Versatility of fluorescent nanodiamonds as free radical quantum sensors: from arthritis and metastasis to potential applications in heart diseases

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CHAPTER 1

General Introduction and Thesis Outline

Fluorescent nanodiamonds

Nanodiamonds (NDs) are carbon-based nanoparticles with sizes ranging from 5 to 500 nm, but most biological applications have been developed with fluorescent NDs (FNDs) ranging from 10 to 140 nm [1,2]. The first NDs were discovered in the Soviet Union during the 1980s as remnants of explosion tests [3]. Since then, the interest of the scientific community in these particles has grown steadily due to the application versatility derived from their chemical and fluorescent properties.

Several methods of obtention have been developed, including nanodiamonds produced by detonation (DNDs) [3] or from milling high-pressure high-temperature (HPHT) microdiamonds [4], as well as nanoparticles produced by chlorination of carbides [5], autoclaving supercritical fluids [6], plasma-assisted chemical vapor deposition (CVD) [7], ultrasound cavitation [8], laser ablation [9], ion irradiation of graphite [10], and electron irradiation of carbon 'onions' [11]. One of the most popular methods nowadays consists in the generation of suitable fluorescent nanodiamonds from HPHT raw microdiamonds. Briefly, the process starts with the electron irradiation of the raw material followed by an annealing step in vacuum under temperatures around 800 °C. Then two consecutive steps of high-energy ball milling are performed, which are known as micronization and nanomilling, respectively. Next, the milled diamonds are decontaminated by treatment with hydrogen fluoride and nitric acid (HF/HNO_3). Ultimately, a series of centrifugations and ultrafiltration render the final FNDs [4]. Logically, the final properties of the FNDs depend on the starting material and the process of obtention.

Properties and biomedical applications

The most upstanding property of FNDs is their fluorescence. Different atomic defects produced during the obtention processes in the nanodiamond lattice provide these particles with dissimilar emission spectra (Figure 1A). For instance, type Ib raw HPHT microdiamonds are produced in a few minutes from metal catalysts. In HPTH reactors, nitrogen (N) atoms provided by the solvent metal, the carbon source, and residual gas in the

reaction chamber, are incorporated into the growing crystals. During the irradiation step, high-energy electrons ‘hit’ and displace carbon atoms in the lattice of the crystals creating vacancies (V). The subsequent annealing process at high temperatures leaves the nitrogen atoms immobile (single Ns) while forcing the migration of the vacancies towards single Ns [4]. As a result, the so-called NV-center defects remain in the resulting nanodiamonds, providing them with a broad fluorescent emission spectrum in the far red. The fluorescent properties of FNDs are far superior to organic dyes since they do not suffer fluorescence blinking or photobleaching, which makes them ideal for real-time imaging. In this regard, Reyes et al. have recently interrogated the capacitation response of living boar sperms using FNDs [12].

Another important property of FNDs is their rich surface chemistry. Several chemical moieties are exposed on the diamond's outermost layer as a result of the treatments they undergo during the generation and purification processes. In general, according to their surface chemistry, FNDs can be classified as hydrophilic, mostly oxygen-terminated diamonds, or hydrophobic, hydrogen-terminated [13]. For instance, the cleaning step with oxidizing acids of nanodiamonds from HPHT raw crystals renders hydrophilic oxygen-terminated FNDs. Besides influencing the fluorescent properties of FNDs (Figure 1B), their surface chemistry makes them ideal platforms for functionalization with macromolecules. Sharmin et al. targeted FNDs to the nucleus and mitochondria of macrophages to study their response to the treatment with acetaminophen [N-acetyl-para-aminophenol (APAP)], one of the most widely used analgesic and antipyretic drugs [14]. They engineered FNDs with antibodies against VDAC2, a channel located in the outer mitochondrial membrane, and used a nuclear localization signal peptide (NLS) derived from the SV40 virus to produce coated FNDs directed to the nuclear pores. In another example, Wu et al. utilized the surface chemistry of FNDs and the biotin-streptavidin technique to produce diamond-virus conjugates and study the response of mammalian kidney cells to viral infections [15].

