Chapter 1

General Introduction

Diamond is a natural substance on earth. It is not only a shining jewel in people's eyes, but also widely applied in industry due to its unique properties including ultra-high hardness, durability, high reflection coefficient and high thermal conductivity.

1.1 Fluorescent nanodiamonds (FNDs)

With the development of nanotechnology, nanodiamonds, especially fluorescent nanodiamonds have gained more and more attention. FNDs are a kind of nano sized carbon-based material with color centers incorporated into the lattice structure. The most studied color defect, also in this thesis, is negatively charged nitrogen vacancy (NV^-) centers[1]. To form nitrogen vacancy centers, a carbon atom is replaced by a nitrogen atom with an adjacent vacancy. Those NV^- centers are well protected in the lattice and responsible for the emission of red fluorescence from 630 to 800 nm[2] without having blinking or bleaching issues. Besides, FNDs are biocompatible materials and suitable for various biological applications for instance drug delivery[3], long time tracking[4], Förster resonance energy transfer (FRET)-based scanning probe techniques[5], imaging[6], diagnosis[7] and so on.

1.1.1 Cellular uptake of FNDs

Successful delivery of nanodiamonds into cells is the first step towards most biological applications. Although the size, shape[8] and surface chemistry[9] of FNDs can affect the cellular uptake, generally bare FNDs are readily internalized by different types of cells. It has been widely reported in immune cells (e.g. macrophages[10], dendritic cells[11], monocytes[12]), cancer cells (e.g. HeLa cells[13], A549 cells[14]) and endothelial cells[15]. Studies have shown that nanodiamonds enter eukaryotic cells mainly via the energy-dependent, clathrin-mediated endocytosis[14, 16] while less popularly via micropinocytosis[17] or caveolae-mediated endocytosis[18].

Sometimes, transportation of FNDs becomes a challenge due to the hinderance of the cell wall or the inherent nature of the cell. In this situation, surface modification[19], chemical treatment[20], electroporation[21], injection[22] and other procedures can facilitate the cellular uptake. However, it is worth to be noted that harsh treatment can cause irreversible damage and the cells should be treated carefully.

If cells are too small and/or don't endocytose particles (e.g. bacterial cells or sperm cells), FNDs still can attach to the cell membrane[23, 24] and effectively label the cells.

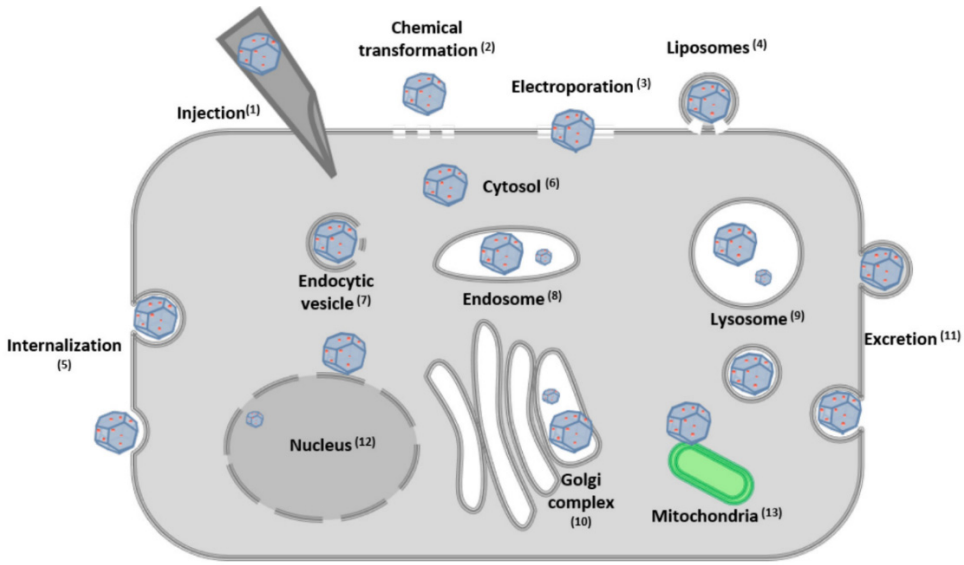

Figure 1. Overview of FNDs' interaction within the cell. Images (1)–(4) show different approaches for artificial internalization of FNDs, while (5) represents endocytosis of FNDs. FNDs can remain in endocytic vesicles (7), endosomes (8) and follow the endocytic pathway to lysosome (9) and the Golgi complex (10), escape to the cytosol (6), or be excreted (11). If FNDs are functionalized it is possible to direct them to different subcellular compartments such as nuclei (12) and mitochondria (13). Reprint from A. Mzyk et al., 2021[25].

