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Free radical detection in living cells with relaxometry

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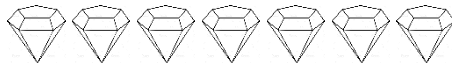
Chapter



7. General Discussion & Future Perspectives

The studies in this dissertation show the usefulness of fluorescent nanodiamonds FNDs in biomedical applications. They have gained popularity over the last few years, due to their unique optical properties and biocompatibility. These properties made them perfect sensors for free radicals. The main focus of this work is to measure free radical species in biological environment. Free radicals are involved nearly in every disease or pathogenic condition. However, there are currently no techniques which can both quantify and localize radicals with subcellular resolution. Thus, it hard to understand their exact role at the molecular level. The objective of this work is to achieve a better understanding of free radicals in biological environments by using diamond magnetometry as shown in **chapters 4 and 5**. This objective requires an optimization of the materials and experimental methods for magnetic sensing. In this chapter we discuss the findings of this research.

To measure free radicals and get meaningful information, FNDs need to be taken up by cells. The fate of FNDs was observed over time in **chapter 2** to have a better understanding of where these FNDs are located after cell division. Since we used yeast cells as a model organism to study the fate of FNDs, we had four possibilities of where FNDs will end up: First the particles stay with the mother cell, second moves to the daughter cell, third particles are excreted, or fourth particles are found in both mother and daughter cells. In addition to the fate of FNDs, we also monitored the particles movement pattern within the cell. This gave us a better understanding of the particle's diffusion and how much the particle moves. We found that in most of the cases the particles are found in daughter cells or are excreted. Moreover, we also observed that particles in yeast cells move less compared to other cell lines. This work opens the possibilities for future investigations of free radical production during cell division.

To have better control over our FNDs location inside cells in **chapter 3** we optimized our nanodiamonds with specific antibodies attached to the surface to achieve targeting. We chose nuclear pour complex (NPC) antibodies specific for yeast cells. This antibody has been chosen because of the key role the nuclear pore complex plays in transporting into the nucleus. Also, targeting the nucleus will give a better understanding of DNA damage once free radical measurements are done in future work. In this study we compare targeting success between bare-FNDs vs FNDs-Antibody. The comparison was based on measuring the distance between a particle and the nucleus. The measurements were done from 0 hours up to 24 hours after incubation. We found that the success rate of targeting was 70% when FNDs-Antibody was used compared to bare FNDs. It was observed that the longer the incubation time was the higher the percentage of targeted particles. This work could

be useful for labeling the nucleus or performing magnetometry measurements in the future.

In **chapter 4 and 5** we performed magnetometry measurements (T_1) to study the free radical production in living cells. The presence of free radicals in healthy cells is balanced and they play an important role in a cell's physiological processes such as cell signaling, immunity, cell death, and induction of mitogenic response [1,2]. We compared detecting free radicals by diamond magnetometry with detection based on fluorescent dyes. We found that diamond magnetometry has the advantage that it is biocompatible and measures the current concentration of free radicals. Thus, it is possible to stimulate cells and readout the dynamic changes in radical concentration in real time with nanoscale resolution. Fluorescent dyes on the other hand offer limited spatial resolution since they can diffuse throughout the cell, and measure the history of free radical production.

In **chapter 4** we investigated the production of free radicals in human umbilical vein endothelial cells (HUVECs) under different shear stress. Here shear stress plays a key role in free radical production in endothelial cells. Cells were incubated with FNDs in a microfluidic channel and exposed to laminar flow causing shear stress. We investigated free radical production at static conditions, during flow (2, 10, and 20 dyne/cm^2), and after flow . These parameters were chosen to mimic the shear stress those cells experience in the vascular system. To measure the radical production, we performed T_1 measurements and compared them with fluorescent dyes specifically for NO^* and O_2^*. We found that NO^* is much higher concentrated than O_2^{*-} which means that NO^* has a more dominant effect on our T_1 measurements as we measure the sum of all radicals. We found that T_1 slightly decreased during exposure to 2 dyne/cm^2. This means that there is a higher concentration of radicals. For 20 dyne/cm^2 we noticed an increase of the T_1 values which indicates a decrease in radical concentration. A flow of 10 dyne/cm^2 had no effect on radical production.

Moreover, we also performed a different set of experiments where we increased the flow rate gradually between 0 to 20 dyne/cm^2 every 30 minutes. Here, we found that by increasing flow rate every 30 minutes during an in total 4 hours experiment T_1 steadily decreased which means more radicals have been formed over time. This was expected due to the increase in NO^* concentration over time under shear stress.

In **chapter 5** we measured free radicals on boar sperm cells during the capacitation process (a process of sperm maturation) where they play a key role. So, the presence of free radicals in this case is important. We explore the affinity of

FNDs to sperm cells and sensitivity to detect radicals. To this end we used commercially available oxygen terminated FNDs. We found that both bare-FNDs and NH_2-FNDs were attached to the acrosome part. We also found that bare-FNDs were more sensitive to detect radicals than NH_2-FNDs with sensitivity of 76% and 66% respectively, as a result we decided to continue our work with bare-FNDs.

When capacitation was induced in sperm, we detected an increase in free radical production as expected. Using diamond magnetometry we were able to measure free radicals on single sperm cells. In addition to that, we altered our system by using different reagents to block free radical production or induce it. We blocked the production of free radicals from NOX5 using apocynin to proof that we are measuring capacitating. We found no significant differences in this experiment which means apocynin did affect NOX5 free radical production. Then, we blocked the free radical production in mitochondria using oligomycin. Once we induced free radical production using progesterone, we noticed that more radicals were formed, but after an hour the effect of progesterone stopped. In conclusion, diamond magnetometry has proven its usefulness to measure small changes in radical production on a single sperm cell level.

In **chapter 6** we explore the commercial aspects of diamond magnetometry. First, we talked about the pros and cons of this technique in comparison to other available techniques for free radical detection, and how this technique can be utilized commercially in different markets. Second, we talked about the possible markets to approach. We first focused on the drug testing market as it is a large market. However, we also show that there are other possibilities which we will explore such as cosmetics, and food industry.

Future Directions

Diamond magnetometry has proven its advantages but there is room for improvements like higher sensitivity, faster measurements. Additionally, more complicated pulse sequences are promising to allow differentiation of radicals. In the meantime, we used the T_1 pulsing sequence which is simple and measures the sum of all radicals within some nanometers from the NV centers. Other sequences might further increase the sensitivity. This has a potential to be applied in the future for diagnosis to differentiate between diseases and infections. Moreover, another challenge is to understand how other factors in a biological system are affecting our T_1 measurements. Such factors include temperature, electric field, or viscosity. Once these challenges are tackled, this will open more opportunities for collaborators and open the door to use diamond magnetometry in different fields not only biomedical applications.

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