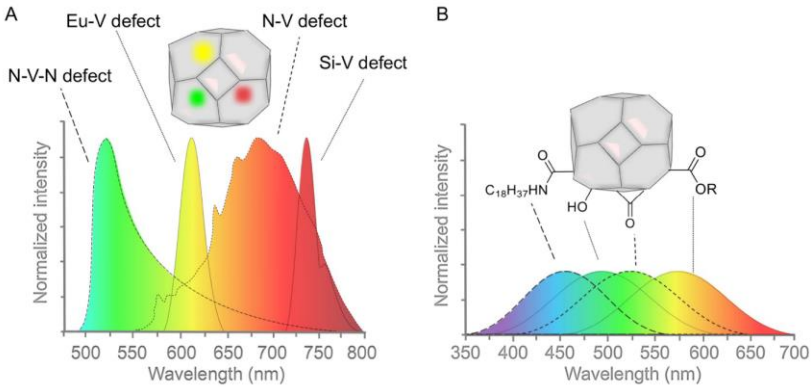

Figure 1: Fluorescent properties of NDs. A) The emission spectrum is characteristic of the type of atomic defect in the diamond lattice. NVN: nitrogen-vacancy-nitrogen, EuV: europium-vacancy, NV: nitrogen-vacancy, SiV: silicon-vacancy. B) The coating of the diamond surface (in this case a DND) with different chemical moieties influences its fluorescent properties. Octadecyl amine ($–CONHC_{18}H_{37}$), hydroxyl (–OH), ketone (–C=O), ester (–C=O–OR). Image taken from reference [16].

Despite the suitability of their superficial chemical groups for molecular grafting and the broad range of functionalization options, FNDs are also widely acknowledged as biologically and chemically inert nanoparticles. This property places them on top of other fluorescent nanoparticles such as quantum dots (QDs) in terms of biomedical applications. QDs, similar to FNDs, show robust fluorescent properties compared to organic dyes. Nevertheless, their extensive application in biomedicine has been hampered by the known toxicity of the heavy metals that form the core of these particles and the cytotoxicity of the compounds used to make their protective shell [17,18]. In contrast, FNDs have been successfully employed in a myriad of cell types, tissues, and organisms [16]. However, toxicity and, more importantly, biocompatibility assays are encouraged in almost every study in the field. This is especially relevant when expanding the application of FNDs to new biological systems of new biological questions where the process to be interrogated might be influenced by the presence of these particles.

Finally, the small diameter of FNDs together with their stable fluorescence makes them versatile sensors with an exceptionally high spatial

resolution, which can be exploited to explore processes at the subcellular level in the nanoscale range [12,14]. Nonetheless, careful selection of the FND size has to be made as the fluorescent properties and colloidal behavior change accordingly with this parameter. For example, NV-center oxygen-terminated FNDs show severe aggregation and precipitation in non-supplemented DMEM (nsDMEM) medium regardless of their size. This behavior complicates its use in certain biological settings that include nsDMEM, one of the most commonly used media in cell culture. In contrast, aggregation in FBS or complete medium (supplemented DMEM) is far less pronounced, especially for relatively big FNDs, *i.e.* 140 nm. Regarding fluorescence, the smaller the FNDs the less fluorescent. This is due to the number of NV centers in the diamond lattice. For instance, an individual 140 nm FND is about 4 orders of magnitude brighter than a single 10 nm particle based on the approximation that around 2700 particles of 10 nm equal the volume of a single 140 nm diamond [2]. Noteworthy, big nanodiamond volumes for more NV centers are not always the most suitable approach for certain biomedical applications. NV centers closer to the core of the particles can be insensitive to environmental changes 'perceived' by NV defects closer to the surface. Additionally, the size of the particle also influences its interaction with the biological system, e.g. the level of cellular uptake by clathrin-coated pits, vacuoles, and micropinocytic vesicles. Lastly, the biological system itself imposes another layer of complexity since, for example, different cell types can uptake particles of the same size at contrasting rates [19,20].