1.1.2 Fate of FNDs

As shown in Figure 1, once entering the cells, FNDs can either be trapped in the vesicles followed by excretion or escape from the endosomes. There is no consensus on

this topic yet due to the limitation of quantifying methods, diversity of the cells and particles[26]. Chu et al. reported that sharp edges can easily rupture the endosome membranes and help flake shape nanodiamonds to be released to the cytoplasm[27].

If FNDs are modified with antibodies, they can target specific cellular compartment and achieve subcellular labeling. Structures that have been labelled are for instance, nucleus[28] and mitochondria[29].

There are limited studies regarding the excretion of nanodiamonds from the body. In rats, it has been observed that NDs are primarily excreted through the urinary tract[30]. However, in mice, the excretion of NDs in urine and feces was found to be nearly undetectable[31].

1.1.3 Biocompatibility of FNDs

Good biocompatibility is the basis for biological applications of materials. The foreign nanoparticles shouldn't cause significant toxic effects or interfere with normal cellular functions when in contact with living cells or tissues[32]. There are some common approaches used to assess the biocompatibility including cytotoxicity assays, cell membrane Integrity, inflammatory response and so on. A combination of different methods, based on the specific purpose, is the way to evaluate the biocompatibility comprehensively.

Due to the inert surface and proper size, it has been widely accepted that FNDs are biocompatible materials in cells[33-35]. Besides, they also do not induce strong adverse effects in organisms[36-38], tissues[39], various animals (rodent[40, 41], zebra fish[42] and monkey[43]).

1.1.4 Diamond relaxometry

In addition to the superior fluorescent characteristic, NV^- centers also bring nanodiamonds exceptional quantum properties and allow them to locally sense pH[44], temperature[45, 46] and so on. Recently, our group has successfully applied diamond

relaxometry to detect free radicals in the mitochondria[47], macrophages[48], yeast cells[49], sperm cells[50], viral[51] and bacterial[52] infection. In brief, free radicals are atoms or molecules that contain an unpaired electron, thus can contribute to the magnetic noise. With diamond relaxometry, the surrounding magnetic noise can be converted to optical signals and FNDs are ultra-sensitive and easy to read.

As shown in Figure 2a, initially, electrons are distributed on three spin sublevels of ground state. After excitation, electrons on the m_s = ± 1 ground states are continuously populated to the excited states, then have a higher chance to decay via metastable state (|s>) and end up on the m_s = 0 ground state. During this process, less photons are emitted. When all the electrons are on the m_s = 0 ground state, known as polarized state or bright state, higher brightness can be obtained due to the radiative transition. Once the laser is off, electrons will automatically relax to the equilibrium state. The transition rate between the polarized state and equilibrium state is the spin-lattice relaxation time T1[53].

T1 values can be estimated by a set of laser pulses with an increment of dark time (Figure 2b). The photoluminescent signals are captured in the short reading windows and plotted against the dark time. Fitting by a biexponential equation, a T1 constant is obtained. When NV^- centers are close to higher magnetic noise originating from species such as gadolinium or free radicals, the T1 is shorter. The fluctuation of T1 values can reflect the dynamics of free radicals in the living bio system.

