds (NDs).[1] Fluorescence arising from NVCs exhibits perfect photo-stability, undergoing neither photo-bleaching nor photo-blinking,[2] a long fluorescence life time[3], and a high quantum yield.[4] These photo-properties, along with the ability to achieve diverse surface modifications [5] and a reduced cytotoxicity[6], make NDs promising candidates as novel bio-imaging agents.[7, 8] In addition, optically detected magnetic resonance (ODMR) has been demonstrated for the detection of a single spin ESR of an NVC under ambient conditions, which has attracted significant attention in the fields of quantum information,[9] magnetic sensing[10, 11], and biomaterials.[12, 13]

For the preparation of NDs containing NVCs, thermal annealing and air oxidation processes are indispensable. Thermal annealing at 800 °C under a vacuum results in the trapping of a moving vacancy by a substitutional nitrogen atom, leading to the formation of an NVC.[14] Subsequent oxidation in air at 500~600 °C removes the graphite on the ND surface, yielding a fluorescent ND. Many scientists have investigated and reported the optical properties of small-sized NDs (<50 nm) with potential bio-applications.[15, 16] However, although annealing and oxidation processes affect physical/chemical properties, such as the particle size, crystal quality, and surface chemical termination, little has been learned about the chemical/physical states of NDs.

Gabel et al reported that air oxidation of NDs is effective for particle size-reduction,[17] with an average crystal size reduction of 10.6 nm/h at 600 °C during air oxidation under atmospheric conditions. Havlik et al reported that annealing at 900 °C for one hour

resulted in 3-fold brighter ND particles compared to particles annealed under commonly used conditions (temperature and time).[18] In addition, several researchers have reported that high-temperature annealing introduces a graphite on the surface, while oxidation effectively removes this graphite.[19, 20] However, a comprehensive understanding of these physical/chemical states for bio-applicable NDs has not been established. Furthermore, the relationship between these states and ND particle properties, such as dispersibility, fluorescence, and ODMR, has not been reported to date. Changes in these states are expected to affect these important particle properties. By elucidating the relationship between the chemical/physical states and the particle properties, it should be possible to readily prepare fluorescent NDs bearing desired properties. For biological investigations, controlling important factors such as the particle size, crystal quality, surface chemical termination, dispersibility, and fluorescence and ODMR signals will strongly facilitate ND research.

Herein, we report the physical/chemical states and particle properties of pristine NDs and NDs annealed and oxidized at various temperatures and for various lengths of time. The properties were investigated using transmission electron microscopy (TEM), Raman and infrared (IR) spectroscopy, x-ray photoelectron spectroscopy (XPS), x-ray diffraction (XRD) and elemental analyses, and dynamic light scattering. Fluorescence and ODMR signals were detected using a home-built microscope.

2. Results and Discussion

2.1 Weight loss of the nanodiamonds

NDs (MD50) purchased from Tomei Diamond Co. Ltd. were used in this study. The NDs were annealed at 800 °C for two hours under a vacuum in order to pair each

nitrogen atom with a vacancy. Subsequently, the NDs were oxidized at 500 °C, 525 °C, 550 °C, 575 °C, or 600 °C for 1, 2, 3, 4, or 5 hours. First, the weight loss of the NDs during annealing and oxidation was investigated. Due to annealing, 7% of the weight of the NDs was lost. Figure 1 shows the weight loss of the annealed NDs versus the oxidation time at various oxidation temperatures. The degree of the weight loss highly

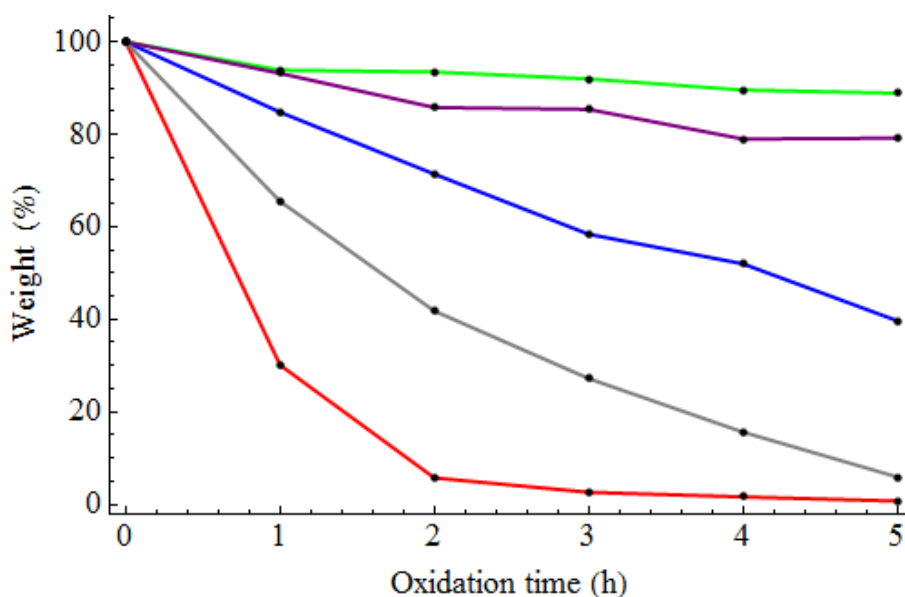


Figure 1. Weight loss of the NDs versus oxidation times with different oxidation temperature.

depended on the oxidation temperature and time. After 5 hours of oxidation, the weight of the NDs remained nearly unchanged at 500 °C, while at 550 °C and 600 °C, significant losses of approximately 40% and 99% were observed, respectively. This weight loss is thought to be due to the isolation of carbon atoms at the surface, which then react with the oxygen and hydrogen in the air to generate carbon oxide. The different degrees of weight loss for the different samples are thought to be related to their physical/chemical states. These three oxidation conditions were thus expected to result in notable differences in the physical/chemical states and were therefore the focus of the present study. Samples are

abbreviated as follows: Pristine NDs: ND-pris; NDs subjected only to thermal annealing at 800 °C for two hours: ND-800; NDs subjected to thermal annealing at 800 °C for two hours and subsequently oxidation at 500, 550, or 600 °C for five hours: ND-500, ND-550, and ND-600, respectively.

2.2 Physical state: Particle size and crystal quality

The ND samples were observed via TEM in order to elucidate any changes in particle size as a function of the annealing and oxidation processes. Figure 2 shows photos, TEM images, and Figure 3 shows size distributions determined from the corresponding TEM images for each of the samples. It can be seen in the photos that ND-pris was a dark-gray color, while ND-800 was black. This change of color may be due to the graphitization of the surface of the NDs during annealing. This graphite was removed by air oxidation, and the degree of whiteness was enhanced with increasing oxidation temperature (a detailed analysis of the surface chemical structure is given in a later section). From the TEM images, it can also be seen that the NDs were not spherical, but had edges. The particle size distributions were similar for ND-pris, ND-800, and ND-500, while the particle sizes for ND-550 and ND-600 were smaller at 35.6 and 14.5 nm, respectively. These results suggested that the surface of ND-800 was graphitized due to annealing, and subsequent oxidation contributed to the removal of the graphite. However, the removal efficiency appeared to depend on the oxidation temperature, because the colors of the different oxidized samples were different. In addition, particle shrinkage occurred when oxidation was performed above 550 °C, and the degree of the size reduction was also temperature dependent.

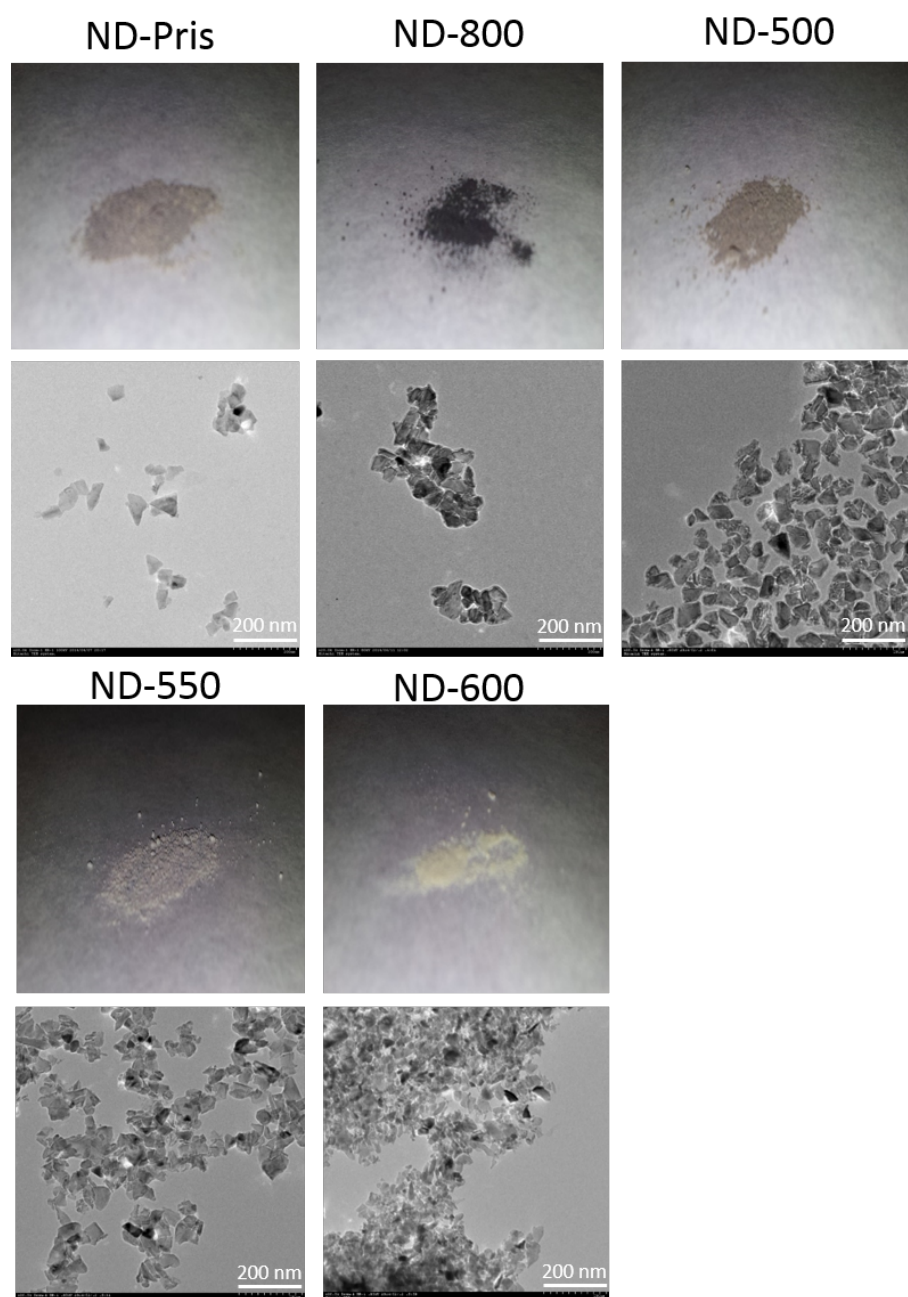


Figure 2. Pictures (upper line), TEM images (lower line) of the samples.

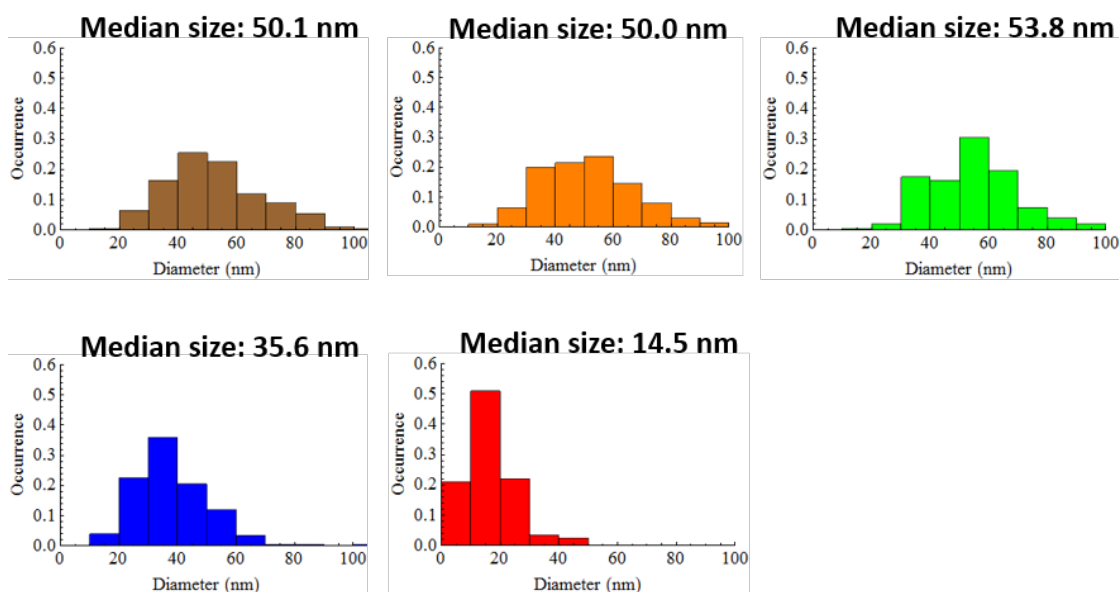


Figure 3. particle size distributions measured form corresponding TEM images of the samples.

To obtain further insight into the particle shrinkage and crystal qualities, a Raman spectroscopic analysis was performed. Figure 4a presents the Raman spectra of the samples. Each spectrum has a D-band (sp^3 carbon), a strain-induced band, and a G-band (sp^2 carbon) near 1332 , 1450 , and 1550 cm^{-1} , respectively. ND-pris exhibited a sharp D-band peak, but the signal for the strain-induced band and the G-band were weak. In contrast, the D-band in the spectrum of ND-800 was drastically enlarged. This result indicated that graphitization of the NDs occurred during annealing, which is supported by the change in color from gray to black (Figure 2). Because this graphite was readily removed by oxidation at 500°C , it is thought that it covered only the surfaces of the NDs. Additional notable changes in the Raman spectra for ND-550 and ND-600 were also observed. The G-band was reduced in the spectra of both ND-550 and ND-600, as was the case for ND-500; on the other hand, the strain-induced bands in the spectra of the former samples were largely enhanced. These results indicated that oxidation above 550°C induced

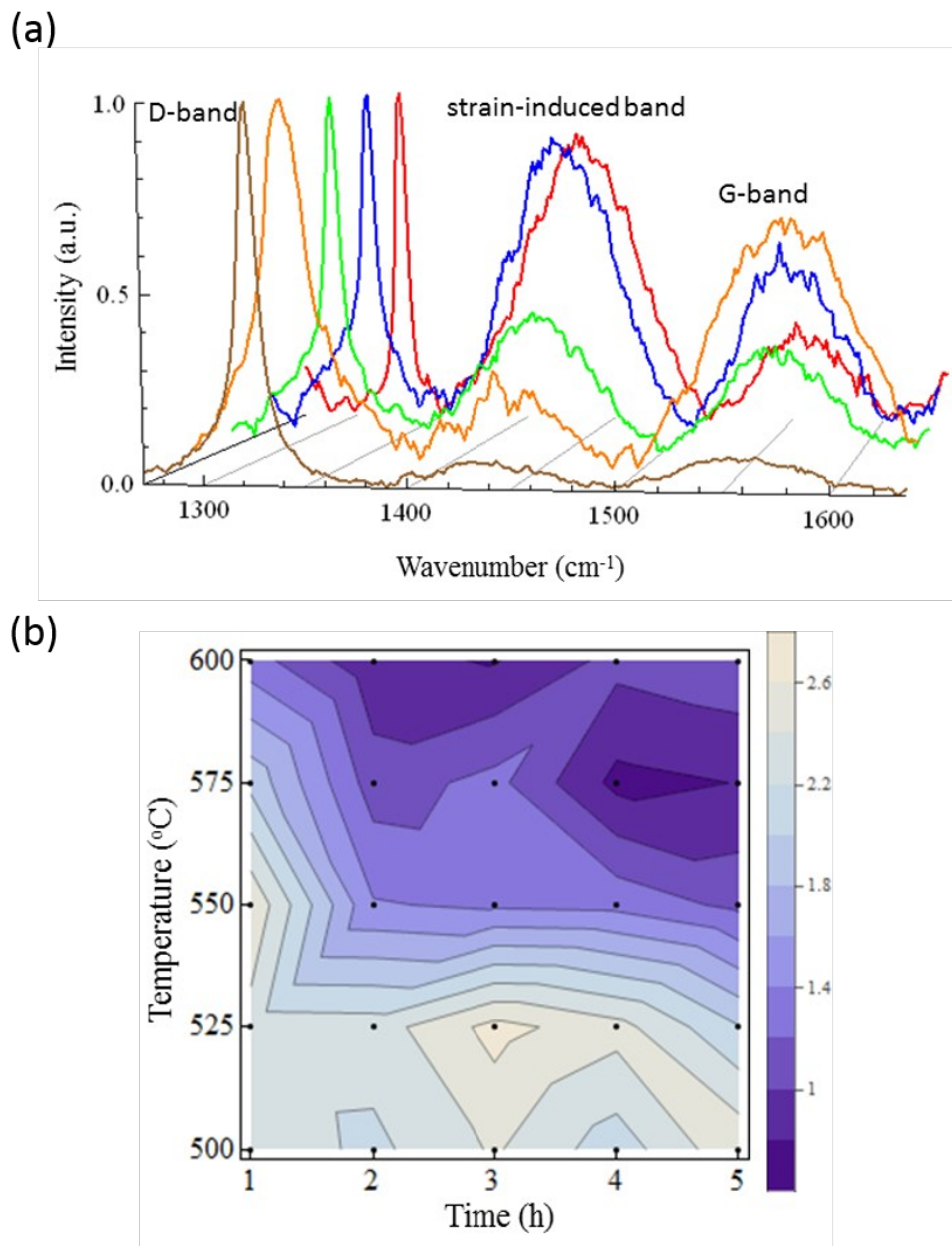


Figure 4. (a) Raman spectra of ND-pris (brown), ND-800 (orange), ND-500 (green), ND-550 (blue) and ND-600 (red). Raman peaks D-band (sp^3 carbon), strain-induced band and G-band (sp^2 carbon) around 1332, 1450 and 1550 cm^{-1} are observed in the spectra. (b) The ratios of Raman signal intensity of diamond's peak divided by that of amorphous carbon's peak intensity calculated for all the prepared samples as function of oxidation time and temperature.