In the experimental chapters of this thesis (Chapters 2 and 3), we used 70 nm oxygen-terminated NV-center FNDs based on the fact that they offer a good ratio size-NV center number. Furthermore, they have been extensively employed by our group in a myriad of biological systems.

Fluorescent properties of NV-center FNDs and free radical detection

NV centers, among other diamond defects, offer a broad emission spectrum from red to near-infrared. This is especially useful because it allows their utilization with a wider number of detection filters when compared to EuV or SiV defects for example (Figure 1A). Additionally, NV

centers can be excited with green-yellow light, which has a low influence on frequent autofluorescence producers in cells such as NAD(P)H, flavins, and lipofuscins [21]. The negatively charged NV centers in FNDs exhibit a high fluorescence yield when excited with a green laser [22 , 23]. Their fluorescence lifetime is relatively long, i.e. around 20 ns, which allows proper discrimination between the FND fluorescent signal and the faster biological autofluorescence in tissues by using temporal gating [24]. Furthermore, NV-FNDs of around 90 nm can be detected with a good signal-to-noise ratio (S/N ratio) in common cell culture media like FBS, nsDMEM, and complete DMEM [2].

Besides their general fluorescent properties, NV centers show peculiar fluorescent features related to the quantum behavior of the six delocalized electrons that result from the juxtaposition of a vacancy to a nitrogen atom (Figure 2A). Upon laser excitation of the NV centers in the FND lattice, the delocalized electrons transition from the ground state to the excited state. After a certain time in the excited state, electrons will return to the ground state and emit a photon. The way electrons come back to the ground state differs and so does the intensity of the corresponding emitted photons. Figure 2B details the quantum process.

After turning the laser off, the re-accommodation (‘relaxation’) of the electrons within the ground state occurs, unmixing the single population at ms=0 (brighter state) into the initial two subpopulations ms=0 and ms=±1 (darker state) (Figure 3). Importantly, spontaneous relaxation is influenced by the magnetic surroundings of the NV centers in FNDs. The more magnetic noise around FNDs, the faster the re-accommodation, meaning a faster transition from the brighter to the darker state [26]. This quantum property of NV centers has been exploited in the past few years to sense free radicals as these molecules carry an unpaired electron that produces magnetic noise when moving inside cells [25,26].

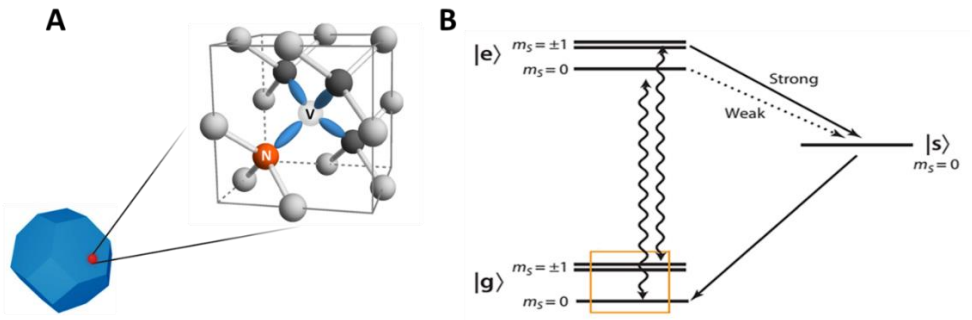

Figure 2: Quantum properties of NV centers. A) Schematic representation of an NV center. Blue ellipsoids represent the six delocalized electrons provided by the nitrogen (N) atom and the three carbon (C) atoms surrounding the vacancy (V). Image modified from [27]. **B)** Jablonski diagram depicting electron transitions between energetic levels in NV centers after laser excitation. Electrons excited from the ms=0 level of the ground state (g) return from the excited state (e) to the same level. Electrons excited from the ms=±1 level of the ground state, usually transition first from the excited state to the dark singlet state (s) before returning to the ground state, but in this case to the ms=0 level (solid straight arrows). The intensity of emitted photons is lower for ms=±1 (g) → e → s → ms=0 (g) transitions than for ms=0 (g) → e → ms=0 (g) transitions. Image modified from [28].