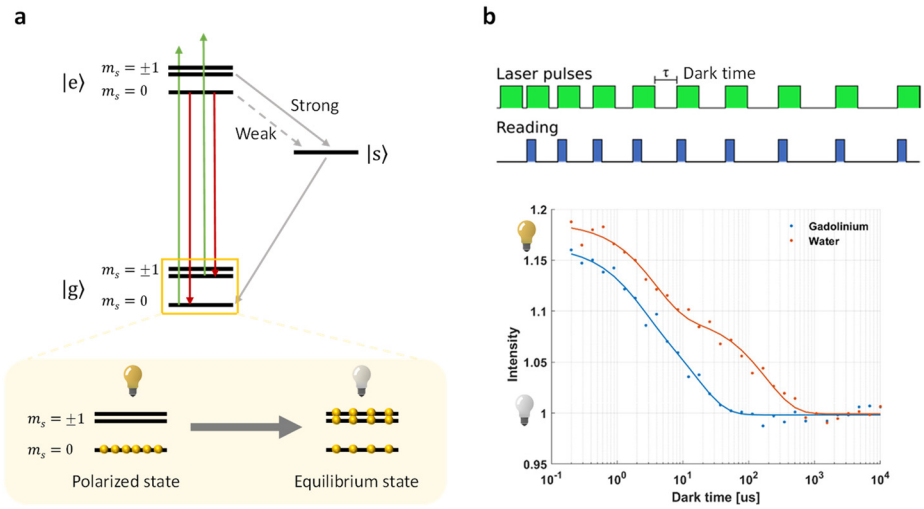

Figure 2. (a) Energy level diagram of NV^- centers and illustration of relaxation time T1. |g> is the ground state, |e> is the excited state, and |s> is the metastable singlet state. (b) Laser pulse sequence and fitting curve of T1 measurements. Reprint from F. Perona Martínez et al., 2020[54].

1.2 Objective and outline of the thesis

The thesis aims to study the internalization, biocompatibility and quantum sensing of nanodiamonds in cells. First of all, in **Chapter 2**, we performed single cell analysis to evaluate the cellular uptake and endosomal escape of FNDs in HeLa cells and Human umbilical vein endothelial cells (HUVECs). In **Chapter 3**, we coated FNDs with a pH sensitive fluorescent dye to not only improve the uptake by the cells, but also indicate the subcellular locations at different stages. In **Chapter 4**, we focused on the biocompatibility of FNDs in boar sperm cells. We systematically evaluated the size and concentration effect on the metabolic activity and cell viability. Later on, in **Chapter 5**, we conduct real-time diamond relaxometry to measure free radicals in primary bronchial epithelial cells upon stimulation with cigarette smoke extract. Finally, in **Chapter 6**, we discussed the importance of our work and further perspective of nanodiamond quantum sensing.

References

1. Davies, G., *A-nitrogen aggregate in diamond - its symmetry and possible structure.* Journal of Physics C-Solid State Physics, 1976. **9**(19): L537-L542.
2. Yu, S.J., et al., *Bright fluorescent nanodiamonds: No photobleaching and low cytotoxicity.* Journal of the American Chemical Society, 2005. **127**(50): 17604-17605.
3. Wang, D.X., et al., *PEGylated nanodiamond for chemotherapeutic drug delivery.* Diamond and Related Materials, 2013. **36**: 26-34.
4. McGuinness, L.P., et al., *Quantum measurement and orientation tracking of fluorescent nanodiamonds inside living cells.* Nature Nanotechnology, 2011. **6**(6): 358-363.
5. Tisler, J., et al., *Highly Efficient FRET from a Single Nitrogen-Vacancy Center in Nanodiamonds to a Single Organic Molecule.* ACS Nano, 2011. **5**(10): 7893-7898.
6. Igarashi, R., et al., *Real-Time Background-Free Selective Imaging of Fluorescent Nanodiamonds in Vivo.* Nano Letters, 2012. **12**(11): 5726-5732.
7. Miller, B.S., et al., *Spin-enhanced nanodiamond biosensing for ultrasensitive diagnostics.* Nature, 2020. **587**(7835): 588-593.
8. Hemelaar, S.R., et al., *The Response of HeLa Cells to Fluorescent NanoDiamond Uptake.* Sensors, 2018. **18**(2): 15.
9. Weng, M.F., et al., *Cellular uptake and phototoxicity of surface-modified fluorescent nanodiamonds.* Diamond and Related Materials, 2012. **22**: 96-104.
10. Huang, K.J., et al., *Phagocytosis and immune response studies of Macrophage-Nanodiamond Interactions in vitro and in vivo.* Journal of Biophotonics, 2017. **10**(10): 1315-1326.
11. Jung, H.S., et al., *Polydopamine Encapsulation of Fluorescent Nanodiamonds for Biomedical Applications.* Advanced Functional Materials, 2018. **28**(33): 9.
12. Niora, M., et al., *Quantitative Evaluation of the Cellular Uptake of Nanodiamonds by Monocytes and Macrophages.* Small, 2023. **19**(11): 2205429.
13. Zheng, M.L., et al., *Endocytic Fluorescence of Nanodiamond in Living Hela Cells Determined by Microscopy Imaging Techniques.* Journal of Nanoscience and Nanotechnology, 2016. **16**(7): 6748-6754.
14. Perevedentseva, E., et al., *Nanodiamond internalization in cells and the cell uptake mechanism.* Journal of Nanoparticle Research, 2013. **15**(8): 12.
15. Solarska-Sciuk, K., et al., *Intracellular transport of nanodiamond particles in human endothelial and epithelial cells.* Chemico-Biological Interactions, 2014. **219**: 90-100.
16. Faklaris, O., et al., *Photoluminescent Diamond Nanoparticles for Cell Labeling: Study of the Uptake Mechanism in Mammalian Cells.* ACS Nano, 2009. **3**(12): 3955-