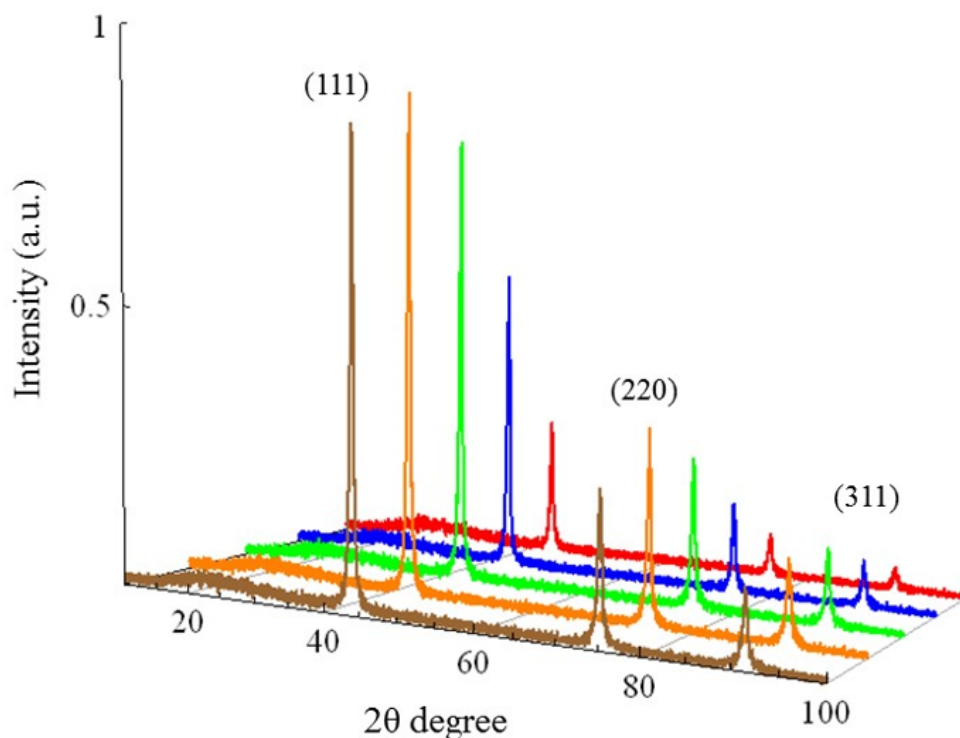


Figure 5. XRD patterns of ND-pris (brown), ND-800 (orange), ND-500 (green), ND-550 (blue) and ND-600 (red).

distortion of the crystal structure, resulting in an increase in the intensity of the strain-induced bands. Such distortion was likely caused by the annihilation of carbon atoms at the surface. Figure 4b shows a contour map of the ratio of the Raman signal intensity for diamond divided by that of the strain-induced band for each of the prepared samples. The black points represent the individual oxidation conditions. It can be seen from the figure that crystal destruction was triggered in the oxidation temperature range from 525 °C to 550 °C and appeared to be more temperature-dependent than time-dependent.

XRD analyses supported the particle shrink and crystal distortion suggested by TEM and Raman spectra. Figure 5 shows XRD spectra of the samples. Each spectrum shows diffraction peaks at 43.8° (111), 75.2° (220), and 91.4° (311). ND-pris, TD-800, and

TD-500 exhibited nearly the same peak intensities, while those of ND-550 and ND-600 were lower. This decrease in the intensities of the XRD signals imply both a reduction in the crystallite size and a distortion of crystal structure. Calculation of the crystallite size using Scherrer's equation from (111) peak was attempted, but proper values were not obtained (Figure 6 and Table 1) because Scherrer's equation does not apply to particles with unusual shapes, such as the NDs used in this study (Figure 2).

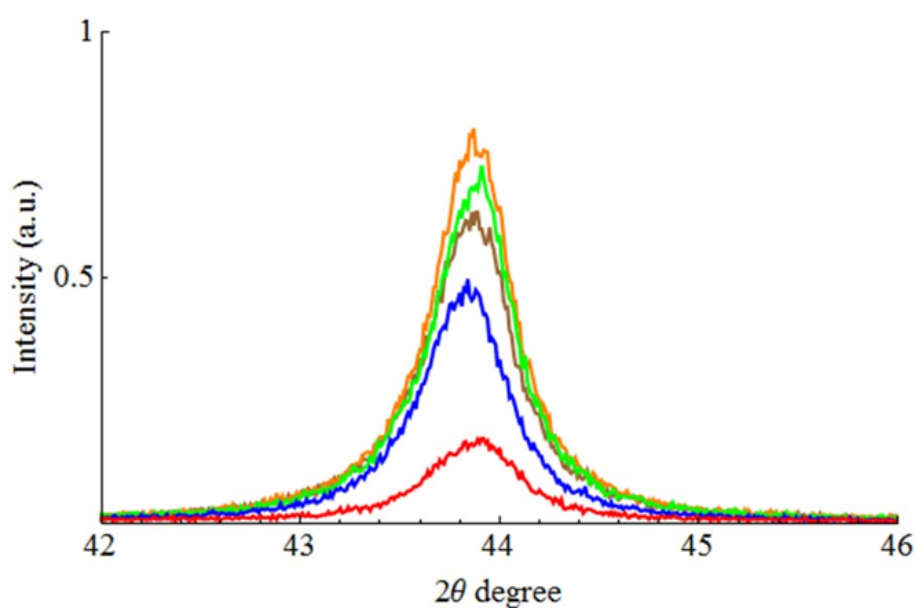


Figure 6. Precisely measured XRD spectra of (111) diffraction pattern of ND-pris (brown), ND-800 (orange), ND-500 (green), ND-550 (blue) and ND-600 (red).

Table 1. Bragg angles and FWHMs of the (111) peaks of the ND samples in Figure 4 determined via Lorentzian fitting and their crystal sizes estimated using Scherrer's equation.

Parameter	Bragg angle (°)	FWHM (°)	Crystal size (nm)
ND-pris	43.85	2.52	41.0
ND-800	43.87	2.36	44.3
ND-500	43.87	2.37	43.9
ND-550	43.82	2.44	42.6
ND-600	43.87	2.46	42.0

To summarize, it was revealed that annealing at 800 °C introduced graphite on the surfaces of the NDs. Air oxidation at 500 °C was effective for removal of the introduced surface graphite, but not for changing the particle size. On the other hand, air oxidation above 550 °C led to both removal of the graphite and particle size reduction, and the degree of each effect increased with increasing oxidation temperature.

2.3 Chemical state: bonding state of carbon

Next, the types and numbers of atoms contained in the samples were investigated. Table 2 shows the elemental analysis results for the samples. All of the NDs contained carbon as the major component, along with minor amounts of oxygen and hydrogen and negligible amounts of nitrogen. The results also indicated that the annealing process reduced the number of oxygen atoms below the detection limits for elemental analysis. However, the desorbed oxygen atoms were recovered following oxidation above 500 °C. These results indicated that nearly all of the oxygen atoms existed on the surfaces of the NDs, which was in agreement with the Raman data. In addition, the oxygen to carbon atomic ratios increased with increasing temperature. To learn in more detail about the

Table 2. Elemental analysis results for the ND samples.

Element	H	C	N	O
ND-pris	0.22	96.28	0.1	2.45
ND-800	0.05	98.89	0.37	0
ND-500	0.24	96.06	0.1	2.03
ND-550	0.06	94.94	0	3.04
ND-600	0.26	76.51	0	2.88

concentration of the different elements at the surfaces of the NDs, XPS spectra were obtained.

XPS is a favorable method for characterizing material surfaces because the escape depth of an electron is localized to within a few nanometers of the target surface. The survey XPS spectra are shown in Figure 7a. Each spectrum exhibited C1s and O1s peaks at approximately 285 eV and 533 eV, respectively, but their intensities were different for the different samples. The atomic concentrations of carbon and oxygen were calculated from the corresponding intensities of the C1s and O1s peaks. Notably, 13% of the carbon atoms on the surface of ND-pris were oxidized, which dropped to 8% for ND-800 and then recovered to 12% for ND-500. ND-600 had a relatively high oxygen to carbon ratio of 32%, which can be attributed to the high surface-area-to-volume ratio induced by size reduction. A plot of the ratios of oxygen to carbon estimated from the elemental analysis and XPS results is displayed in Figure 8. It can be seen in the figure that the XPS and elemental analysis results were similar, although the XPS data were larger than the elemental analysis data. This difference occurred because XPS only measures the surface area of a particle, while elemental analysis evaluates the entire ND particle. It should also be noted that even for the most oxidized ND sample (ND-600), the oxygen accounted for only 32% of the total atoms on the surface. This low concentration of oxygen limits the surface functionalization of the NDs.

Figure 7b presents the deconvoluted high resolution C1s spectra of the samples along with a comparison plot of each component for all of the samples. Each spectrum was calibrated against the 374 eV Ag 3d peak as an internal standard, and survey spectra are shown in Figure 9. The C1s spectra were curve-fitted into three peak components with

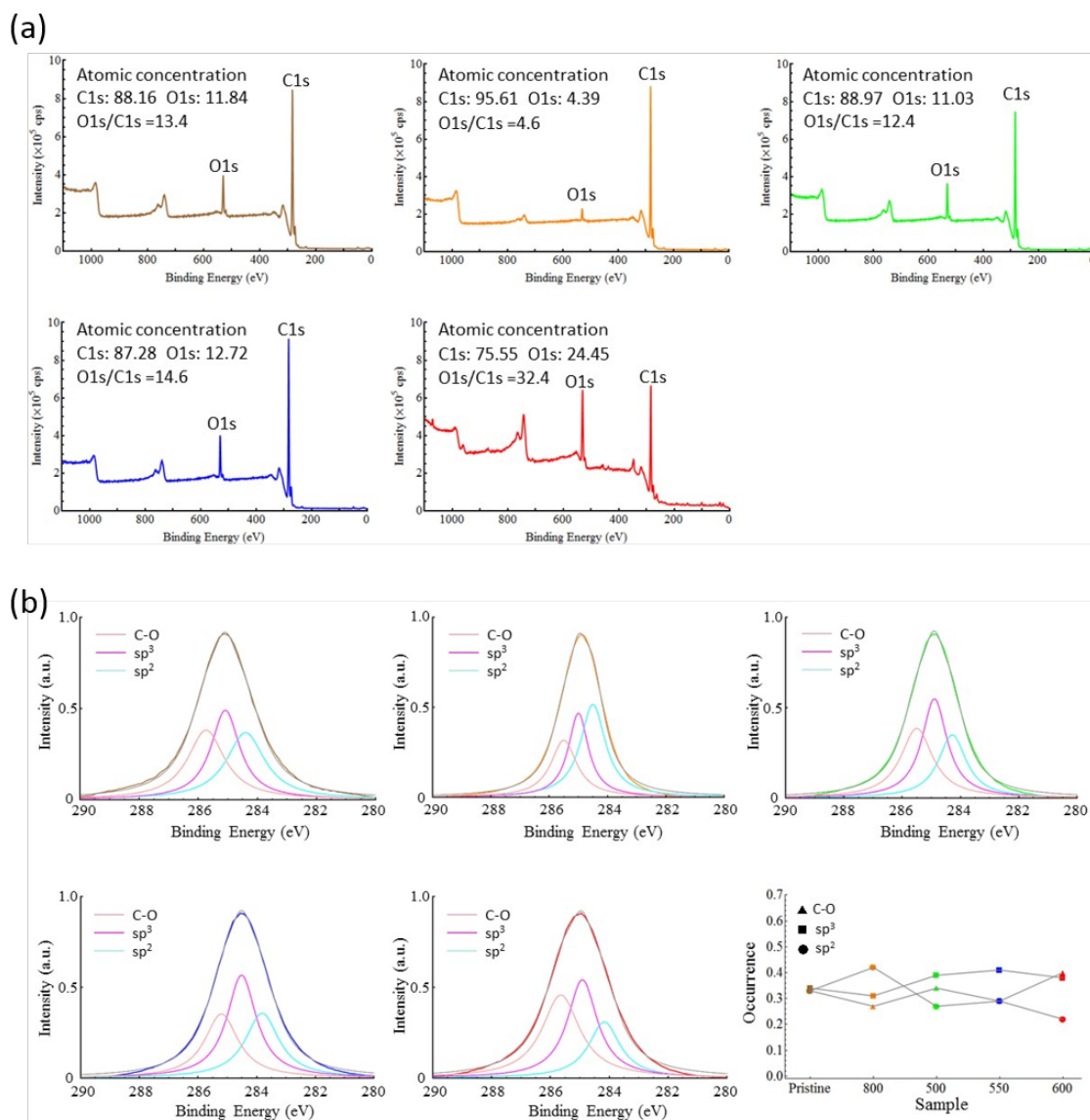


Figure 7. (a) Survey XPS spectra of ND-pris (brown), ND-800 (orange), ND-500 (green), ND-550 (blue) and ND-600 (red). Atomic concentration is calculated form the peak intensities of C1s and O1s. (b) Deconvoluted high resolution C1s spectra of ND-pris (brown), ND-800 (orange), ND-500 (green), ND-550 (blue) and ND-600 (red), and comparison plot of each component among samples.

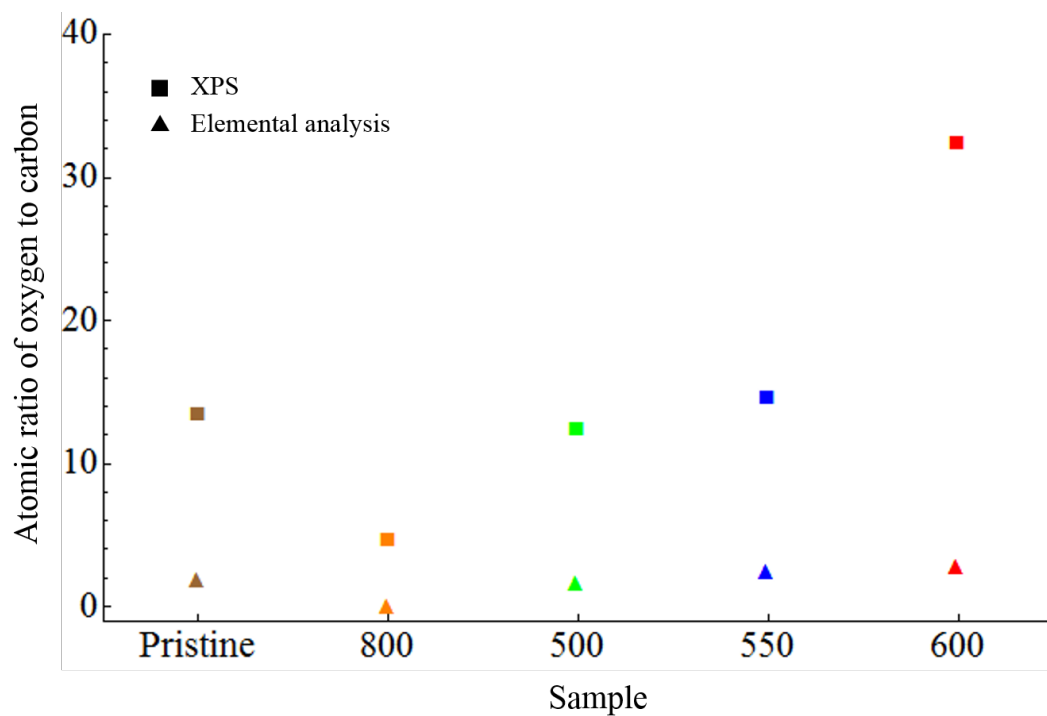


Figure 8. Ratios of oxygen to carbon of the samples estimated from elemental analysis (square) and XPS (triangle).

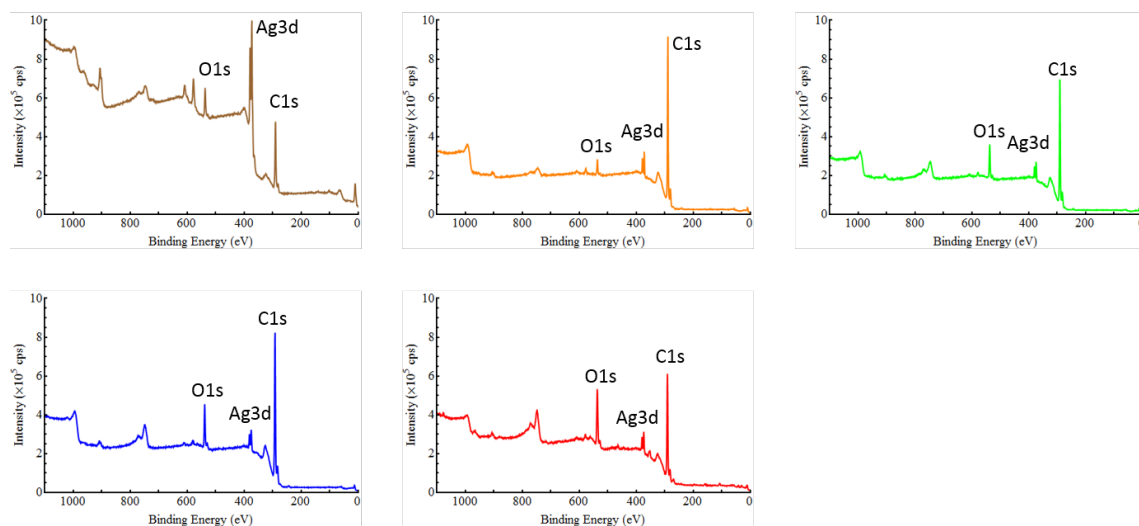


Figure 9. Survey XPS spectra of the samples. Ag 3d is contained in the samples as internal standard.

binding energies of 284 ± 0.5 , 285 ± 0.5 , and 286 ± 0.5 eV, which were attributed to sp^2 , sp^3 , and C-O bonds. Each of the peak components was also fitted using the Lorentz model, and the existing ratios were estimated. Table 3 shows the probability for each component in the different ND samples. The major component for ND-pris, ND-500, and ND-550 was sp^3 bonding, while those for ND-800 and ND-600 were sp^2 bonding and C-O groups, respectively. As mentioned above, the annealing process introduced graphite, which was then removed during oxidation. Oxygen-containing groups were simultaneously introduced, and the graphite-removal/oxygen-introduction efficiency appeared to be temperature dependent.

