Biomedical Applications of Nanodiamonds in Microbiology

Norouzi, Neda

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Chapter V

General Discussion

The studies in this dissertation represent stepping stones toward the potential applications of nanodiamonds in a microbiological environment. Different ways of synthesizing nanodiamonds lead to diamond materials with different sizes, purities, and entirely different properties, which are discussed in more detail in **chapter 1.** A clear distinction should be made between milled fluorescent nanodiamonds (FNDs) and detonation nanodiamonds (DNDs), which also have an expanding range of potentially interesting applications in biology and materials science. The usefulness of FNDs as sensors for detecting free radical species in bacteria as well as DNDs potential as antibactericidal coating have been considered in this thesis. In this chapter, the findings have been discussed, specifically in light of the mission of our research team.

5.1 Free radical detection in microbiological systems

Over the last decades, the role of free radicals in biological processes has become more and more recognized. Several techniques are currently available to detect free radicals indirectly and directly in a cellular environment. Free radicals can be measured indirectly by their effect on the cells (e.g., DNA damage [1] or lipid peroxidation [2]) or via quantitative polymerase chain reaction (PCR) for enzymes involved in stress responses. Direct methods alternatively rely on the interaction of the radicals with fluorescent dyes as detector molecules. However, most of these dyes react with all kinds of reactive oxygen species (ROS). Since non-radical species are more abundant, the signal is usually dominated by these. Even the ones that are radical specific have certain flaws. There are also a few modern dyes with specificity for certain molecules which have already been used to follow ROS production in bacteria. [3,4] Often the dyes are somewhat toxic and can diffuse away from where they were created, which reduces spatial resolution. [5,6]

All of these approaches have their advantages, however, they possess a set of drawbacks, such as relatively low selectivity, and low sensitivity. Basically, most of the available methods involve the reaction of the sensor with the radical species of interest, and potentially interfere with its natural function. Moreover, the methods are destructive, and some prior knowledge is required about which pathways are triggered by certain radicals. However, very little is known about this factor due to a lack of methods to assess radical

formation at the nanoscale by giving spatial information. While diamond magnetometry, which emerged from the quantum information field, is already established in physics, it is entirely new in the biomedical areas. Diamond magnetometry based on a diamond defect, the nitrogen-vacancy (NV) center, does not react with radicals and one can read out a local magnetic resonance by measuring the fluorescence of the defect since their fluorescence strongly depends on magnetic fields in its surrounding. This new technique has already been successfully used in physics to measure magnetic vortices, [7] fields caused by nanostructures [8] or molecules on the diamond surface. [9–11]

Relaxometry, a specific mode of diamond magnetometry, is also extremely sensitive and selective to free radicals. The unique properties can be advantageous if one is interested specifically in the free radical species generated *in situ*, inside the cells. There are also a few articles bio-applications of FNDs as nanosensors to free radical detection in the biological environment, [12,14] however, primarily focused on Eukaryote cells. Recently, we have demonstrated that it is possible to measure free radical formation in living yeast cells and radical production from mitochondria. [12,13]

Bacteria and their resistance to antibiotics are among the biggest health concerns to date. Free radical generation occurs in all classes of antibiotics during the killing process regardless of the antibiotic's working mechanism. Thus, this is an important factor to assess. This work is the first demonstration of relaxometry in assessing antibiotics or their interaction with bacteria (**chapter 3**). Relaxometry, which allows measuring the radical response on a single bacteria level, offers a powerful tool to gain insights into this important process. However, this method, as any novel technique, requires that certain things are considered when applying it to a complex system, such as the optimization of the materials and experimental methods for magnetic sensing.

Nanodiamonds are promising candidates for biomedical applications, due to their high biocompatibility as compared to other (carbon-based) nanomaterials.[15,16] To validate the use of nanodiamond applications in microbiological systems, determining their lack of toxicity and biocompatibility in interaction with bacterial cultures is needed. In our earlier publication toward this objective, we consider milled HPHT

nanodiamonds' interaction with Gram-negative and Gram-positive bacteria for the first time. [17] The results showed that for a Gram-positive strain, these nanodiamonds' presence leads to a sharp reduction in colony-forming ability under optimal conditions. Whereas, no significant adverse impacts of milled ND for a Gram-negative strain was observed. It was shown that the colony forming ability of *S. aureus* ATCC 12600 was sharply reduced when exposed to concentrations of 10–100 mg mL^{-1} of milled nanodiamonds in phosphate buffered saline. [17] However, observations made in experiments with higher ND concentrations, or in DI water as a suspension medium did not yield results consistent with a 'contact killing' model. Furthermore, metabolic rates were not reduced under conditions that significantly reduced the colony count. Therefore, killing on contact is deemed an unlikely mechanism. In contrast, the effects of NDs on Gram-positive strain (*S. aureus*) viability were found to depend on many factors, including the concentration and size of nanoparticles, the suspension medium, and incubation time. Therefore, the biocompatibility assessment of nanodiamonds using different bacterial models in medium was subject of **chapter 2**.