3962.

17. Liu, K.K., et al., *Endocytic carboxylated nanodiamond for the labeling and tracking of cell division and differentiation in cancer and stem cells.* Biomaterials, 2009. **30**(26): 4249-4259.
18. Moscariello, P., et al., *Unraveling In Vivo Brain Transport of Protein-Coated Fluorescent Nanodiamonds.* Small, 2019. **15**(42): 12.
19. Nie, L.Y., et al., *pH Sensitive Dextran Coated Fluorescent Nanodiamonds as a Biomarker for HeLa Cells Endocytic Pathway and Increased Cellular Uptake.* Nanomaterials, 2021. **11**(7): 11.
20. Hemelaar, S.R., et al., *Generally Applicable Transformation Protocols for Fluorescent Nanodiamond Internalization into Cells.* Scientific Reports, 2017. **7**: 7.
21. Tzeng, Y.K., et al., *Superresolution Imaging of Albumin-Conjugated Fluorescent Nanodiamonds in Cells by Stimulated Emission Depletion.* Angewandte Chemie-International Edition, 2011. **50**(10): 2262-2265.
22. Hebisch, E., et al., *Nanostraw-Assisted Cellular Injection of Fluorescent Nanodiamonds via Direct Membrane Opening.* Small, 2021. **17**(7): 12.
23. Ong, S.Y., et al., *Interaction of nanodiamonds with bacteria.* Nanoscale, 2018. **10**(36): 17117-17124.
24. San-Martin, C.R., et al., *Fluorescent nanodiamond labels: Size and concentration matters for sperm cell viability.* Materials Today Bio, 2023. **20**: 7.
25. Mzyk, A., et al., *Diamond Color Centers in Diamonds for Chemical and Biochemical Analysis and Visualization.* Analytical Chemistry, 2022. **94**(1): 225-249.
26. Martens, T.F., et al., *Intracellular delivery of nanomaterials: How to catch endosomal escape in the act.* Nano Today, 2014. **9**(3): 344-364.
27. Chu, Z.Q., et al., *Rapid endosomal escape of prickly nanodiamonds: implications for gene delivery.* Scientific Reports, 2015. **5**: 8.
28. Morita, A., et al., *Targeting Nanodiamonds to the Nucleus in Yeast Cells.* Nanomaterials, 2020. **10**(10): 10.
29. Schirhagl, R., et al., *Intracellular Quantum Sensing of Free-Radical Generation Induced by Acetaminophen (APAP) in the Cytosol, in Mitochondria and the Nucleus of Macrophages.* ACS Sensors, 2022. **7**(11): 3326-3334.
30. Rojas, S., et al., *Biodistribution of Amino-Functionalized Diamond Nanoparticles. In Vivo Studies Based on F-18 Radionuclide Emission.* ACS Nano, 2011. **5**(7): 5552-5559.
31. Yuan, Y., et al., *Biodistribution and fate of nanodiamonds in vivo.* Diamond and Related Materials, 2009. **18**(1): 95-100.
32. Zhu, Y., et al., *The Biocompatibility of Nanodiamonds and Their Application in Drug*

Delivery Systems. Theranostics, 2012. **2**(3): 302-312.