The ratios of the sp^3 to sp^2 carbons in the different ND samples estimated from their Raman and XPS spectra were then compared. Because Raman spectroscopy provides information about the carbon bonding state, the ratios of the Raman signal intensities for the sp^3 and strain-induced bands divided by that of the sp^2 band were calculated for quantitative comparison of the carbon bonding states in the different samples. Values of 4.1, 1.4, 3.1, 3.8, and 9.5 were obtained for ND-pris, ND-800, ND-500, ND-550, and ND-600, respectively. A plot of the ratios of the sp^3 to sp^2 bonded carbons in the

Table 3. Existence probabilities for the sp^2 , sp^3 , and CO components estimated from Figure 4b.

Component	sp^2	sp^3	CO
ND-pris	0.33	0.34	0.33
ND-800	0.42	0.31	0.27
ND-500	0.27	0.39	0.34
ND-550	0.29	0.41	0.29
ND-600	0.22	0.38	0.40

ND samples estimated from the elemental analysis and XPS data is shown in Figure 10.

In both cases, the ratio decreased following annealing and increased as the oxidation

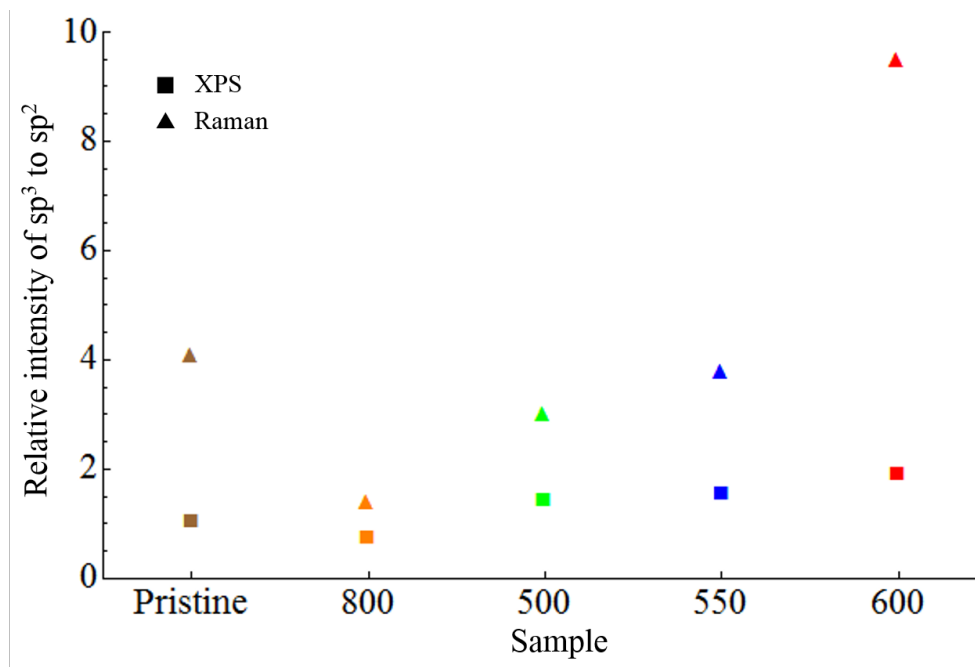


Figure 10. Ratios of sp^3 to sp^2 of the samples estimated from Raman and XPS.

temperature increased. The differences in the XPS and Raman plots can be attributed to the differences in the observation depths of these analytical techniques.

2.4 Chemical state: bonding state of oxygen

Given the experimental results described above, it was anticipated that oxygen species reacted with the carbon in the graphite removed from the ND surface to form alcohol, carboxyl, lactone, and/or epoxy groups. To determine which types of oxygen-containing species were formed in the oxidized samples, FT-IR, and zeta potential analyses were performed. FT-IR spectra of the ND samples are shown in Figure 11a. A stretch vibration of O-H around 3400 cm^{-1} ; adsorbed water derived band around 3200 cm^{-1} ; aliphatic

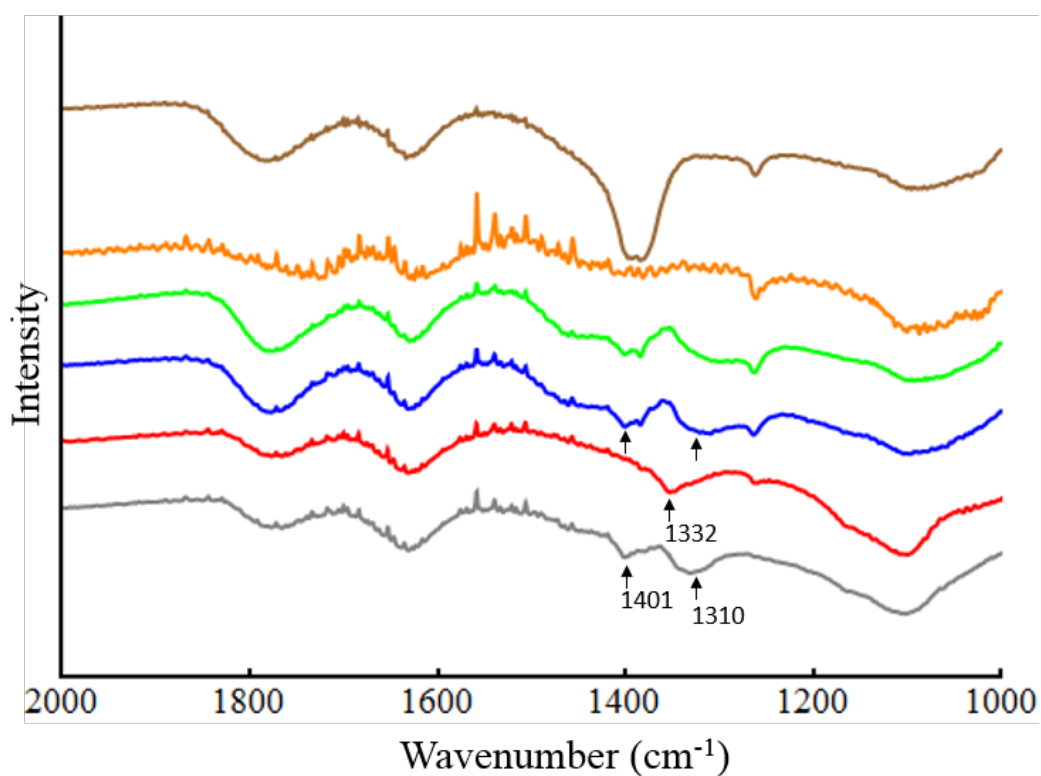
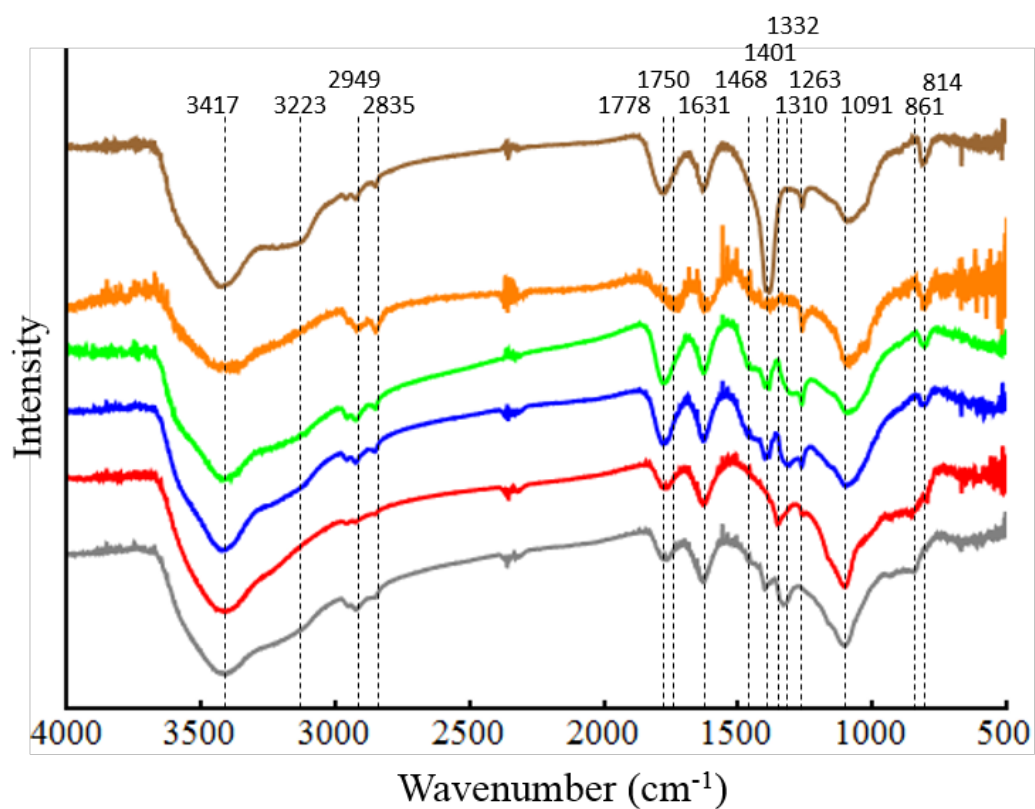


Figure 11. (a) FT-IR spectra of of ND-pris (brown), ND-800 (orange), ND-500 (green), ND-550 (blue) and ND-600 (red). (b) in the spectral range 1000-2000 cm^{-1} .

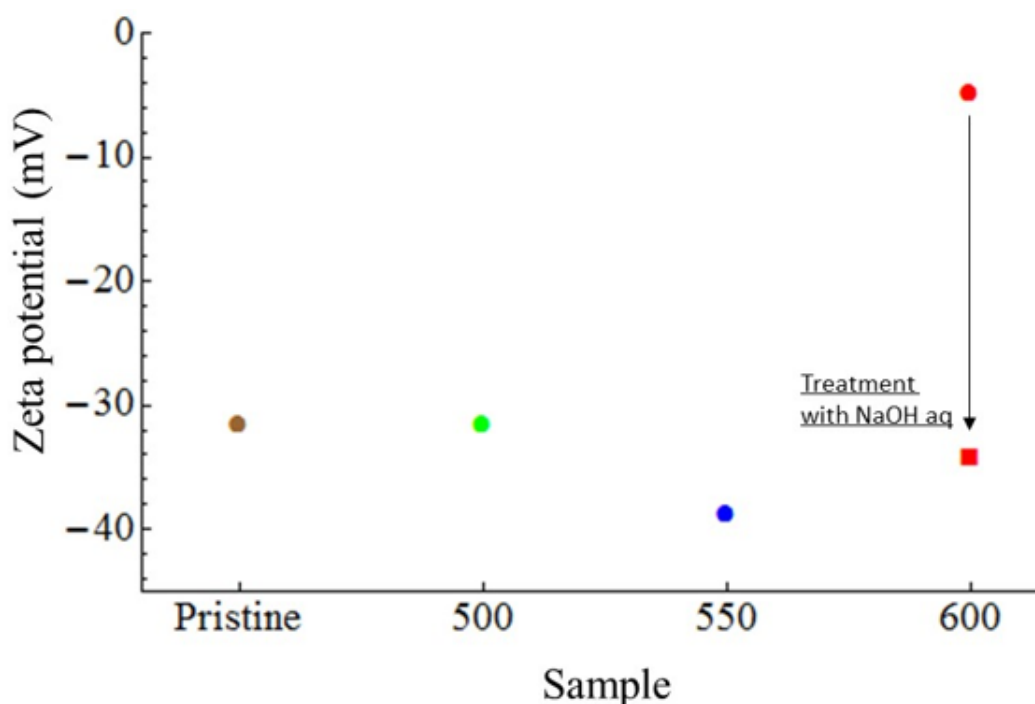


Figure 12. Zeta potential of the prepared samples (dot). Square plot indicate the NaOH aqueous solution treated sample.

C-H stretching vibrations at $2835\text{--}2849\text{ cm}^{-1}$; carbonyl C=O peak at $1750\text{--}1778\text{ cm}^{-1}$; C=C and/or adsorbed waters peak at 1631 cm^{-1} ; COO^- peak at $1401\text{--}1468\text{ cm}^{-1}$; C-O peak at $1263\text{--}1310\text{ cm}^{-1}$; C-O-C stretching peak at 1091 cm^{-1} ; and C-H bending peak at $814\text{--}861\text{ cm}^{-1}$ are observed in the samples. Interestingly, significant differences in the FTIR spectra of ND-500 and ND-550 were not observed, while notable changes were seen in the spectrum of ND-600. Figure 11b shows the spectra in the range $1000\text{--}2000\text{ cm}^{-1}$. The relative intensity of the C-O at 1310 cm^{-1} and COO^- at 1401 cm^{-1} are diminished, while C-O at 1332 cm^{-1} are emerged. We assumed that these spectral changes are attributed to the changes of the oxygen containing groups, possibly due to the conversion from carboxyl groups to a different species such as carboxylic anhydrides. To confirm this, ND-600 was then treated with an aqueous NaOH solution in order to facilitate the

hydration of the surface anhydride groups and thus the formation of free COOH groups. Gray spectrum in Figures 11a and 11b show the NaOH treated ND-600. It is observed that the disappeared peaks at 1310 and 1401 cm^{-1} are regenerated, and the emerged peaks at 1332 cm^{-1} are disappeared. This data supported the conclusion that the surface COOH groups were dehydrated in the ND-600. To gain further evidence for this dehydration, the zeta potentials of the ND samples were also determined.

Figure 12 shows plots of the zeta potentials for the samples. Note that it was not possible to disperse ND-800 in water, and thus no data was obtained for this sample (Figure 13). The zeta potentials were determined to be -31.5 , -31.5 , -38.8 , and -4.9

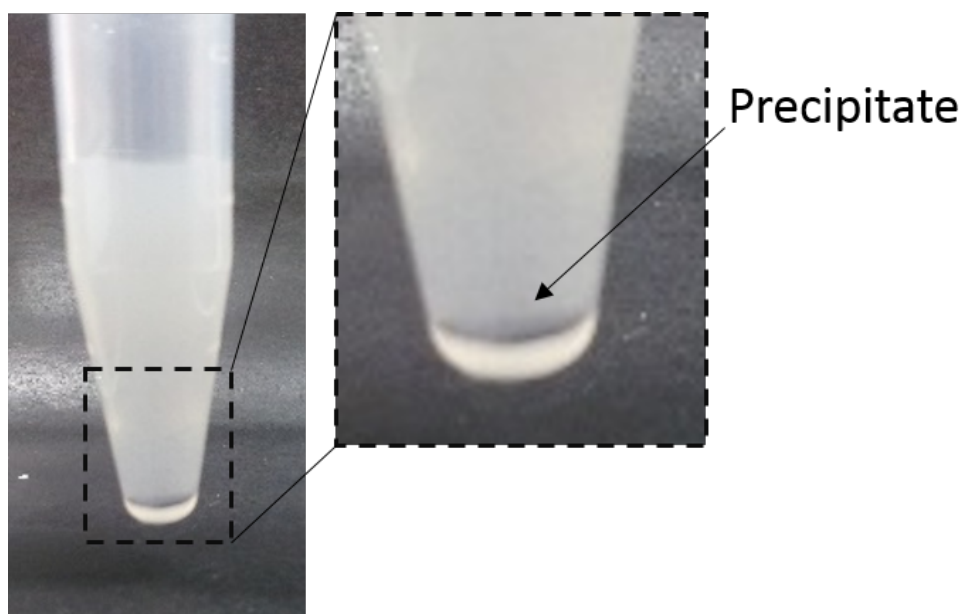


Figure 13. Picture of dispersion state of ND-800. This sample is immediately precipitate in water before measurement, so the proper experimental data is not obtained.

mV for ND-pris, ND-500, ND-550, and ND-600, respectively. The negative charges are attributed to the presence of COOH groups on the surfaces of the NDs. The very small zeta potential value for ND-600 indicated that dehydration of the COOH groups occurred,

leading to the formation of carboxylic anhydrides. After this treatment, the zeta potential was found to be -34.2 mV (square point in Figure 12). Based on these results, it was concluded that excess oxidation led to size reduction accompanied by the introduction of carboxylic anhydride groups on the surfaces of the NDs, which in turn had a lower zeta potential. We further experimented XPS analysis, and the results are given in Figure 14 and Table 4. Thus, the overall changes in the physical/chemical states of NDs due to

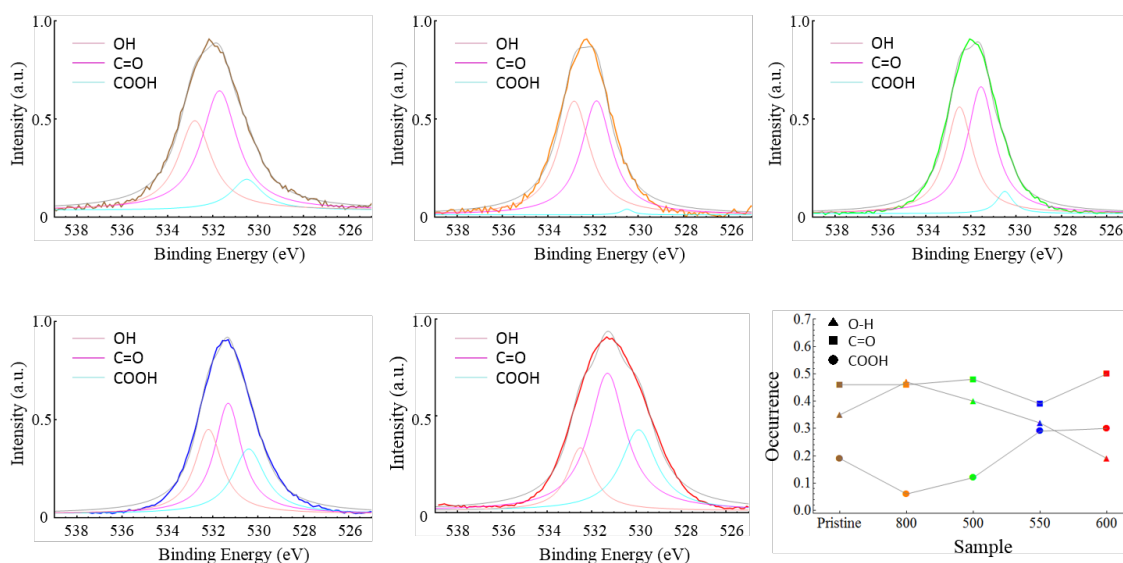


Figure 14. Schematic illustration of the changes of physical/chemical state of the annealing and oxidation process.