Free radical diamond-based quantum sensing is a novel technique that combines the excellent fluorescent, size, and biocompatibility features of NV-center FNDs to interrogate biological systems in terms of free radical generation. The technique is also known as diamond magnetometry and uses T1 relaxometry measurements to detect free radicals in dissimilar experimental settings [12,14,15,27]. By translating nanoscale magnetic noise into optical signals, this technique offers a uniquely high sensitivity.

During T1 relaxometry measurements, laser pulses separated by increasing dark times (laser off) are used to detect whether the NV centers in FNDs are in their brighter or darker state and how fast the switch between states has occurred (Figure 4A). Because of the unpaired electron in free radicals, the more free radicals moving around FNDs the more magnetic noise around NV centers. The more magnetic noise the shorter the transition time from the brighter to the darker state (Figure 4B). In summary, the amount of free radicals is inversely proportional to the relaxation (T1) times, i.e. more free radicals result in shorter T1 times [29].

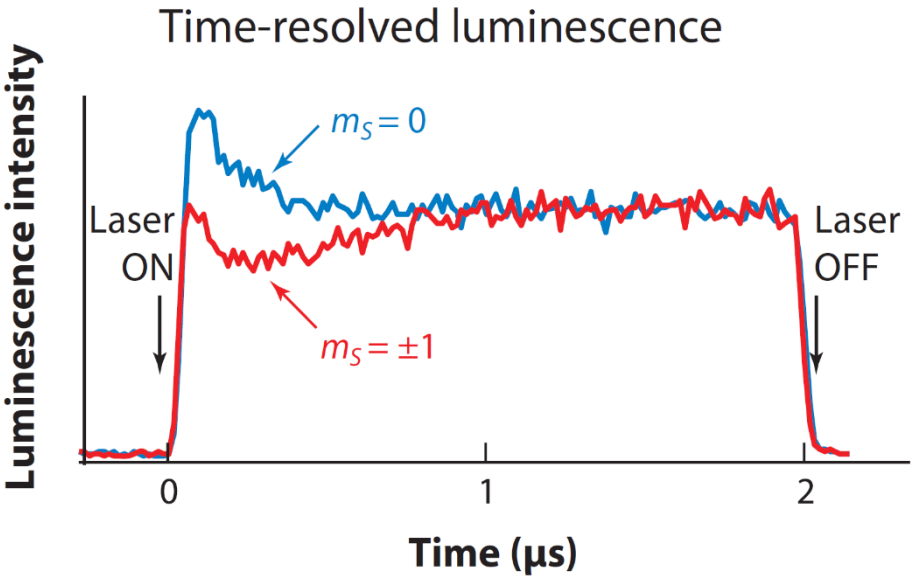

Figure 3: Optical contrast between NV center states. Different luminescence intensities are observed when excited electrons come from a single population in the ground state (ms=0, blue line) or two subpopulations (ms=0 and ms=±1, red line). The different intensities between these two states generate an optical contrast that dissipates after continuous laser excitation (overlapping between blue and red lines). Blue line: NV center previously excited ('pumped'). Red line: NV center excited for the first time. Image taken from reference [26].