33. Vaijayanthimala, V., et al., *The long-term stability and biocompatibility of fluorescent nanodiamond as an in vivo contrast agent.* Biomaterials, 2012. **33**(31): 7794-7802.
34. van der Laan, K.J., et al., *Evaluation of the Oxidative Stress Response of Aging Yeast Cells in Response to Internalization of Fluorescent Nanodiamond Biosensors.* Nanomaterials, 2020. **10**(2): 13.
35. Hsu, T.C., et al., *Labeling of neuronal differentiation and neuron cells with biocompatible fluorescent nanodiamonds.* Scientific Reports, 2014. **4**: 11.
36. Mohan, N., et al., *In Vivo Imaging and Toxicity Assessments of Fluorescent Nanodiamonds in Caenorhabditis elegans.* Nano Letters, 2010. **10**(9): 3692-3699.
37. Fujiwara, M., et al., *Real-time nanodiamond thermometry probing in vivo thermogenic responses.* Science Advances, 2020. **6**(37): 9.
38. Hui, Y.Y., et al., *Single particle tracking of fluorescent nanodiamonds in cells and organisms.* Current Opinion in Solid State & Materials Science, 2017. **21**(1): 35-42.
39. Zhang, Q.W., et al., *Fluorescent PLLA-nanodiamond composites for bone tissue engineering.* Biomaterials, 2011. **32**(1): 87-94.
40. Yuan, Y., et al., *Pulmonary toxicity and translocation of nanodiamonds in mice.* Diamond and Related Materials, 2010. **19**(4): 291-299.
41. Lin, Y.W., et al., *Targeting EGFR and Monitoring Tumorigenesis of Human Lung Cancer Cells In Vitro and In Vivo Using Nanodiamond-Conjugated Specific EGFR Antibody.* Pharmaceutics, 2023. **15**(1): 23.
42. Tseng, P.H., et al., *Identification of Two Novel Small Compounds that Inhibit Liver Cancer Formation in Zebrafish and Analysis of Their Conjugation to Nanodiamonds to Further Reduce Toxicity.* Advanced Therapeutics, 2019. **2**(12): 14.
43. Moore, L., et al., *Biocompatibility Assessment of Detonation Nanodiamond in Non-Human Primates and Rats Using Histological, Hematologic, and Urine Analysis.* ACS Nano, 2016. **10**(8): 7385-7400.
44. Fujisaku, T., et al., *pH Nanosensor Using Electronic Spins in Diamond.* ACS Nano, 2019. **13**(10): 11726-11732.
45. Neumann, P., et al., *High-Precision Nanoscale Temperature Sensing Using Single Defects in Diamond.* Nano Letters, 2013. **13**(6): 2738-2742.
46. Wu, Y.K., et al., *Nanodiamond Theranostic for Light-Controlled Intracellular Heating and Nanoscale Temperature Sensing.* Nano Letters, 2021. **21**(9): 3780-3788.
47. Nie, L., et al., *Quantum monitoring of cellular metabolic activities in single mitochondria.* Science Advances, 2021. **7**(21): 8.
48. Sigaeva, A., et al., *Diamond-Based Nanoscale Quantum Relaxometry for Sensing*

Free Radical Production in Cells. Small, 2022. **18**(44): 13.

49. Morita, A., et al., *Detecting the metabolism of individual yeast mutant strain cells when aged, stressed or treated with antioxidants with diamond magnetometry.* Nano Today, 2023. **48**: 13.
50. Reyes-San-Martin, C., et al., *Nanoscale MRI for Selective Labeling and Localized Free Radical Measurements in the Acrosomes of Single Sperm Cells.* ACS Nano, 2022. **16**(7): 10701-10710.
51. Wu, K.Q., et al., *Applying NV center-based quantum sensing to study intracellular free radical response upon viral infections.* Redox Biology, 2022. **52**: 11.
52. Schirhagl, R., et al., *Diamond Relaxometry as a Tool to Investigate the Free Radical Dialogue between Macrophages and Bacteria.* ACS Nano, 2023. **17**(2): 1100-1111.
53. Schirhagl, R., et al., *Nitrogen-Vacancy Centers in Diamond: Nanoscale Sensors for Physics and Biology*, in *Annual Review of Physical Chemistry, Vol 65*, M.A. Johnson and T.J. Martinez, Editors. 2014, Annual Reviews: Palo Alto. p. 83-105.
54. Martinez, F.P., et al., *Nanodiamond Relaxometry-Based Detection of Free-Radical Species When Produced in Chemical Reactions in Biologically Relevant Conditions.* ACS Sensors, 2020. **5**(12): 3862-3869.