Table 4. Existence probabilities of the COOH, C=O, and OH components estimated from Figure 14.

Element	COOH	C=O	OH
ND-pris	0.19	0.46	0.35
ND-800	0.06	0.46	0.47
ND-500	0.12	0.48	0.40
ND-550	0.29	0.39	0.32
ND-600	0.30	0.50	0.19

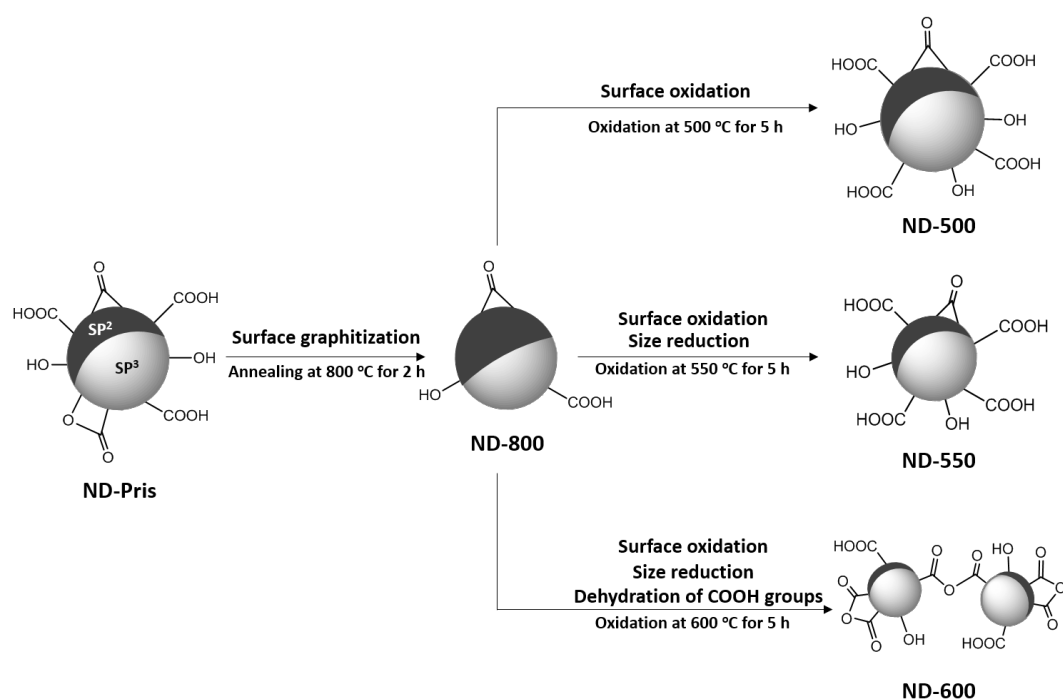


Figure 15. Schematic illustration of the changes of physical/chemical state of the annealing and oxidation process.

annealing and oxidation have been revealed, and a schematic illustration of the results is presented in Figure 15.

2.5 Dispersibility

The colloidal stability of nanoparticles is well described using Derjaguin and Landau, Verwey and Overbeek (DLVO) theory.[21, 22] This theory treats the interactions among particles in terms of a balance between attractive van der Waals forces and repulsive electrical double layer forces. According to this theory, the size and zeta potential of particles, as well as the ionic strength of a solution of the particles, are important factors for determining their dispersibility. Therefore, the dispersibility of the ND samples was investigated from both theoretical and experimental points of view. Figure 16 shows a

plot of the interaction forces calculated using DLVO theory and experimental data for the time-dependent changes in the intensities of dynamic light scattering for the NDs in salt-containing aqueous solutions. Photos of their dispersion states are presented in Figure 17.

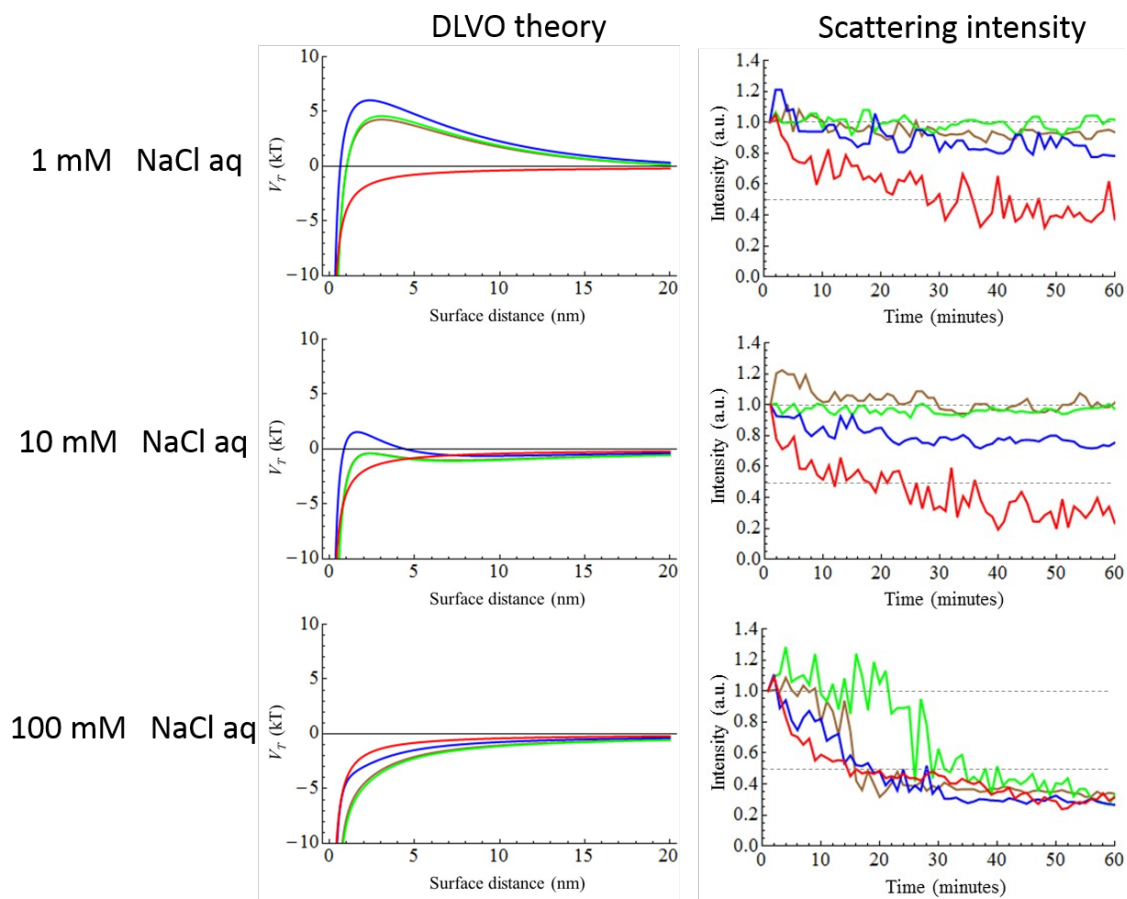


Figure 16. Plot of interaction forces calculated by DLVO theory, and experimental data of time-dependent changes of intensity of dynamic light scattering in 1, 10 and 100 mM salt-containing aqueous solution. ND-pris, ND-500, ND-550 and ND-600 are represented by brown, green, blue and red color plot, respectively.

The DLVO plots are given in units of kT versus the separation distance between particle surfaces. In a 1 mM salt-containing solution, all of the samples except for ND-600 exhibited a positive local potential energy maximum at a surface distance of approximately 2 nm (plots of the surface distance versus solid fraction for the ND samples are shown in Figure 18). These samples may exhibit good dispersibility because the energy beyond

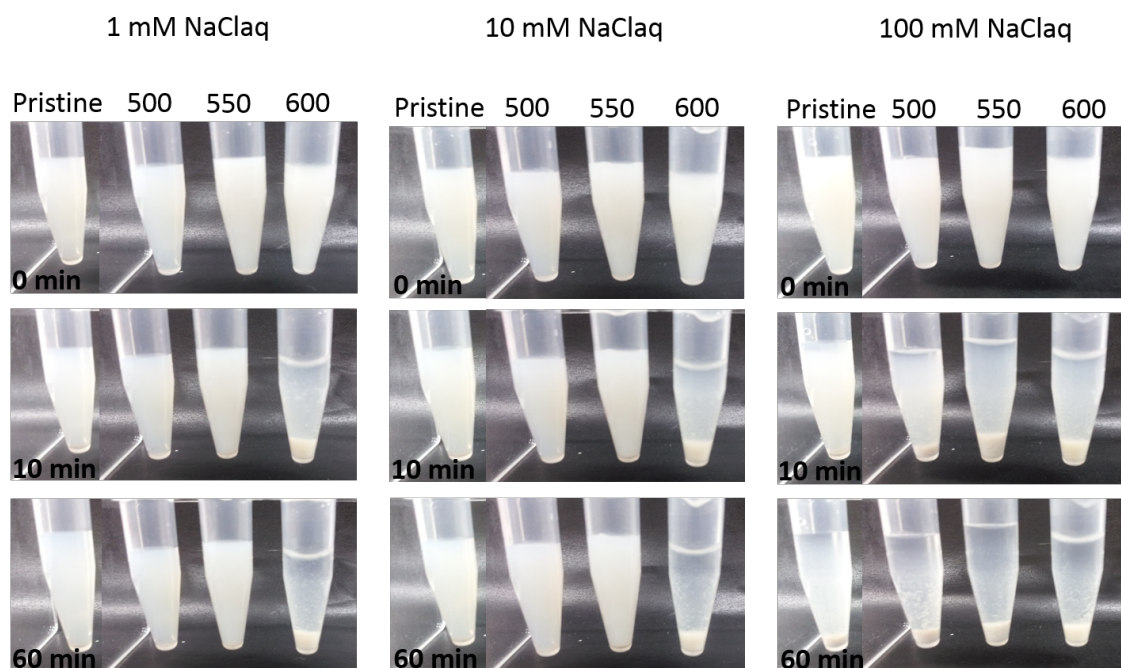


Figure 17. Pictures of time-dependent changes of dispersion state.

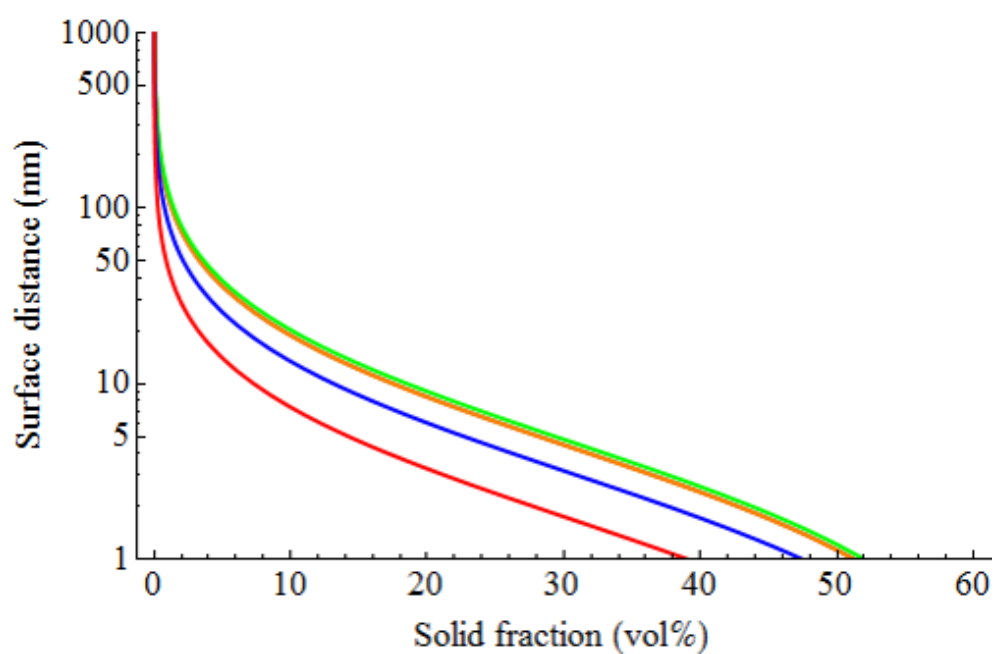


Figure 18. Surface distance versus solid fraction of ND-pris (brown), ND-800 (orange), ND-500 (green), ND-550 (blue) and ND-600 (red) calculated by woodcock theory.

this maximum is required for aggregation. On the other hand, ND-600 would be expected to readily aggregate, because the potential energy of the encountered particles dropped to a primary minimum. Notably, the experimental data was in agreement with the theoretical results. ND-pris, ND-500, and ND-550 exhibited constant scattering intensities, even after 60 minutes, indicating good dispersibility in solution. In contrast, ND-600 displayed a lower dispersibility in three different salt solutions (1 mM, 10 mM, and 100 mM). In addition, for the calculations in a 10 mM salt solution, ND-pris, ND-500, and ND-550 each exhibited a local maximum near zero, and thus the samples were assumed to be rapidly dispersed and then gradually aggregated over an extended period. The experimental data also supported this result; the samples did not precipitate in as short a period. For the 100 mM solutions, the samples were predicted to precipitate immediately, and this result confirmed experimentally. Few literature reports mention the dispersibility of NDs in terms of DLVO theory. These results demonstrated that this theory provides valid information on the dispersion state of NDs. Furthermore, it can be concluded from the results that in order to maintain dispersion in high-concentration salt-containing solutions, chemical modification of the surface is required. Dendrimer and lipid coatings have been reported to enhance the dispersibility of NDs.[23, 24]

2.6 Fluorescence and ODMR properties

Next, the fluorescence and ODMR spectra of the ND samples were evaluated. There are two types of nitrogen-vacancy centers in NDs, each with a different charged state. One is neutral (NV^0), while the other is negative (NV^-), and they appear as zero-phonon lines (ZPLs) at 575 nm and 637 nm, respectively.[25] Both NVCs emit fluorescence, but only

NV^- can be detected using ODMR.[26, 27] The ground state of NV^- is a spin-triplet, and the spin sublevels $m_s = 0$ and $m_s = \pm 1$ are split by 2870 MHz. In addition, the spin sublevel $m_s = \pm 1$ is degenerate in the absence of an external magnetic field. Microwave (MW) irradiation at this frequency induces electron spin magnetic resonance (ESR) between $m_s = 0$ and the degenerate $m_s = \pm 1$ spin levels, which results in a decrease in the fluorescence intensity from NV^- due to spin-dependent intersystem crossing. ODMR functions via detection of ESR through changes in the fluorescence intensity. ODMR signals are assumed to be affected by the surface chemical termination, crystal quality, and particle size of NDs. Thus, the fluorescence and ODMR signals for the NDs prepared in this study were investigated. Figure 19 shows the fluorescence spectra of the bulk samples. The data for ND-pris is not shown because this sample was not subjected to annealing, and thus no NVCs were introduced into the NDs. ND-800 exhibited a very weak fluorescence intensity, possibly due to absorption of the fluorescence from the NVCs by the surface graphite. The other samples exhibited similar fluorescence spectra, with ZPLs for NV^0 and NV^- at 575 and 637 nm, respectively. The ODMR spectra of ND-800, ND-500, ND-550, and ND-600 are shown in Figure 19b. Each sample clearly exhibited MW-dependent reduction of the fluorescence intensity at 2870 MHz. Notably, in the spectrum of ND-800, the signal was weaker and broader than that in the spectrum of ND-500, which was likely caused by the graphite. The strongest ODMR signal of 4.7% was observed for ND-500. The ODMR signal then decreased for ND-550 and ND-600 to 3.6% and 2.4%, respectively. This decrease in the intensity is assumed to derive from the decrease in the total number of NVCs (both NV^0 and NV^-) contained in the NDs due to their reduction in size. However, it must be considered that the ODMR signal is

