Fluorescent nanodiamonds quantum sensing free radicals in bio-samples

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General Discussion

Chapter 5

Free radicals play a crucial role in numerous pathogenic conditions including the diseases responsible for most deaths worldwide (such as cancer, cardiovascular disease, infections, Alzheimer's disease, Huntington's disease). Right now, very little is known about where and when radicals are built, how they work, or which ones play a role. Also, their short lifetime and reactivity pose a problem for the current methods.

Thus, measuring free radicals has drawn researchers' attention over the years. Currently, there are several methods available to detect free radicals inside cells, including direct and indirect methods. However, none of them can determine the location and identity of radicals at high time resolution.

FNDs have great potential in biomedical and biological applications due to their remarkable physicochemical characteristics and low toxicity. The diversity of functional groups on the diamond surface enables them to bind bioactive molecules and allows surface engineering (i.e for drug delivery or diagnosis). Their excellent optical properties make them efficient tools for imaging, or as biomarkers for biology. In this thesis, we make use of the Nitrogen-Vacancy (NV centers) inside FNDs to sense free radicals in cells or organelles.

NV centers can convert magnetic noise into an optical signal by changing their brightness according to the magnetic surroundings. Since optical signals can be read out very sensitively, this method is orders of magnitude more sensitive than conventional MRI. Due to these characteristics, diamond magnetometry allows us to detect free radicals (unpaired electrons) in a high spatial resolution and in real-time.

To fully utilize NV centers in diamond particles in free radical sensing, a diamond particle should be close to its target. In our case, this target was mitochondria for various reasons. Mitochondria are not only the cells' energy plants, but also play crucial roles in cell communication, cell signaling, maintaining cell function and the immune system. Additionally, mitochondria are vital organelles in redox signaling and dominant in free radical production. In **chapter 2**, we demonstrated using diamonds to detect mitochondrial free radicals both in single cells and isolated single mitochondria.

In this study, the biggest obstacle and the first step was to prove that diamond particles reached mitochondria. Macrophage J774 A.1 cells were used in this

work, this cell line plays vital roles in the first line of defense in the immune system. Thus macrophages were chosen in this study. To bring diamonds to mitochondria, antiVDAC2 antibodies were adsorbed to diamond particles. To prove that diamonds reached mitochondria inside macrophage J774 A.1 cells, we have first tried colocalization. However, after staining mitochondria with MitoTracker green we found that mitochondria are very abundant in these cells. With the resolution limits of confocal microscopy, mitochondria appear to be “everywhere” and the whole cell is bright. Two experiments were conducted instead: First, we tested the diamond uptake ability of macrophages. We found that the number of diamond particles per cell reached the maximum after 14h of incubation. We incubated antiVDAC2 antibodies adsorbed to FNDs (antiVDAC2-FND) with cells for different time points, then isolated mitochondria from cells, we found the diamond particles attached to isolated mitochondria after 14h incubation. We assume we targeted diamond to the mitochondrial surface by antibodies adsorbed diamond after 14h incubation.

We further tested and compared radical generation in macrophages, with bare FND and antiVDAC2-FND incubated with cells for 14h respectively. Our results show significantly higher radical generation when the nanodiamonds were attached to mitochondria (aVDAC2-FNDs), than when they were not (FNDs). This result indicates that coating with antiVDAC2 antibodies, causes diamonds to reach the mitochondrial surface. With these targeted diamonds, we next looked into free radical generation in both cells or isolated mitochondria in presence of triggers (different concentrations of Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)) or antioxidants (superoxide dismutase (SOD) and catalase (CAT)). Free radical generation in cells or isolated mitochondria was detected with targeted diamonds by T1. Our results show that T1 can follow free radical generation in mitochondria that were triggered by high concentration CCCP (10μM) or decreased by antioxidants. Cellular reactive species (ROS) were generally measured by fluorescence probes, such as Amplex ultra red. This is a well-established method for measuring mitochondrial H2O2. In this project were used for comparison with T1 measurements. Unlike T1 measurements, these probes measure the total amount of ROS in the cell, but not free radicals. They are also facing several difficulties in long term measurement due to their short photostability and thus are not suitable for long term measurements. Our results demonstrate that diamond magnetometry is a useful tool for long-term monitoring radical changes in real time in biological samples such as living cells or organelles (eg. mitochondria) compare to these traditional tools. In addition, we also excluded several potential factors which might influence T1 measurements in complex biological samples, such as temperature, pH and viscosity.

Diamond transport inside a cell can offer a lot of useful information for drug delivery or targeting. We have shown in **chapter 2** that a diamond can reach mitochondria in macrophages by using antibody coatings. However, most cells do not ingest particles as quick as macrophages, or in such great numbers. Thus, in **chapter 4** we investigated a strategy to increased uptake and introduced pH sensitivity. Hela cells do not readily to uptake diamonds in large numbers even after a quite long incubation periods around 8 hours. The low diamond uptake ability poses a problem for researchers who perform diamond related researchers. To this end, we used pHrodo green dextran coated FNDs to study their transport pathway in Hela cells. pHrodo green dextran has a positive charge. Thus it adsorbs on the negatively charged diamond surface. pHrodo green dextran coating, increased cellular uptake by 5.3 times compared to naked diamonds. With more diamonds inside Hela cells, we were able to investigate the diamond's fate inside Hela cells. The coated diamond particles aggregate slightly in cells, while there are no differences in size before and after coating without cells. For these aggregates we observed transportation in Hela cells. They follow the endo-lysosomal pathway and are excreted by exocytosis. There are emerging therapeutic delivery platforms using FNDs to enhance drug delivery[1] or release to specific targets[2]. For such applications it is important to understand and control the location of diamonds within cells.

While the previous chapters were concerned with cell lines, in **chapter 3** we performed the first measurements in primary human cells. Dendritic cells are in the first line of defense, they recognize and engulf pathogens, thus activate NADPH (NOX2), leading to massive superoxide anion generation and thus kill pathogens. Dendritic cells are antigen-presenting cells that link innate and adaptive immunity[3]. In this chapter, we investigated human primary dendritic cells' uptake of diamond, cell viability and subcellular location of diamonds. With diamonds located inside endo-phagosomes, we investigated NOX2 related production of free radicals. By using the NOX inhibitor- Diphenyleneiodonium chloride (DPI), or antioxidants- SOD and CAT, we confirmed that diamond magnetometry can map free radical generation in phagosomes in human primary dendritic cells. Primary cells exhibit several advantages over laboratory animals or cell lines. They are often closer to a clinical setting due to more-relevant morphological, physiological and biochemical properties, including receptors[4]. As discussed earlier in the general introduction, free radicals are related with several diseases. Thus, monitoring free radicals while biological samples are under stress might be an indicator for early phase diagnosis. The study of free radical detection in human primary cells takes our application one more step closer to clinical trials, and supports future investigation into novel therapeutic applications utilizing FNDs.

The study contributes to a clearer understanding of radical generation in biological samples. Diamond magnetometry has been widely used in physics, however, it is still new in biology. In this project, we built a connection between physics and biology application using diamond magnetometry.

Overall, diamond magnetometry is a useful tool in free radical measurements either in single living cells, single organelles or primary cells. With diamond magnetometry, free radical measurements can be done over time. This way it is possible to follow the same cell before and after an intervention (for instance by an inhibitor or trigger). However, at the moment, we can only measure the sum of all radicals, but can't distinguish which radical is generated. To achieve this goal, further steps are possible using the spectroscopic ability of magnetic resonance methods which requires more complex pulsing sequences.

References

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