Our publication in **chapter 2** [18] is a systematic experimental study of different strains, nanodiamond sizes, and surface chemistries in bacterial growth medium. We identify aggregation as an important factor in reducing the *in vitro* colony forming ability of *S. aureus* when exposed to milled nanodiamonds. Most notably, particle aggregation behaviour and bacterial clumping are considered to explain reduced colony counts in the presence of FNDs, which can be wrongly interpreted as a bactericidal effect. Furthermore, it might be an explanation why apparent contradictions are abundant in the literature on this topic. Throughout these experiments, it became clear that the apparent "antibacterial effect" of nanodiamonds depends strongly on the type of material used, its surface chemistry, the suspension medium in which the experiment is performed, the bacterial strain and exposure time. The experiments show that no mechanism can be linked to a specific material property, but prove that aggregation and bacteriostatic effects of nanodiamond attachment play a significant role in the reported results.

This finding formed a motivation to apply nanodiamonds as fluorescent sensors, since it has been shown that nanodiamonds were not damaging the cells in any way. Thus, they fulfil two desired conditions for proceeding with

sensing experiments in living cells: they are biocompatible to the cell and can be immobilized on the cell wall. Therefore, exploring the potential use of FNDs as sensing probes for e.g. magnetic noise of free radical molecules in *S. aureus* was aimed for in **chapter 3**.

While mammalian cells may ingest nanodiamonds via the endocytosis pathway, bacteria lack this reliable way to endocytose nanodiamonds. Therefore, detecting free radicals in bacteria using nanodiamond-based relaxometry seems challenging. The consequence of this is that the use of diamond and nanodiamond sensing methods stays limited to the extracellular environment for now.

As Diamond magnetometry is based on optical readout and heavily affected by the optical properties of the samples, the sample should be transparent enough to allow for the excitation of the probe and the detection of the probe's emission. Consequently, to avoid fluorescent background from the components of the bacterial growth medium and avoid growing a thick layer of bacteria, phosphate buffered saline (PBS) was used as a medium in which T1 relaxometry is performed in **chapter 3**.

We investigated the possibility of free radical detection in bacteria during stress using the new approach of relaxometry. We use the antibiotic Vancomycin (in different concentrations) and UV irradiation as stressors. Based on the literature, it was hypothesized that exposure to antibiotics induces ROS production in bacterial cells. It is also well established that bacteria are susceptible to UV irradiation which leads to structural cell damage, by providing favorable conditions for producing highly harmful free radicals. FNDs can sense the spin noise generated by free radicals in their surroundings and thus allow nanoscale T1 relaxometry. The differences observed in T_1 for UV-treated (UVC) and antibiotic-treated samples (at 20 and 340 μg mL^{-1}) were significant at the beginning ($p<0.001$), however, at the end of treatment, T_1 remained constant (probably due to saturation,, below this concentration our equipment is unable to differentiate shorter T_1 values since the minimal distance between pulses we can generate is reached). This could be resolved by using equipment with a faster pulse rate. In general, our relaxometry results are consistent, and T_1 decreasing was continuous after adding antibiotics. UV-treated cells show slightly higher T_1 and a significant decrease in T_1 was measured after 1 hour of treatment.

The results of the present study confirmed that the lack of FND uptake by bacterial cells did not prevent relaxometry application in microbiology, providing a valuable strategy for free radical detection. Since the ND is on the surface of the bacterial cells, the increase in free radicals might be due to the lysis/destruction of the bacterial cell wall by antibiotics or within or through the cell wall. As this reduction is dose dependent, relaxometry allowed us to differentiate between free radical levels in different antibiotic concentrations and UV treatment.

The results of this study demonstrate that relaxometry can be used to perform a measurement before and during the reaction on the exact same bacterium and exact same particle similar to what has been shown in other cell types. [13,19] Additionally, we have shown that the sensitivity of relaxometry is sufficient to detect the free radicals. [19]

Moreover, free radicals can be stabilized by so-called spin traps, which again chemically react with the radicals. The accumulating spins can then be read out by using electron spin resonance (ESR) techniques. Recently, ESR/spin trapping was examined as an approach to monitor the intracellular and extracellular free radical formation in *S. aureus* while treating with antibiotics by Wang et al. [20] It should be mentioned that ESR is relatively insensitive and requires relatively high concentrations of spin traps. They could detect the extracellular free radicals formed in broth medium through Fenton reaction and radical formation in the presence of H_2O_2 and iron. However, assessing the amount of intracellular free radical inside the *S. aureus* cells is still not accessible using the ESR/spin trapping approach. Both ESR and fluorescent dyes measure the history of the sample rather than the current state and are thus complementary to proposed magnetometry technique we present in **chapter 3**.