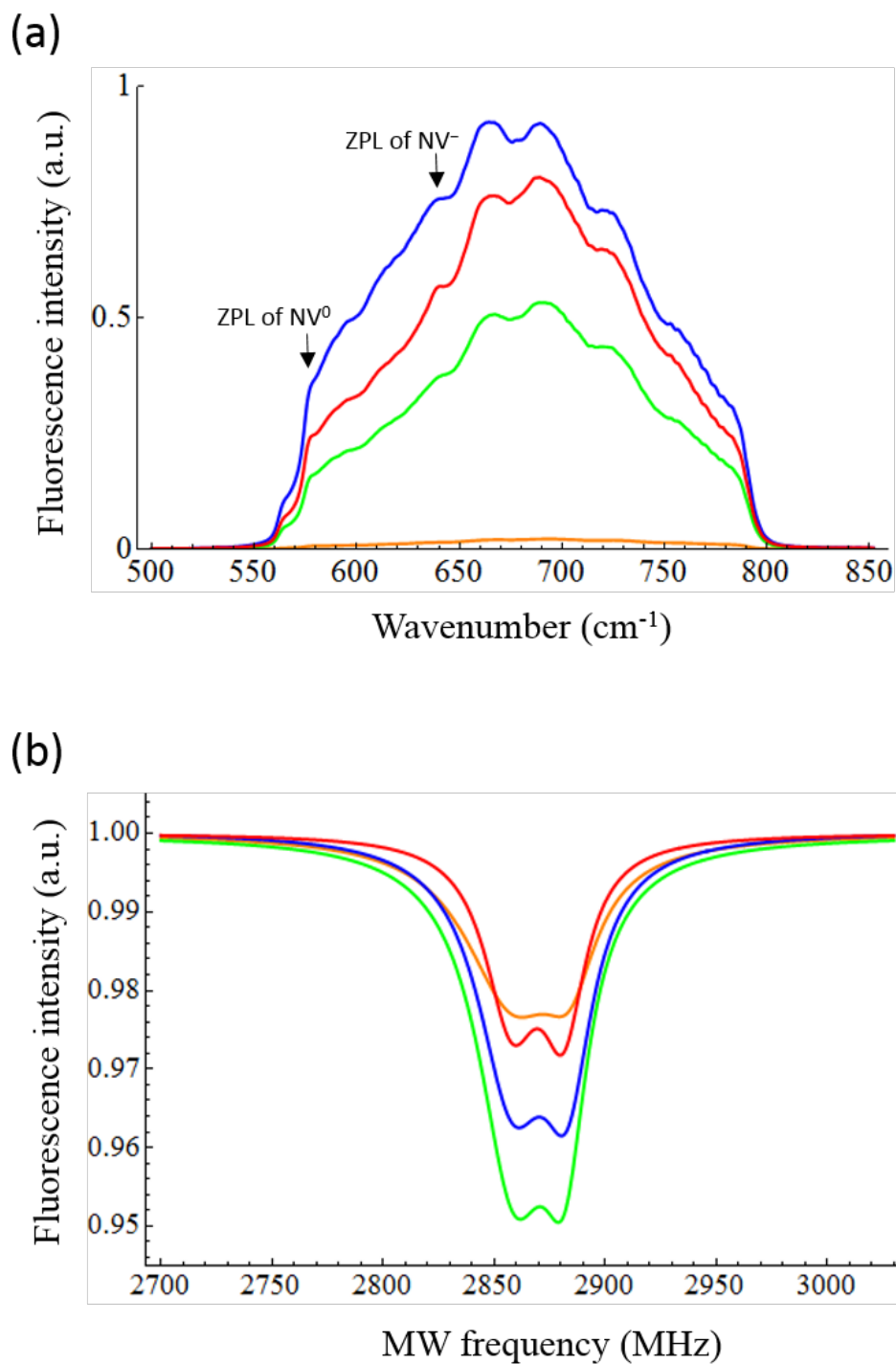


Figure 19. (a) Fluorescence and (b) ODMR spectra of ND-800 (orange), ND-500 (green), ND-550 (blue), and ND-600 (red).

determined as shown in Equation (1):

$$I_{ODMR} = (I_{OFF} - I_{ON}) / I_{OFF} \quad (1)$$

where I_{ODMR} , I_{OFF} , and I_{ON} indicate the intensity of the ODMR, and the peak intensities of the distribution when the MW irradiation are off and on, respectively. Thus, the signal may not be influenced by the number of NVCs if NV^0 and NV^- randomly exist in the NDs. In addition, the strain induced splitting coefficient values (E -values) of the ND samples were notable. The spin Hamiltonian of an NV^- is expressed using Equation (2):

$$H = D[S_z^2 - \frac{1}{3}S(S + 1)] + E(S_x^2 - S_y^2), \quad (2)$$

where S , D , and E are the electron spin vector ($S = 1$ for the triplet state), the fine-structure zero-field splitting, and the strain induced splitting coefficient, respectively. Thus, crystal distortion near the NVCs is estimated by measuring the E -value. Table 4 shows the intensity, half-width at half-maximum (HWHM), and E -values for the ODMR spectra of the ND samples. The values were estimated by fitting the ODMR spectra us-

Table 5. ODMR signal data for Figure 16b.

Element	Intensity (%)	HWHM (MHz)	E -value (MHz)
ND-800	2.3	16.8	13.0
ND-500	4.7	13.7	11.5
ND-550	3.6	14.5	12.3
ND-600	2.4	12.9	11.6

ing two Lorenz functions. Unlike for the results of the Raman spectroscopic analysis,

no dependence of the crystal distortion on the oxidation process was observed, These results implied that ODMR-active NV^- sites that caused distortion of the crystal structure rarely existed close to the surface. In other words, the charge states of the NVCs were converted from NV^- to NV^0 close to the surfaces of the NDs, resulting in lower-intensity ODMR spectra. It has been reported that surface termination using fluorine enhances the NV^-/NV^0 ratio. Fluorine termination may thus result in restriction of such an undesirable conversion of NVCs.[28] Finally, the ODMR intensities of all of the prepared samples were plotted as a function of the oxidation time and temperature (Figure 20). A discrete

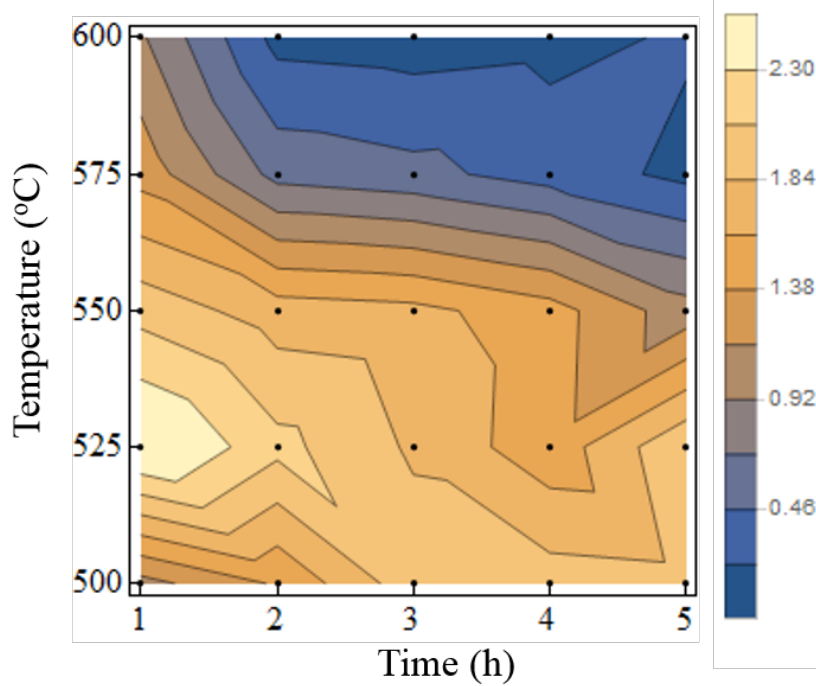


Figure 20. ODMR intensities for all of the prepared ND samples as a function of the oxidation time and temperature.

maximum for the ODMR intensity was identified in the samples oxidized at 525 °C for 1 hour. Oxidation at temperatures above 575 °C resulted in NDs with a weak ODMR intensity of less than half the maximum value. It is possible, therefore, that particle shrinkage

may critically affect the ODMR intensity.

3. Conclusion

Changes in the physical/chemical states of NDs due to annealing and oxidation have been quantitatively analyzed, and several key points have been elucidated: Weight loss in the NDs occurred via several processes; a surface graphite introduced during annealing was oxidized in air at temperatures above 500 °C; distortion of the crystal during oxidation at 525–550 °C was accompanied by particle shrinkage; the sp^3/sp^2 ratio increased with increasing temperature, and COOH groups introduced at lower oxidation temperature were dehydrated at higher oxidation temperatures, resulting in NDs with low zeta potentials.

In addition to these results, it was found that the dispersibility, fluorescence, and ODMR properties of the NDs are related to their physical/chemical properties. Notably, there is a strong relationship between the dispersibility of NDs and their physical/chemical states, and the dispersibility can be estimated in advance using DLVO theory. In addition, it was determined that the ODMR signal intensity for an ND may be influenced by its sp^2 ratio and particle size; the ODMR signal decreased with decreasing particle size, but crystal distortion around NV^- was not observed, indicating that an NVC existing at the surface was converted from NV^- to NV^0 . Furthermore, the optimal oxidation conditions for a discrete maximum ODMR intensity were determined. These experimental results suggest that it should be possible to control the physical/chemical states and properties of NDs through adjustment of the annealing and oxidation processes, and will thus open avenues for facilitating their bio-application.

4. Experimental Section

TEM images were obtained using a Hitachi H-7650. Raman spectra were obtained using a Horiba XploRa in the range from 1200 to 1650 cm^{-1} in order to evaluate the distribution of the diamond sp^3 band (1332 cm^{-1}), the strain-induced band (1450 cm^{-1}), and the graphite sp^2 band (1550 cm^{-1}). A 488-nm excitation laser was used. X-ray diffraction patterns were recorded using a power X-ray diffractometer (MultiFlex DR, Rigaku, Tokyo Japan) with a $\text{CuK}\alpha$ source (x-ray wavelength 0.15405 nm) and an acceleration voltage and anode current of 30 kV and 30 mA, respectively. XPS spectra were recorded using an ULVAC PHI 5500MT system. Samples mounted on indium foil were analyzed using $\text{MgK}\alpha$ radiation (15 kV, 400 W) in a vacuum chamber ($<1 \times 10^{-8}$ Torr). Precise measurement and characterization of the energies of the C1s and O1s peaks were achieved by referencing them to the 3d peak of Ag at 374 eV. FTIR spectra were obtained on a JASCO FT/IR-4200 using standard KBr-pellets with a resolution of 2 cm^{-1} . The zeta potentials of the samples in neutral water were measured using a Malvern Zeta sizer Nano instrument. The concentration of each ND sample was 1 mg/ml in a salt-containing aqueous solution, and the samples were sonicated for 5 minutes prior to measurement. Time-dependent changes in the dynamic light scattering intensities of the samples (1 mg/ml in Milli-Q water) were determined using a Wyatt DynaPro NanoStar. Fluorescence and optically detected magnetic resonance spectra were recorded using a home-built microscope established by Igarashi et al.[29] The field of view was illuminated using a 532-nm Nd:YAG laser at 7 mW, and fluorescence images ($40 \mu\text{m} \times 40 \mu\text{m}$) were obtained using an electron multiplying charge-coupled device (EMCCD) camera, while fluorescence spectra were

recorded using a Hamamatsu photonics PMA-12. The emitted light was collected using an oil immersion 100 × objective lens (Numerical Aperture = 1.49) and passed through a dichroic mirror centered at 575 nm and a long (short)-wave pass filter longer than 590 nm (shorter than 842 nm) to detect the intrinsic signal. The microscope was equipped with a computer-controlled moving stage. Treatment of ND-600 in an NaOH aqueous solution was performed using the following procedure. NaOH (0.1 M) was added to the NDs and the suspension was stirred for two hours at 90 °C. After washing three times with Milli-Q water, the NDs were stirred in 0.1 M HCl for two hours at 90 °C. The NDs were then centrifuged and washed three times with Milli-Q water.

References

- [1] I. Aharonovich, A. Greentree, and S. Prawer. Diamond photonics. *Nature Photonics*, Vol. 5, pp. 397–405, 2011.
- [2] S. Yu, M. Kang, H. Chang, K. Chen, and Y. Yu. Bright fluorescent nanodiamonds: no photobleaching and low cytotoxicity. *Journal of the American Chemical Society*, Vol. 127, No. 50, pp. 17604–5, December 2005.
- [3] G. Liaugaudas, G. Davies, K. Suhling, R. Khan, and D. Evans. Luminescence lifetimes of neutral nitrogen-vacancy centres in synthetic diamond containing nitrogen. *Journal of physics. Condensed matter : an Institute of Physics journal*, Vol. 24, No. 43, p. 435503, October 2012.
- [4] F. Jelezko and J. Wrachtrup. Single defect centres in diamond: A review. *Physica Status Solidi (a)*, Vol. 203, No. 13, pp. 3207–3225, October 2006.
- [5] A. Krueger and D. Lang. Functionality is Key: Recent Progress in the Surface Modification of Nanodiamond. *Advanced Functional Materials*, Vol. 22, No. 5, pp. 890–906, March 2012.
- [6] A. Schrand, H. Huang, C. Carlson, J. Schlager, E. Osawa, S. Hussain, and L. Dai. Are diamond nanoparticles cytotoxic? *The journal of physical chemistry. B*, Vol. 111, No. 1, pp. 2–7, January 2007.
- [7] Y. Hui, C. Cheng, and H. Chang. Nanodiamonds for optical bioimaging. *Journal of Physics D: Applied Physics*, Vol. 43, No. 37, p. 374021, September 2010.

- [8] J. Say, C. Vreden, D. Reilly, L. Brown, J. Rabeau, and N. King. Luminescent nanodiamonds for biomedical applications. *Biophysical Reviews*, Vol. 3, No. 4, pp. 171–184, October 2011.
- [9] H. Bernien, B. Hensen, W. Pfaff, G. Koolstra, M. S. Blok, L. Robledo, T. Taminiau, M. Markham, D. Twitchen, L. Childress, and R. Hanson. Heralded entanglement between solid-state qubits separated by three metres. *Nature*, Vol. 497, No. 7447, pp. 86–90, May 2013.
- [10] T. Staudacher, F. Shi, S. Pezzagna, J. Meijer, J. Du, C. Meriles, F. Reinhard, and J. Wrachtrup. Nuclear magnetic resonance spectroscopy on a (5-nanometer)³ sample volume., February 2013.
- [11] M. Geiselmann, M. Juan, J. Renger, J. Say, L. Brown, F. Javier G. Abajo, F. Koppens, and R. Quidant. Three-dimensional optical manipulation of a single electron spin., March 2013.
- [12] Y. Hui and H. Chang. Recent Developments and Applications of Nanodiamonds as Versatile Bioimaging Agents. *Journal of the Chinese Chemical Society*, Vol. 61, No. 1, pp. 67–76, January 2014.
- [13] R. Schirhagl, K. Chang, M. Loretz, and C. Degen. Nitrogen-vacancy centers in diamond: nanoscale sensors for physics and biology. *Annual review of physical chemistry*, Vol. 65, pp. 83–105, January 2014.
- [14] X. Song, G. Wang, X. Liu, F. Feng, and J. Wang. Generation of nitrogen-vacancy

- color center in nanodiamonds by high temperature annealing. *Applied Physics Letters*, Vol. 102, p. 133109, 2013.
- [15] A. Schrand, S. Hens, and O. Shenderova. Nanodiamond Particles: Properties and Perspectives for Bioapplications. *Critical Reviews in Solid State and Materials Sciences*, Vol. 34, No. 1, pp. 18–74, January 2009.
- [16] J. Tisler, G. Balasubramanian, B. Naydenov, R. Kolesov, B. Grotz, R. Reuter, J. Boudou, P. Curmi, M. Sennour, A. Thorel, M. Börsch, K. Aulenbacher, R. Erdmann, P. Hemmer, F. Jelezko, and J. Wrachtrup. Fluorescence and spin properties of defects in single digit nanodiamonds. *ACS nano*, Vol. 3, No. 7, pp. 1959–65, July 2009.
- [17] T. Gaebel, C. Bradac, J. Chen, J.M. Say, L. Brown, P. Hemmer, and J.R. Rabeau. Size-reduction of nanodiamonds via air oxidation. *Diamond and Related Materials*, Vol. 21, pp. 28–32, January 2012.
- [18] J. Havlik, V. Petrakova, I. Rehor, V. Petrak, M. Gulka, J. Stursa, J. Kucka, J. Ralis, T. Rendler, S. Lee, R. Reuter, J. Wrachtrup, M. Ledvina, M. Nesladek, and P. Cigler. Boosting nanodiamond fluorescence: towards development of brighter probes. *Nanoscale*, Vol. 5, No. 8, pp. 3208–11, April 2013.
- [19] J. Cebik, J. McDonough, F. Peerally, R. Medrano, I. Neitzel, Y. Gogotsi, and S. Osswald. Raman spectroscopy study of the nanodiamond-to-carbon onion transformation. *Nanotechnology*, Vol. 24, No. 20, p. 205703, May 2013.
- [20] S. Osswald, G. Yushin, V. Mochalin, S. Kucheyev, and Y. Gogotsi. Control of

sp²/sp³ carbon ratio and surface chemistry of nanodiamond powders by selective oxidation in air. *Journal of the American Chemical Society*, Vol. 128, No. 35, pp. 11635–42, September 2006.