Free radicals and oxidative stress: implications in Biomedicine

Free radicals are highly reactive molecules with at least one unpaired electron. They are a crucial group of reactive oxygen species (ROS) that have both physiological and pathological roles in biology. At physiological levels, they perform as signaling molecules and regulate pivotal processes. For instance, in knee chondrocytes, the production of free radicals, mainly by NADPH oxidases (NOXs), is fundamental for intracellular signaling mechanisms contributing to the maintenance of cartilage homeostasis as they modulate chondrocyte apoptosis, gene expression, extracellular matrix (ECM) synthesis and breakdown, and cytokine production [29]. Conversely,

beyond a certain threshold, they become hazardous for the cell as they rapidly generate other ROS (Figure 5A), producing oxidative damage on cellular constituents such as DNA, proteins, and lipids (Figure 5B) [30]. The accumulation of oxidative damage leads to a deleterious state known as oxidative stress in which an imbalance in the redox conditions of the cell prevents it from homeostatic functioning. Oxidative stress is a 'biomedical hub' since it is present in practically all 'worldwide killers', e.g. cancer, obesity, diabetes, and cardiopathies [31]. This is associated with the fact that human physiology is based on an aerobic metabolism where the production of free radicals and ROS is inherent to the utilization of oxygen in cellular respiration [31,32].

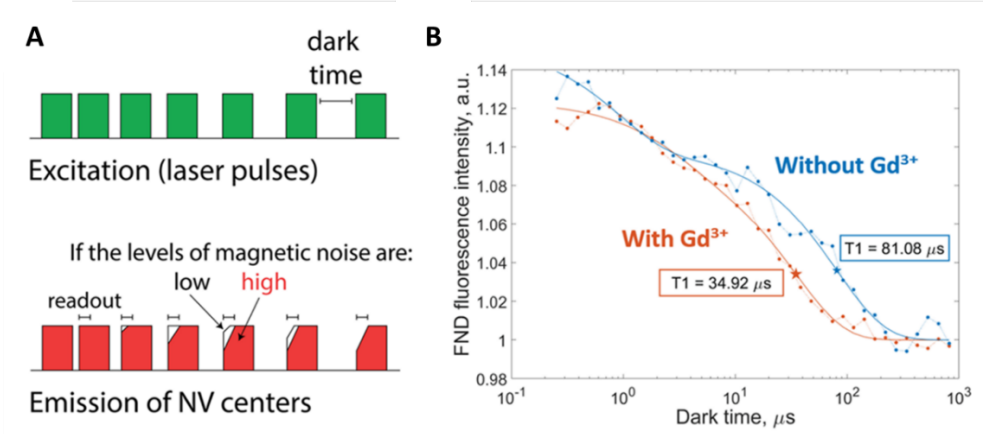

Figure 4: T1 relaxometry in diamond-based quantum sensing. A) Typical sequence of laser pulses to interrogate the state (brighter or darker) of NV centers in FNDs and the readout depending on the magnetic noise in the surroundings of the particles. The first laser pulse pumps all electrons to the ms=0 (g) level (brighter state). The interrogation of the fluorescence intensity with later pulses shows if the magnetic noise is low (slow relaxation to the darker state) or high (fast relaxation to the darker state). **B)** Relaxation (T1) times of an ensemble of NV centers in the presence (red line) or absence (blue line) of the paramagnetic ion gadolinium (Gd3+). Image taken from reference [32].

Importance of free radical detection

Currently, the scientific community is armed with a plethora of assays and commercial kits to interrogate the oxidative status of different biological systems. Nevertheless, a common impediment to the progress of knowledge

is the interpretation and communication of results when some scientists are unfamiliar with the complexities of ROS. The term ROS is often discussed as one molecular entity while in fact refers to radical and non-radical species. Additionally, some of them contain nitrogen besides oxygen and are known as reactive nitrogen species (RNS). Examples of free radicals are the superoxide radical anion ($O_2^{\bullet -}$) and the hydroxyl radical ($OH^{\bullet}$). Hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are part of the group of non-free radical ROS, and examples of RNS are peroxynitrite ($ONOO^-$/ONOOH) and the nitrogen dioxide radical ($NO_2^{\bullet}$). The dissection of ROS into different subgroups is not a matter of nomenclature but of our ultimate understanding of redox biological processes. Different ROS species show dissimilar features in terms of their lifetime, subcellular compartmentalization, target molecules, etc. [33]. Therefore, the implementation of novel approaches with specific sensitivity for certain ROS groups (e.g. free radicals) and the possibility of targeting the detection to subcellular locations represent a major need in the field of Redox Biology.