It is known that T1 relaxation of nitrogen-vacancy centers can be affected by other paramagnetic species. Thus, the paramagnetic form of iron might have an influence on T1. However, since we did our experiments in PBS, we did not include iron compounds as seen by Wang et al. [20] in electron spin resonance (ESR) spectroscopy. Wang *et al.* have also measured the internal iron concentration of *S. aureus* with and without 6 h of antibiotic treatment. They did not find any significant differences in iron concentration in the supernatant from antibiotic treated compared to untreated samples. Thus,

we do not expect significant iron release by using higher concentration of vancomycin.

The use of relaxometry has provided reasonable sensitivity, spatial resolution and temporal resolution for free radical generation in biological environments. In principle, relaxometry offers a very high spatial resolution which means that NDs report on their immediate environment (10-20 nanometers from the particle's surface). In practice, however, the setup used for this thesis is based on confocal microscopy which does not allow us to use this unprecedented resolution. Consequently, the resolution is limited which might be resolved by future optimization and various control studies over the nanodiamond's intracellular localization. The temporal resolution of our technique can be high on the order of milliseconds-seconds. However, further optimization of the sensing protocols can make it possible to detect the changes in intracellular concentrations of free radicals on a very short time scale.

To summarize, diamond is a versatile material for applications in biomedical engineering. NV centers in nanodiamonds can be used for sensitive detection of free radicals in biology. However, one needs to adjust and optimize the protocols to achieve the best resolution and highly targeted measurements. In this work we show that nanodiamonds of median sizes up to 125 nm inhibit proliferation of gram positive bacteria in suspension due to their tendency to aggregate individual cells (**chapter 2**). Furthermore, since no toxicity was observed, these results encourage further steps toward the development of nanodiamond magnetometry in microbiology. We introduced relaxometry with nanodiamonds as a promising, sensitive, and valuable tool to detect free radical generation induced by various stress conditions on the surface of bacterial cells of a Gram-positive pathogen model (**chapter 3**). We report here that exposing bacterial cells to stress conditions leads to free radical generation. This radical response is also dose-dependent. We further observed the dynamics of radical generation at the single-cell level. This kind of information is valuable for assessing the working mechanisms of bacterial killing as well as the formation of resistance to antibiotics. Therefore, nanodiamond-based magnetometry can be a very efficient tool, complementing other techniques.

5.2 Diamond Thin Films

Biofilm formation on a material surface due to bacterial adhesion is a severe problem in health and economics. [21] In many applications, such as the marine environment or food industry, biofilm formation can be a part of a major factor in industrial process control or economic loss. [22,23] In the human body, bacterial adhesion and biofilm formation can also cause serious health problems since such infections are challenging to treat, especially biomaterial-associated infections. [24]

Biofilm formation is initiated by bacterial adhesion to a substratum surface. Upon adhesion, bacterial cells produce a viscoelastic Extracellular Polymeric Substance (EPS) matrix consisting of polysaccharides, proteins, nucleic acids and lipids. The EPS production as well as the adhesion, serves as a survival strategy for the microorganisms against biological, mechanical and chemical stresses.

Some methods of treatment and prevention including antibiotic therapy have been considered a solution to mechanically remove biofilms in biomedical applications. However, with the rise in antibiotic-resistant bacteria, a high demand for anti-bacterial, anti-fouling, and bactericidal materials that are not based on antibiotics is also increasing. [25] Accordingly, different approaches have been developed to provide infection-resistant biomaterials such as cationic or polymeric coatings. [26] However, the antibactericidal efficacy of such release coatings might be decreased over time.

Recently, mechanically or chemically engineering of surface properties has been explored as a promising approach to directly repel bacteria through engineered roughness or hydrophobicity. Among the engineered surfaces, nanostructured surfaces are new. [27,28] However, their possible merits as anti-adhesive surfaces in general need to be explored further. Hence, it would be a logical scheme to explore the new coating on top of a mechanically and chemically engineered nanostructured surface which would yield a unique possibility of minimal contact between bacteria and surface, and may keep bacteria in their antibiotic-susceptible state (planktonic).

Black diamond (nanostructured black silicon surface with a diamond coating) has previously been proven to kill Gram-negative bacteria on

contact. Through the last chapter of this thesis (**chapter 4**), the antiadhesive properties of several bD and bSi surfaces (with F- or H- surface termination) are evaluated with two pathogenic Gram-positive bacteria (*staphylococcal* EPS-producing and non EPS-producing strains) associated with biomaterial associated, infections including biofilm formation. Preliminary data shows that both Gram-positive bacteria are repelled by black diamond surfaces, and in the case of fluorine-terminated surfaces up to 50% of *S. aureus* and 74% of *S. epidermidis* are killed on contact. However, caution is needed if the surfaces are exposed to conditions that support biofilm growth for extended periods of time. Biofilm formation after 24 hours showed that while all surfaces outperform glass over the long-term (24 h), diamond coated surfaces of fluorine and hydrogen-terminated surfaces significantly inhibit biofilm formation. In conclusion, fluorine and hydrogen diamond-coated surfaces with and without nano-needles have repelling, bactericidal and biofilm-inhibiting effects on Gram-positive bacterial strains and are promising antimicrobial surfaces.

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