- [21] E. Verwey and J. Overbeek. Theory of the stability of lyophobic colloids. *Elsevier*, 1948.
- [22] B. V. Deryagyn and L. Landau. Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solution of electrolytes. *Acta Phys. Chem.*, Vol. 15, p. 633, 1941.
- [23] Y. Hui, B. Zhang, and Y. Chang. Two-photon fluorescence correlation spectroscopy of lipid-encapsulated fluorescent nanodiamonds in living cells. *Optics Express*, Vol. 18, No. 6, pp. 2135–2143, 2010.
- [24] L. Zhao, T. Takimoto, M. Ito, N. Kitagawa, T. Kimura, and N. Komatsu. Chromatographic separation of highly soluble diamond nanoparticles prepared by polyglycerol grafting. *Angewandte Chemie (International ed. in English)*, Vol. 50, No. 6, pp. 1388–92, February 2011.
- [25] Y. Chang, H. Lee, K. Chen, C. Chang, D. Tsai, C. Fu, T. Lim, Y. Tzeng, C. Fang, C. Han, H. Chang, and W. Fann. Mass production and dynamic imaging of fluorescent nanodiamonds. *Nature nanotechnology*, Vol. 3, No. 5, pp. 284–8, May 2008.
- [26] P. Maletinsky, S. Hong, M. Grinolds, B. Hausmann, M. Lukin, R. Walsworth, M. Loncar, and A. Yacoby. A robust scanning diamond sensor for nanoscale imag-

ing with single nitrogen-vacancy centres. *Nature nanotechnology*, Vol. 7, No. 5, pp. 320–4, May 2012.

- [27] B. Horowitz, V. Alemán, D. Christle, A. Cleland, and D. Awschalom. Electron spin resonance of nitrogen-vacancy centers in optically trapped nanodiamonds. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 109, No. 34, pp. 13493–7, August 2012.
- [28] S. Cui and E. Hu. Increased negatively charged nitrogen-vacancy centers in fluorinated diamond. *Applied Physics Letters*, Vol. 103, No. 5, p. 051603, 2013.
- [29] R. Igarashi, Y. Yoshinari, H. Yokota, T. Sugi, F. Sugihara, K. Ikeda, H. Sumiya, S. Tsuji, I. Mori, H. Tochio, Y. Harada, and M. Shirakawa. Real-time background-free selective imaging of fluorescent nanodiamonds in vivo. *Nano letters*, Vol. 12, No. 11, pp. 5726–32, November 2012.

Chapter IV

Suppression of Non-Specific Protein-Nanodiamond Adsorption Enabling Specific Targeting of Nanodiamonds to Bio-molecules of Interest

Abstract

Applications of nanodiamonds (NDs) to a bio-imaging are restricted because of the low dispersibility in aqueous solution and non-specific adsorption of bio-molecules on the surface of NDs. Here, we quantitatively elucidated that a hyper-branched polyglycerol modified nanodiamond realizes both excellent dispersibility and marked suppression of non-specific adsorption to proteins. Moreover, selective targeting to the proteins of interest, and a measurement of optically detected magnetic resonance from this nanodiamond on living cells were demonstrated.

1. Introduction

Nanodiamonds (NDs) have attracted much attention in the fields of biology, and several applications to fluorescence imaging were reported.[1–5] Fluorescence imaging is a powerful tool for visualizing how biological systems function at the single-molecular level. Nitrogen-vacancy center (NVCs), lattice defects in NDs, emit fluorescence in the near-IR region.[6, 7] NVCs show neither photo-bleaching nor photo-blinking,[6] which cause major problems in the use of conventional fluorophores such as quantum dots, fluorescent organic dyes and fluorescent proteins. Such a favorable photo-stability of NVCs enables a long-term imaging of biomolecules in vivo.[2, 8] However, there are considerable problems in the application of NDs to bio-imaging. The surface of NDs is highly hydrophobic, resulting in low dispersibility in aqueous solution, and leads to nonspecific adsorption of biomolecules on the surface through hydrophobic interaction. In terms of bioimaging, these undesirable properties lead to decreased reliability for target specificity.

To solve these problems, we focused on hyper-branched polyglycerol (HPG) modification on ND surface. HPG are commonly used for surface modification of nanoparticles. [9, 10] This highly-branched structure is expected to maintain dispersion state and restrict nonspecific protein adsorption. Zhao *et al.* have reported that hyper-branched polyglycerol modified NDs (ND-HPG) show good dispersibility even in physiological media.[11, 12] However, quantitative data of dispersion state have not been presented. Besides, the ability of non-specific adsorption of ND-HPG has not been investigated. Here, we report quantitative results that HPG surface modification is effective for exhibited both excellent dispersibility even in physiological solution and suppression of nonspecific ad-

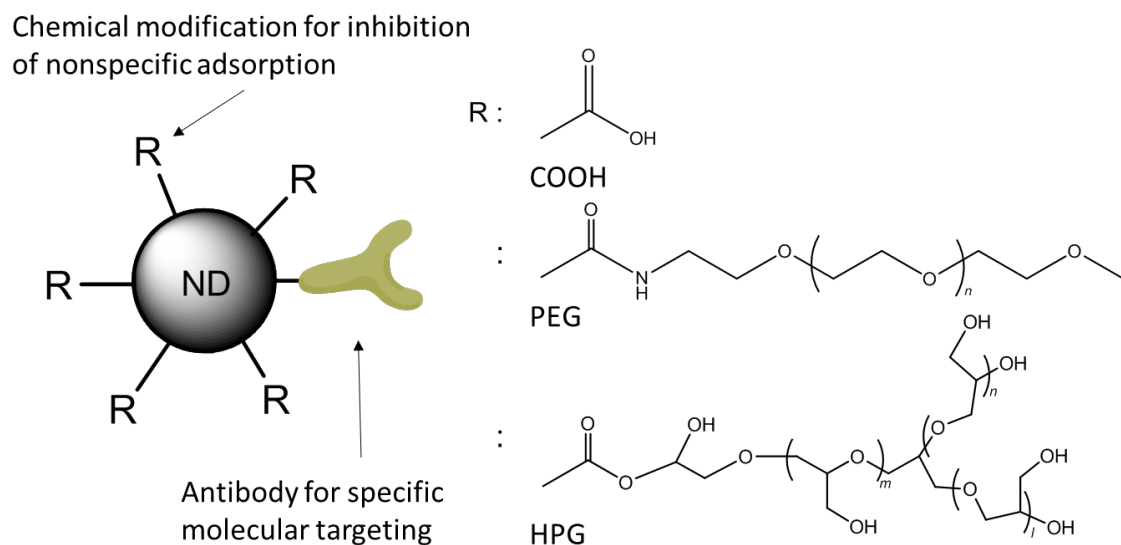


Figure 1. Schematic illustration of surface modified nanodiamond.

sorption to proteins. Furthermore, we present a method for specific molecular targeting with suppressing the nonspecific adsorption.

2. Results and Discussion

In this study, we first chemically modified the surface of NDs by hydrophilic groups, such as carboxyl (ND-COOH), polyethylene glycol (ND-PEG) or hyper-branched polyglycerol (ND-HPG). ND particles with a median size of 53 nm were used in all experiments (Figure 1). Synthesis of these surface-modified NDs were confirmed by IR spectroscopy, Raman spectroscopy, thermogravimetry and elemental analysis.

First, we investigated the dispersibility of each sample in aqueous solution. Figure 2 shows time courses of the dynamic light scattering intensity of 1 mg/ml of ND-COOH, ND-PEG and ND-HPG in milli-Q water and phosphate-buffered saline (PBS). Note that the concentration of NDs was calculated in terms of core diamond particles without con-

sidering the surface-modified groups. As a result, ND-COOH and ND-PEG gradually precipitated in milli-Q water, on the other hand, a precipitation was observed immediately and they totally precipitated after 120 minutes in PBS. By contrast, ND-HPG was found to be totally dispersed even after 120 minutes. These differences in dispersibility were presumably due to low concentrations of surface chemical groups. From the results of elemental analysis, COOH groups, which were also used for substitution with PEG or HPG groups, covered single-digit percentages of the surface area under the assumption that the ND is perfectly spherical. This data is coincide with the data previously reported.[13] This low proportion of COOH or PEG groups would provide insufficient surface hydrophilicity to overcome the effect of hydrophobic interaction among NDs, resulting in aggregation and precipitation. Note that the poor dispersibility was not improved even by modification with 20 times longer PEG chains. By contrast, the highly-branched HPG group patched the exposed hydrophobic surface area sufficiently to prevent aggregation, resulting in excellent dispersibility of ND-HPG.

Surface hydrophobicity also causes nonspecific adsorption of proteins onto NDs. In order to determine the amount of protein adsorption, we measured the adsorption isotherms

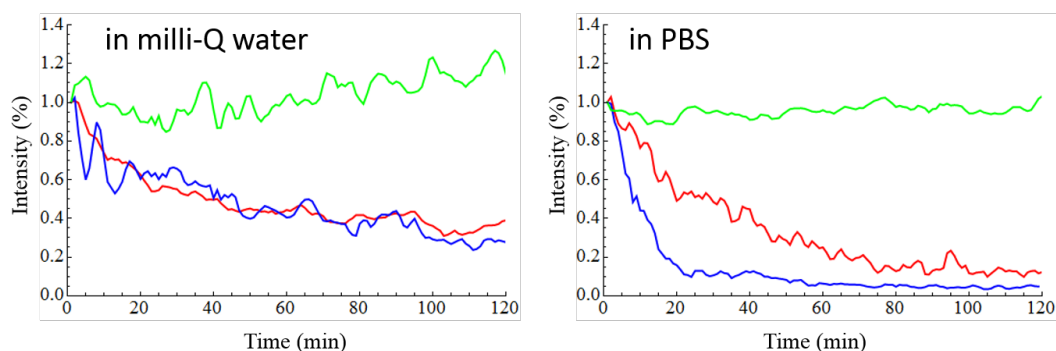


Figure 2. Time courses of scattering intensity of 1 mg/ml of ND-COOH (red), ND-PEG (blue), ND-HPG (green), after 120 minutes in milli-Q water (left) and PBS(right).

of lysozymes on the surface of NDs as shown in Figure 3. The adsorption isotherms were

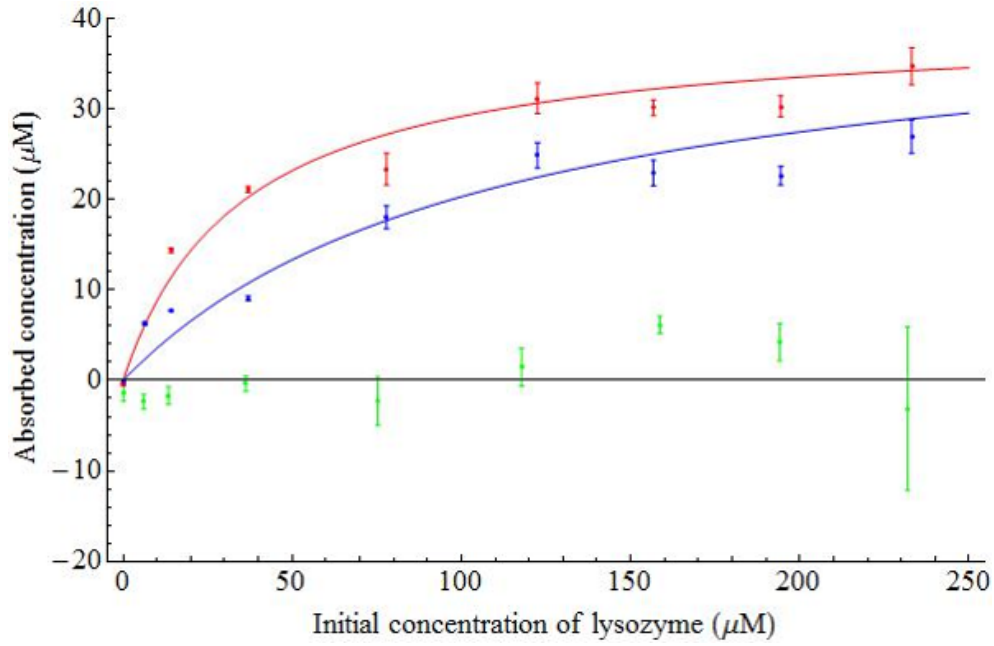


Figure 3. Adsorption isotherm of lysozyme on ND-COOH (red), ND-COOH (blue) and ND-HPG (green). The approximation was performed with the Langmuir method of fitting.

best fit by the Langmuir adsorption model:

$$\theta = \theta_{max} \frac{C}{1 + KC} \quad (1)$$

where θ is the number of occupied adsorption sites relative to the total number of sites at saturation, θ_{max} is the total number of adsorption sites, K is the Langmuir equilibrium constant, and C is the equilibrium protein concentration. A single adsorption plateau at concentrations of 50-100 μM was shown in the isotherms of ND-COOH and ND-HPG, which indicated that approximately 4000 lysozymes are adsorbed on the surface of an ND. By contrast, the adsorption of lysozyme on ND-HPG was markedly suppressed on ND-HPG, thereby making it impossible to fit of the isotherm. Furthermore, we also in-

investigated the non-specific adsorption of NDs onto cell membranes. Each type of surface-modified ND was added to A431 cell culture media at a final concentration of 1 mg/ml. After incubation for two hours, free NDs were removed by washing three times with PBS, and the NDs that were nonspecifically adsorbed onto the cell membrane were observed by bright-field microscopy (Figure 4). The bright-field images of A431 cells treated with ND-COOH or ND-PEG at 1 mg/ml clearly revealed the non-specific adsorption of NDs, observed as dark-black agglomerates, on the cell surface. It has been reported that PEG modification on surface of the quantum dots and gold nanoparticles is effective for the suppression of nonspecific adsorption of bio-molecules[11, 14], but it is not effective for NDs. Contrary to this, no non-specifically bound ND-HPG particles were observed on the cell surface. Notably, the same results were also obtained with a tenfold higher concentration (10 mg/ml) of ND-HPG.

Collectively, HPG surface modification of ND highly enhanced the water dispersibility and markedly suppressed the non-specific adsorption of bio-molecules. Therefore, ND-HPG is a potential candidate for the in situ specific targeting to bio-molecules. To confirm this, we further modified the surface of ND-HPG by the covalent attachment of an anti-epidermal growth factor receptor (EGFR) monoclonal antibody, and performed immunohistochemical targeting of EGFR on the cell membrane of A431. To obtain anti-EGFR-ND-HPG, unreacted COOH groups remaining on the surface of ND-HPG were reacted with anti-EGFR. Synthesis of anti-EGFR-ND-HPG was confirmed by fluorescence spectroscopy and fluorescent microscope using fluorophore-conjugated secondary antibodies.

The 50-nm sized ND is difficult to image by conventional fluorescence microscopy

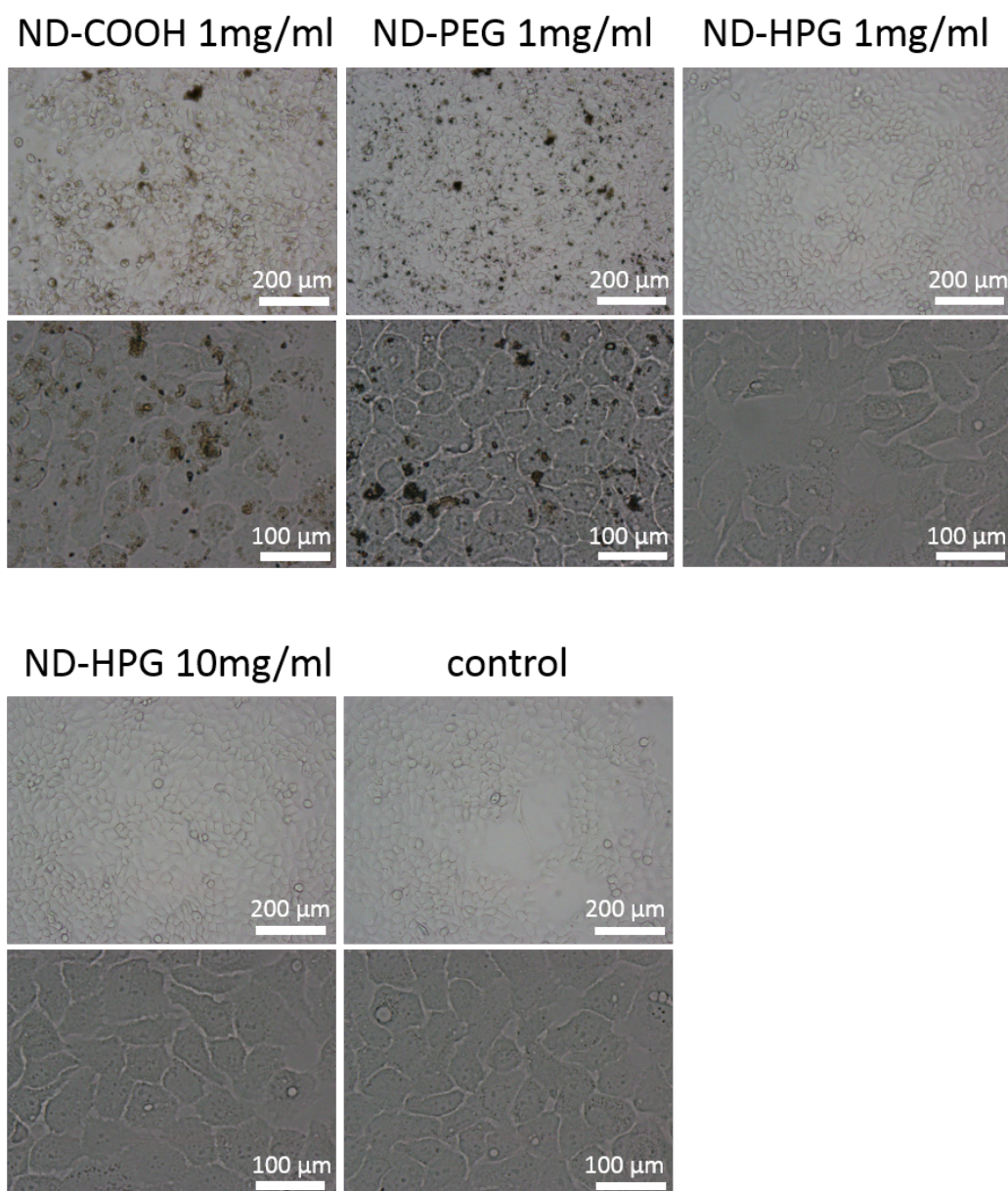


Figure 4. Bright field images of A431 cells after incubation with ND-COOH 1 mg/ml, ND-PEG 1 mg/ml, ND-HPG 1 mg/ml, ND-HPG 10 mg/ml and control in DMEM for 2 h, and then washed three times with PBS.

because it contains very few NVCs unless ion irradiation is applied (Chapter I).[15] We therefore labeled anti-EGFR-ND-HPG with fluorescein as an imaging agent. In this experiment, the ND suspension was added to A431 cell culture media to a final concentration of 10 $\mu\text{g/ml}$ before incubation at 37 °C for 1 hour. Unbound NDs were removed by washing the cells three times with PBS. Figure 5 shows the bright-field images and fluorescence images of living A431 cells treated with the NDs. Green fluorescence spots, indicating the presence of fluorescein-labeled NDs, were observed on the cells treated with anti-EGFR-ND-HPG, whereas almost no spots were observed on the cells treated with ND-HPG. This result indicated that antibody-conjugated ND-HPG was specifically bound to the target receptor, and that the HPG surface modification is feasible for in situ molecular-specific targeting. In particular, the specific targeting of ND-HPG can be readily be used for fluorescence molecular imaging. For example, NVCs in an ND are potentially an ideal single-molecule tracking probe because of their perfect photo-stability, which allows long-term tracking over days.