Among the challenges of detecting free radicals in biological systems are their extremely short average lifetime ranging from 10^{-9} to 10^{-6} sec, their concentration on the nanomolar scale, and their multiple intracellular sources and locations [33,34]. By using NV center FNDs for quantum detection of free radicals, diamond magnetometry overcomes these challenges, providing a versatile platform that can be adapted to dissimilar biological settings. It combines real-time measurements at a single-cell level and subcellular resolution with high specificity and sensitivity for the free radical populations of ROS.

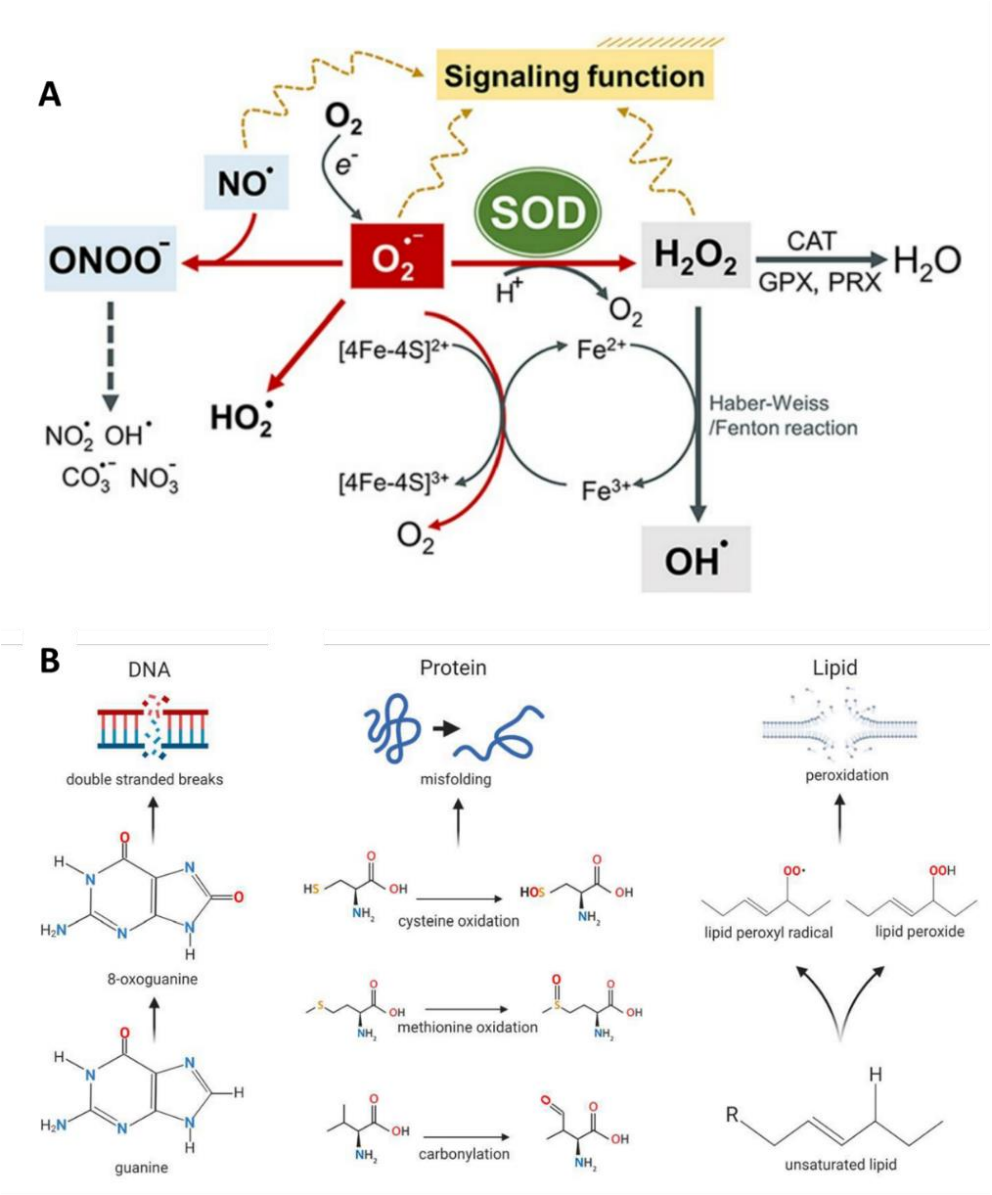

Figure 5: Free radicals and ROS as key players in cellular homeostasis. A) Common reactions leading to ROS generation from free radicals. Image taken from reference [35]. **B)** Free radical- and ROS-mediated damage to cellular components. Image taken from reference [31].

Thesis objectives and outline

This thesis's general aim was the implementation of diamond-based quantum sensing of free radicals in two *in vitro* settings of increasing biomedical relevance (Chapters 2 and 3). Noteworthy, in these experimental chapters, we paved the ground for future applications of the technique where the detection of free radicals can be targeted to specific subcellular locations. Functionalization of the FNDs and further standardization of the experimental conditions must be performed to accomplish this goal. On the other hand, we also explored knowledge gaps in the intersection between the fields of Redox Signaling and Mechanobiology (Chapter 4), where the application of diamond magnetometry could be pivotal to unraveling new mechanisms related to cardiac diseases.

In this thesis, we focused on three major diseases, *i.e.* arthritis, cancer, and cardiovascular diseases, that are often displayed as comorbidities in a high number of patients. Several studies have focused on the greater risk associated with suffering one of them and the subsequent appearance of the others. For instance, rheumatoid arthritis patients show a higher risk of developing various site-specific cancers compared to the general population, while survivors