We further measured the fluorescence and optically detected magnetic resonance (ODMR) signal arising from NVCs in the selectively-targeted ND. The ODMR signal is a potential means to obtain valuable information in living cells such as dynamics of bio-molecules, electromagnetic fields, and temperature.[16–19] However, ODMR measurements of NDs specifically targeted to organelles or proteins have not previously been reported. Therefore, we examined whether an ODMR signal would be detectable from an ND specifically bound to an EGFR molecule on a living A431 cell. Figure 6a and 6b shows bright field and fluorescence (532 nm excitation) images of an ND on A431 cells, respectively. The red bright spots represent the fluorescence emitted by the ND. The NVC-selective image

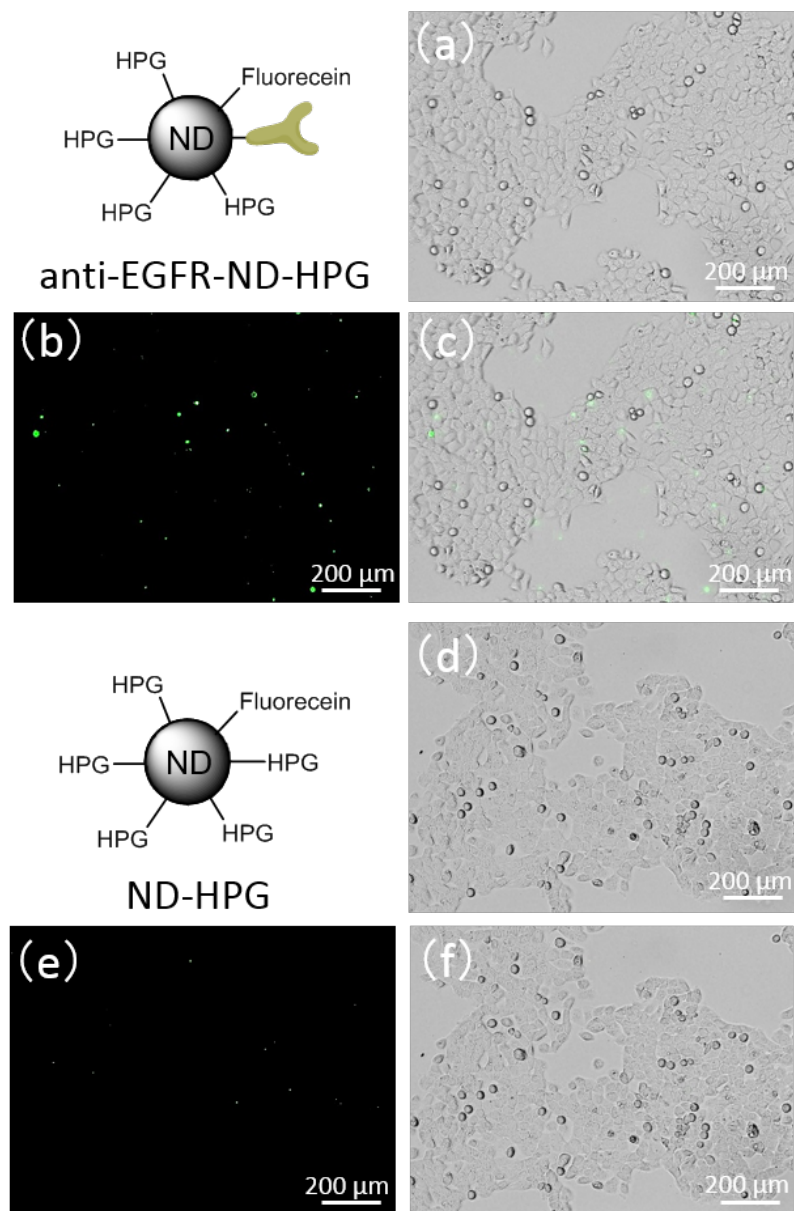


Figure 5. (a) Bright field images, (b) fluorescence images of A431 cells after incubation with 10 $\mu\text{g}/\text{ml}$ of fluorescein conjugated anti-EGFR-ND-HPG. (c) is merge of (a) and (b). (d) Bright field images, (e) fluorescence images of A431 cells after incubation with 10 $\mu\text{g}/\text{ml}$ of fluorescein conjugated ND-HPG. (f) is merge of (e) and (f). Green spot indicate the fluorescence from Fluorescein.

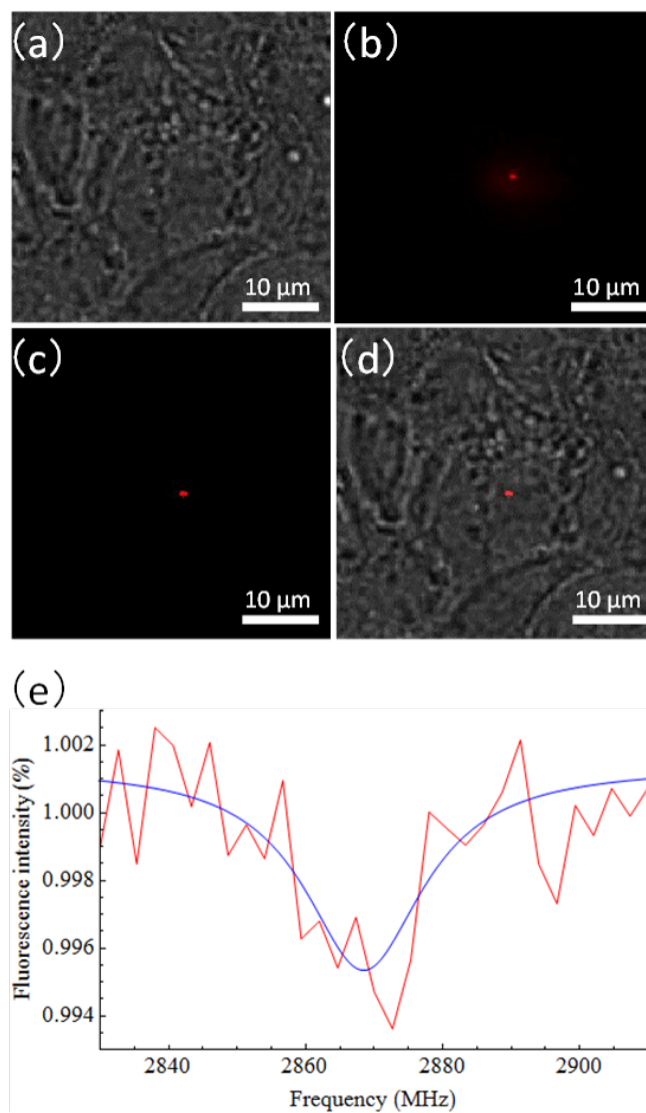


Figure 6. (a) Bright field images, (b) fluorescence images, (c) SIP image and (d) merge of (a) and (c) of A431 cells after incubation with 100 $\mu\text{g/ml}$ of anti-EGFR-ND-HPG. Red dot in (b), (c), and (d) shows the fluorescence from NVCs. (e) ODMR spectrum of this ND.

at 2.87 GHz of microwave (MW) [20] is shown in Figure 6c, and merged on the bright field image in Figure 6d. Figure 6e shows the ODMR spectrum acquired from this ND by sweeping the MW range from 2830 to 2910 MHz. A decrease in fluorescence intensity, derived from spin-dependent intersystem-crossing, was observed when the MW frequency was irradiated at approximately 2870 MHz, as expected. As a result, an ODMR signal was successfully obtained from anti-EGFR-ND-HPG bound to EGFR. Hence, we conclude that this specific molecular targeting technique using ND-HPG is likely to be applicable to a wide variety of biological experiments via ODMR measurement.

In summary, we synthesized three types of ND surface-modified with COOH, PEG or HPG, and tested them for dispersibility and non-specific adsorption of bio-molecules two properties that have been considered as serious obstacles in utilizing NDs for biological applications such as bio-imaging. ND-HPG was found to exhibit excellent water dispersibility and did not precipitate even after 120 minutes in milli-Q water and PBS. The HPG surface modification of NDs also markedly suppressed the nonspecific adsorption of biomolecules including cell membrane. In addition, ND-HPG could be used for in situ specific molecular targeting to EGFR on A431 cells via the conjugation of anti-EGFR antibody. Moreover, we demonstrated that ODMR measurement is possible for an ND that is selectively targeted to bio-molecules of interest. Our specific targeting strategy will provided a way to obtain bio-applicable NDs, will contribute to improving bio-imaging systems, and will open up new horizons for bio-application of NDs.

3. Experimental Section

3.1 Surface modification of NDs

NDs (size: 53 nm, Tomei Diamond) were annealed at 800 °C for two hours under vacuum to pair nitrogen atoms and vacancies, and then oxidized at 550 °C for two hours to remove the surface graphitic layer. By these processes, NVCs are introduced into the diamond lattice and emit fluorescence. The NDs were treated with a mixture of H₂SO₄ : HNO₃ (9:1 v/v) at 70 °C for three days, and then centrifuged for 30 minutes at 15000 g, and the supernatant was discarded. Next, 0.1 M NaOH was added and the suspension was stirred for two hours at 90 °C. After centrifugation, the NDs were stirred in 0.1 M HCl for two hours at 90 °C. Lastly, the NDs were centrifuged and washed three times with milli-Q water. The surface of the NDs was first modified by COOH groups to produce ND-COOH. Next, ND-COOH was dispersed in PBS buffer, and then water soluble carbodiimide (WSC, Dojindo Molecular Technologies, Inc) and N-hydroxysuccinimide (NHS, Wako pure chemical Industries) were added to activate the COOH groups. The mixture was allowed to react with amino-terminal polyethylene glycol (PEG, Mw: 2000, NOF) at ambient temperature in the dark for 8 hours. After the reaction, PEG-modified NDs (ND-PEG) were collected by centrifugation for 30 minutes at 15000 g, and then rinsed three times with milli-Q water. ND-COOH was also then dispersed in glycidol (Sigma-Aldrich), sonicated for two hours, and then stirred for 10 hours at 140 °C. The resulting gel was diluted with methanol, and centrifuge at 40000 g for 30 minutes to correct hyper-branched polyglycerol-grafted NDs (ND-HPG). Then ND-HPG was washed four times to remove free polyglycerol. Lastly, this product was dialyzed against Milli-Q water to replace residual methanol with water. The synthesis of each surface-modified NDs was confirmed by IR (Figure 7a), TGA (Figure 7b), Raman (Figure 7c), and elemental analysis (Table 1). In the IR spectra, absorption corresponding to C-H₂ and C-O-C stretching

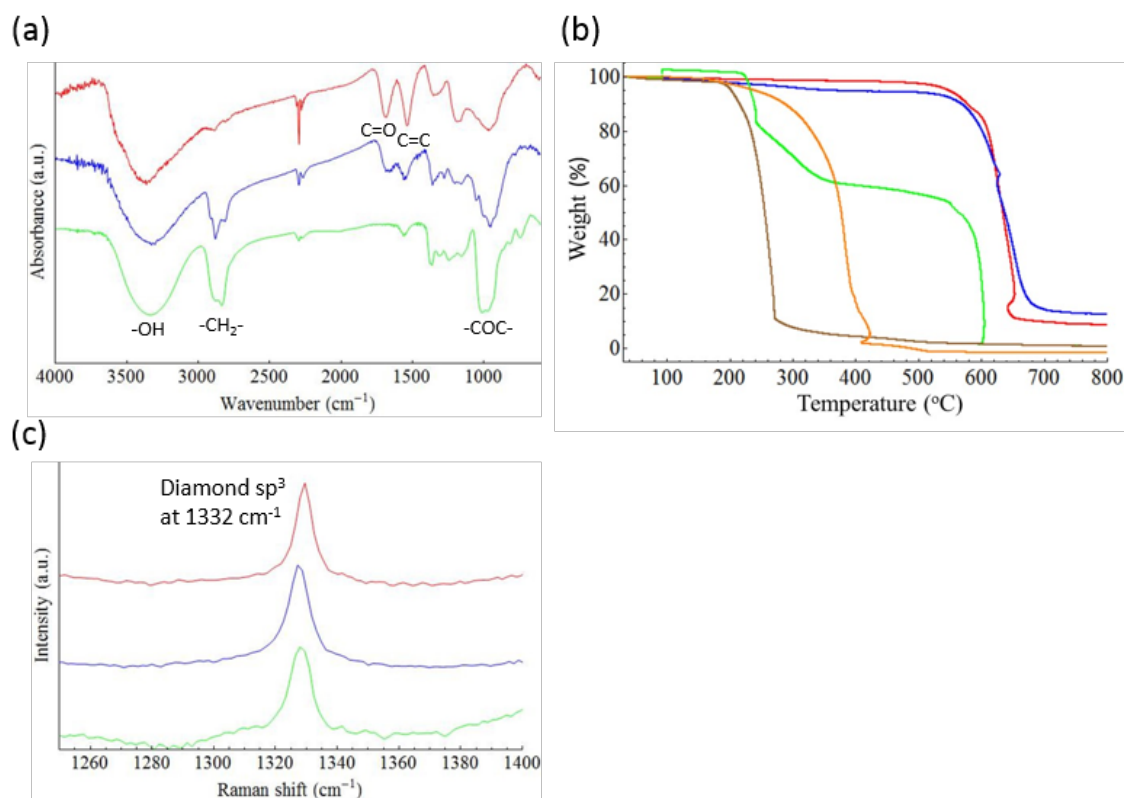


Figure 7. (a) FTIR spectra of ND-COOH (red), ND-PEG (blue) and ND-HPG (green). (b) TGA profile of ND-COOH (red), ND-PEG (blue), ND-HPG (green), free PEG (brown) and free HPG (orange). (c) Raman spectra of ND-COOH (red), ND-PEG (blue) and ND-HPG (green).

increased after PEG and HPG modification. Thermogravimetric analysis shows decomposition of the PEG and HPG, which grafted on the surface of NDs, and Raman spectrum indicate sharp diamond peak at 1332 cm^{-1} . Elemental analysis also support these modification: Nitrogen and oxygen atoms are increased by the PEG modification, and oxygen atoms are highly increased by the HPG modification.

3.2 Dispersibility test of NDs modified with long-chain PEG (Mw: 40 k) modified NDs

From our experiment, PEG (Mw 2k) modification of the ND surface with PEG (Mw 2k) was not effective for improving dispersibility. However, this low dispersibility might

Table 1. Elemental analysis of the samples.

Element	H weight%	C weight%	N weight%	O weight%
ND-COOH	0	96.84	0	2.15
ND-PEG	1.01	94.62	0.3	2.18
ND-HPG	7.58	49.82	0	42.44

be improved by modifying the surface of NDs with longer PEG chain. Thus, we synthesis NDs modified with PEG (Mw 40k) modified NDs, then investigated their dispersibility of water and PBS. At the same time, we examined branched PEG (2 or 4 generation). Figure S3 shows time-dependent changes in the states of dispersion for each type of surface-modified ND. Each sample had relatively good dispersibility for water, but showed precipitation within 10 minutes in PBS (Figure 8). This results indicate that PEG is not sufficient to enhance the dispersibility of NDs regardless of the length of chain.