of most site-specific cancers have increased medium- to long-term risk for one or more cardiovascular diseases [36,37,38]. A common factor in these three diseases is the development of an oxidative stress condition in the cells and tissues of the patients. Therefore, the implementation of novel techniques such as diamond-based quantum sensing can contribute to the understanding of the connections between these three different pathologies where free radicals might play a crucial role in the risk of developing one from the precedents of the other.

The objective of **Chapter 2** was the interrogation of the free radical load in synovial fluid samples from osteoarthritis and rheumatoid arthritis patients. We additionally evaluated the response of these samples in terms of free radical generation upon treatment with piroxicam, a common nonsteroidal anti-inflammatory drug. This study represents the first application of the technique in clinical samples.

The objective of **Chapter 3** was the measurement of free radical levels during the migration of highly metastatic triple-negative breast cancer cells. Furthermore, we performed a thorough biocompatibility assessment of the specific particle-sample system under study by reporting features of intracellular diamond dynamics and the cell's migratory potential. This study highlights the importance of measuring free radicals as a ROS subgroup to unravel the complexities of redox-dependent cellular processes such as migration.

The objective of **Chapter 4** was the identification of potential applications of diamond magnetometry to study redox signaling pathways connected to mechanotransduction in cardiac cells. Here we summarized the importance of free radicals and ROS for heart functioning and development as well as for the pathogenesis of cardiac diseases. A holistic and hierarchical view is provided ranging from the subcellular level to aging and lifestyle as factors affecting the whole organism.

Altogether, this thesis contributes to the understanding of free radical biology and expands the applications of diamond-based quantum sensing in the biomedical field.

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