3.3 Conjugation of anti-EGFR on ND-HPG

We confirmed that anti-EGFR (Sigma-Aldrich) was conjugated to ND-HPG via a secondary antibody (HiLyte Fluor 488, Funakoshi). 10 μ l of Hilyte fluor 488 was added to a 1 ml solution of ND-HPG or anti-EGFR-ND-HPG at an ND concentration of 1 mg/ml, respectively. Each sample was incubated at 37 °C for one hour, and then centrifuged at 40000 g for 30 minutes to remove unreacted Hilyte fluor 488. This washing process was repeated until fluorescence derived from Hilyte fluor 488 was no longer observed in the supernatant. After these process, each ND pellet was resuspended in milli-Q water and its fluorescence spectrum was measured (Figure 9). Fluorescence signal of Hilyte fluor 488 was observed only from anti-EGFR-ND-HPG (Red), indicating the successful

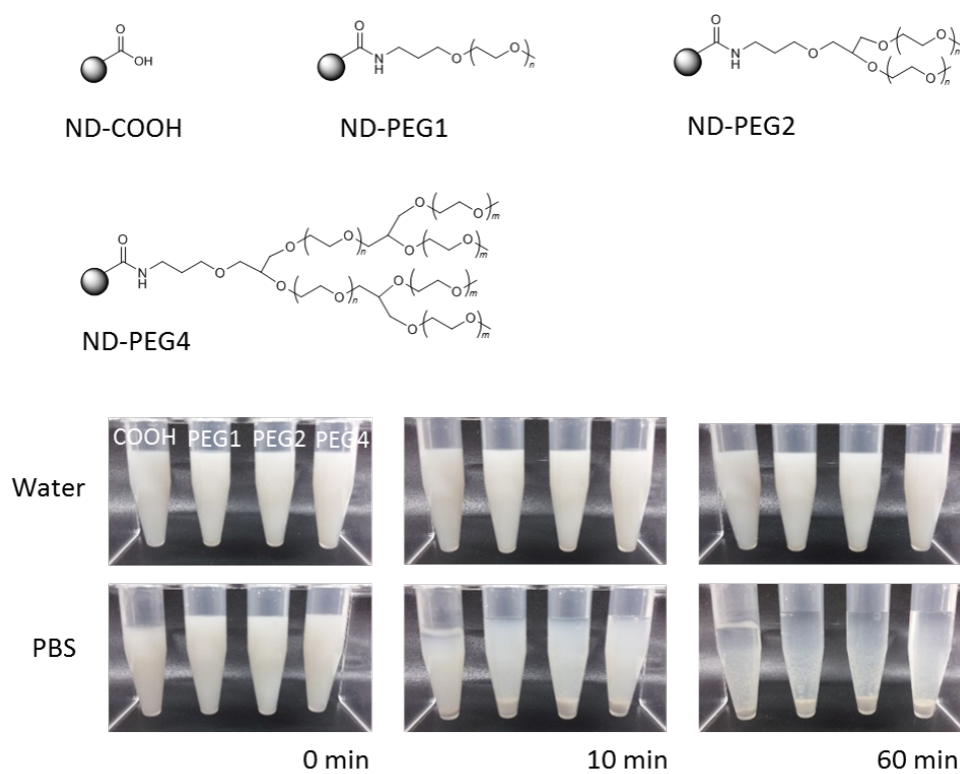


Figure 8. Time-dependent dispersion state of ND-COOH (1 mg/ml), ND-PEG (1 mg/ml), ND-PEG2 (1 mg/ml), ND-PEG4 (1 mg/ml), after 0 min, 10 min and 60 min. The solvents were water (upper row) and PBS (lower row).

conjugation of anti-EGFR and ND-HPG.

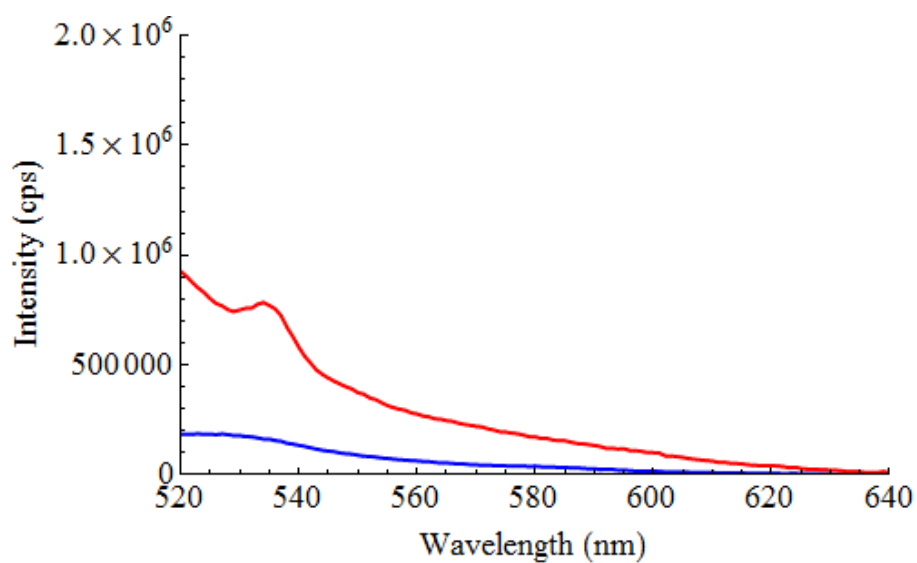


Figure 9. Fluorescence spectrum of anti-EGFR-ND-HPG (red) and ND-HPG reacted with Hilyte fluor 488. After the reaction, free Hilyte fluor 488 was removed by repeated washing. The fluorescence signal around 539 nm indicates the fluorescence from Hilyte fluor 488.

References

- [1] T. Wu, Y. Tzeng, W. Chang, C. Cheng, Y. Kuo, C. Chien, H. Chang, and J. Yu. Tracking the engraftment and regenerative capabilities of transplanted lung stem cells using fluorescent nanodiamonds., September 2013.
- [2] Y. Chang, H. Lee, K. Chen, C. Chang, D. Tsai, C. Fu, T. Lim, Y. Tzeng, C. Fang, C. Han, H. Chang, and W. Fann. Mass production and dynamic imaging of fluorescent nanodiamonds. *Nature nanotechnology*, Vol. 3, No. 5, pp. 284–8, May 2008.
- [3] N. Mohan, C. Chen, H. Hsieh, Y. Wu, and H. Chang. In vivo imaging and toxicity assessments of fluorescent nanodiamonds in *Caenorhabditis elegans*. *Nano letters*, Vol. 10, No. 9, pp. 3692–9, September 2010.
- [4] M. Mkandawire, A. Pohl, T. Gubarevich, V. Lapina, D. Appelhans, G. Rödel, W. Pompe, J. Schreiber, and J. Opitz. Selective targeting of green fluorescent nanodiamond conjugates to mitochondria in HeLa cells. *Journal of biophotonics*, Vol. 2, No. 10, pp. 596–606, October 2009.
- [5] M. Weng, S. Chiang, N. Wang, and H. Niu. Fluorescent nanodiamonds for specifically targeted bioimaging: Application to the interaction of transferrin with transferrin receptor. *Diamond and Related Materials*, Vol. 18, No. 2-3, pp. 587–591, February 2009.
- [6] S. Yu, M. Kang, H. Chang, K. Chen, and Y. Yu. Bright fluorescent nanodiamonds: no photobleaching and low cytotoxicity. *Journal of the American Chemical Society*, Vol. 127, No. 50, pp. 17604–5, December 2005.

- [7] V. Petráková, A. Taylor, I. Kratochvílová, F. Fendrych, J. Vacík, J. Kučka, J. Štursa, P. Cígler, M. Ledvina, A. Fišerová, P. Kneppo, and M. Nesládek. Luminescence of Nanodiamond Driven by Atomic Functionalization: Towards Novel Detection Principles. *Advanced Functional Materials*, Vol. 22, No. 4, pp. 812–819, February 2012.
- [8] C. Fang, V. Vijayanthimala, C. Cheng, S. Yeh, C. Chang, C. Li, and H. Chang. The exocytosis of fluorescent nanodiamond and its use as a long-term cell tracker. *Small (Weinheim an der Bergstrasse, Germany)*, Vol. 7, No. 23, pp. 3363–70, December 2011.
- [9] A. Zill, A. Rutz, R. Kohman, and A. Alkilany. Clickable polyglycerol hyperbranched polymers and their application to gold nanoparticles and acid-labile nanocarriers. *Chem. Commun.*, Vol. 47, pp. 1279–1281, 2011.
- [10] S. Wang, Y. Zhou, S. Yang, and B. Ding. Growing hyperbranched polyglycerols on magnetic nanoparticles to resist nonspecific adsorption of proteins. *Colloids and surfaces. B, Biointerfaces*, Vol. 67, No. 1, pp. 122–6, November 2008.
- [11] L. Zhao, T. Takimoto, M. Ito, N. Kitagawa, T. Kimura, and N. Komatsu. Chromatographic separation of highly soluble diamond nanoparticles prepared by polyglycerol grafting. *Angewandte Chemie (International ed. in English)*, Vol. 50, No. 6, pp. 1388–92, February 2011.
- [12] L. Zhao, Y. Xu, T. Akasaka, S. Abe, N. Komatsu, F. Watari, and X. Chen. Polyglycerol-coated nanodiamond as a macrophage-evading platform for selective

- drug delivery in cancer cells. *Biomaterials*, Vol. 35, No. 20, pp. 5393–406, July 2014.
- [13] T. Nguyen, H. Chang, and V. Wu. Adsorption and hydrolytic activity of lysozyme on diamond nanocrystallites. *Diamond and Related Materials*, Vol. 16, No. 4-7, pp. 872–876, April 2007.
- [14] E. Bentzen, I. Tomlinson, J. Mason, P. Gresch, M. Warnement, D. Wright, E. Sanders-Bush, R. Blakely, and S. Rosenthal. Surface modification to reduce nonspecific binding of quantum dots in live cell assays. *Bioconjugate chemistry*, Vol. 16, No. 6, pp. 1488–94, 2005.
- [15] S. Sotoma, Y. Yoshinari, R. Igarashi, A. Yamazaki, S. Yoshimura, H. Tochio, M. Shirakawa, and Y. Harada. Effective production of fluorescent nanodiamonds containing negatively-charged nitrogen-vacancy centers by ion irradiation. *Diamond and Related Materials*, Vol. 49, pp. 33–38, October 2014.
- [16] L. P. McGuinness, Y. Yan, A. Stacey, D. Simpson, L. Hall, D. Maclaurin, S. Prawer, P. Mulvaney, J. Wrachtrup, F. Caruso, R. Scholten, and L. Hollenberg. Quantum measurement and orientation tracking of fluorescent nanodiamonds inside living cells. *Nature nanotechnology*, Vol. 6, No. 6, pp. 358–63, January 2011.
- [17] M. Geiselmann, M. Juan, J. Renger, J. Say, L. Brown, F. Javier G. Abajo, F. Koppens, and R. Quidant. Three-dimensional optical manipulation of a single electron spin., March 2013.
- [18] G. Balasubramanian, I. Chan, R. Kolesov, M. Al-Hmoud, J. Tisler, C. Shin, C. Kim,

- A. Wojcik, P. Hemmer, A. Krueger, T. Hanke, A. Leitenstorfer, R. Bratschitsch, F. Jelezko, and J. Wrachtrup. Nanoscale imaging magnetometry with diamond spins under ambient conditions. *Nature*, Vol. 455, No. 7213, pp. 648–51, October 2008.
- [19] G. Kucsko, P. Maurer, N. Yao, M. Kubo, H. Noh, P. Lo, H. Park, and M. Lukin. Nanometre-scale thermometry in a living cell. *Nature*, Vol. 500, No. 7460, pp. 54–8, August 2013.
- [20] R. Igarashi, Y. Yoshinari, H. Yokota, T. Sugi, F. Sugihara, K. Ikeda, H. Sumiya, S. Tsuji, I. Mori, H. Tochio, Y. Harada, and M. Shirakawa. Real-time background-free selective imaging of fluorescent nanodiamonds in vivo. *Nano letters*, Vol. 12, No. 11, pp. 5726–32, November 2012.

Part III

Summary and General Conclusion

Effective Production of Fluorescent Nanodiamonds Containing Negatively-Charged Nitrogen-Vacancy Centers by Ion Irradiation

In Chapter I of Part II, I demonstrated ion irradiation is effective method for improving the concentration of nanodiamonds containing negatively-charged nitrogen-vacancy centers. It is revealed that He^+ ion irradiation at 1×10^{13} is the most effective condition for producing nanodiamonds containing negatively-charged nitrogen-vacancy centers. ODMR observations of the nanodiamonds showed that ion irradiation increased the number of such nanodiamonds up to 200 ppm. In addition, I elucidated that ion irradiation leads a competition in two opponent roles of vacancies; effective for pairing with nitrogen atoms and as defects for developing damage in crystalline.

Optically Detected Magnetic Resonance Signals Arising from Nitrogen-Vacancy Centers in Nanodiamonds as Small as 5-nm

In Chapter II, I elucidated surface termination of OH groups on nanodiamonds is effective for enhancing ODMR signal. ODMR intensity is increased 2.7-fold for OH-termination as compared with non-surface modification. We further demonstrated that single spin detection of an OH-terminated detonation synthesized nanodiamonds (size: 5 nm), and determined the orientation of nitrogen-vacancy axis towards external magnetic field.

Comprehensive and Quantitative Analysis for Controlling the Physical/Chemical States and Particle Properties of Nanodiamonds for Biological Applications

In Chapter III, I comprehensively and quantitatively analyzed the physical/chemical states such as particle size, crystal quality and chemical bonding state of carbon and oxygen, and particle properties such as dispersibility, fluorescent and optically detected magnetic resonance of annealed and oxidized nanodiamonds. We then elucidated following things: Weight loss in the NDs occurred via several processes; a surface graphene layer introduced during annealing was oxidized in air at temperatures above 500 °C; distortion of the crystal during oxidation at 525–550 °C was accompanied by particle shrinkage; the sp^3/sp^2 ratio increased with increasing temperature, and COOH groups introduced at lower oxidation temperature were dehydrated at higher oxidation temperatures, resulting in NDs with low zeta potentials. Furthermore, it was determined that the ODMR signal intensity for an ND may be influenced by its sp^2 ratio and particle size; the ODMR signal decreased with decreasing particle size, but crystal distortion around NV^- was not observed, indicating that an NVC existing at the surface was converted from NV^- to NV^0 . Furthermore, the optimal oxidation conditions for a discrete maximum ODMR intensity were determined.

Suppression of Non-Specific Protein-Nanodiamond Adsorption Enabling Specific Targeting of Nanodiamonds to Bio-molecules of Interest

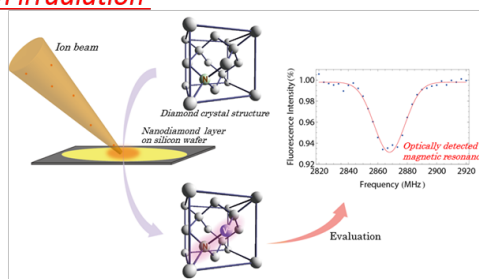
In Chapter IV, I demonstrated hyper-branched polyglycerol (HPG) modification on nanodiamond surface realize both excellent dispersibility, even in salt-containing solution, and marked suppression of non-specific adsorption to proteins. These feasible effects were not obtained from COOH or polyethylene glycol (PEG) surface termination, which are commonly used for surface modification of nanoparticles. I further modified the sur-

face of ND-HPG by the covalent attachment of an anti-epidermal growth factor receptor (EGFR) monoclonal antibody, and performed immunohistochemical targeting of EGFR on the cell membrane of A431. As a result, antibody-conjugated ND-HPG was specifically bound to the target receptor, and that the HPG surface modification is feasible for in situ molecular-specific targeting. Moreover, I demonstrated that ODMR measurement is possible for an ND which is selectively targeted to a bio-molecule of interest.

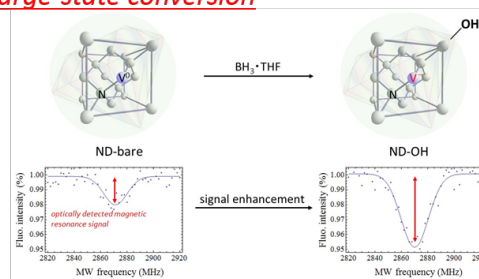
Concluding remarks

In this thesis, I demonstrated preparation of bioinvestigation-applicable fluorescent/ODMR-active nanodiamonds. In the theme, I showed the optimal condition of ion irradiation for producing nanodiamonds containing negatively-charged nitrogen-vacancy centers; ODMR enhancement by surface OH-termination; chemical/physical states and particle properties of nanodiamonds subjected to annealing and oxidation; selective targeting method for proteins of interest. These works are expected to open up new horizons for application of this novel material in nanobiology.

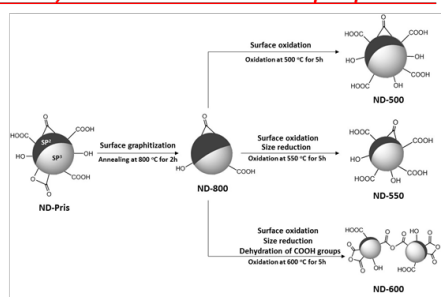
Ion irradiation



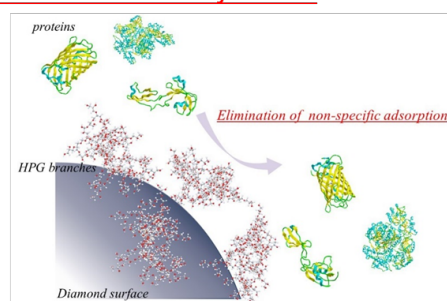
Charge-state conversion



Physical, chemical states and properties



Surface chemical modification



List of Publications

Effective Production of Fluorescent Nanodiamonds Containing Negatively-Charged Nitrogen-Vacancy Centers by Ion Irradiation

Shingo Sotoma, Yohsuke Yoshinari, Ryuji Igarashi, Akiyoshi Yamazaki, Shige H. Yoshimura, Hidehito Tochio, Masahiro Shirakawa and Yoshie Harada

Diamond and Related Materials, **2014**, 10, 1106-1115.

Optically Detected Magnetic Resonance Signals Arising from Nitrogen-Vacancy Centers in Nanodiamonds as Small as 5-nm

Shingo Sotoma, Ryuji Igarashi, Hidehito Tochio, Yoshie Harada and Masahiro Shirakawa

Scientific Reports, under review.

Comprehensive and Quantitative Analysis for Controlling the Physical/Chemical States and Particle Properties of Nanodiamonds for Biological Applications

Shingo Sotoma, Ken-ichi Akagi, Saburo Hosokawa, Ryuji Igarashi, Hidehito Tochio, Yoshie Harada and Masahiro Shirakawa

RSC Advances, **2015**, 5, 13818-13827.

Suppression of Non-Specific Protein-Nanodiamond Adsorption Enabling Specific
Targeting of Nanodiamonds to Bio-molecules of Interest

Shingo Sotoma, Ryuji Igarashi, Jun Iimura, Yuta Kumiya, Hidehito Tochio, Yoshie
Harada and Masahiro Shirakawa

Chemistry Letters, in press.

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