Fluorescent Nanodiamonds for cell tracking and nanoscale temperature sensing in breast carcinoma

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Abstract

Fluorescent nanodiamonds (FNDs) have recently developed into a key element in the interdisciplinary fields between biology, chemistry, physics and materials sciences. The FND is a promising nanoprobe, owing its properties to a fluorescent defect, the so-called nitrogen-vacancy (NV^-) center. This NV^- center has a number of impressive optical and magnetic properties, for instance emitting bright fluorescence at 685 nm. The produced fluorescence has excellent photo stability. The past decade, studies confirmed that FNDs are also biocompatible and nontoxic. Altogether, these features make FNDs a potential candidate for long term imaging *in vivo* and got them up for debate in different fields of medicine. One of those fields is radiotherapy, which is an important component of anti-cancer treatment. Some patients do not respond to radiotherapy and up to now clinicians are unable to predict which patients have high risk at reappearance after therapy. In this review, breast cancer will be used as a model for introducing FNDs as a marker for bio-imaging. Breast cancer is the most common cancer affecting women from all over the world, therefore early detection and more knowledge about this type of cancer will provide a brighter future for these women. This paper provides a summary of the used 'triple assessment', which includes clinical examination, imaging and needle biopsy, the cancer therapy and more knowledge about the tumor's microenvironment. Additionally, the problem of targeting the DNA damage is mentioned and in which manner more knowledge about this can be obtained. This will involve long-term cell tracking and nanoscale temperature sensing using FNDs.

Introduction

A useful tool for life science research nowadays is fluorescence microscopy, it is a noninvasive way to visualize biological processes. In the fluorescence field, nanoscale carbon materials such as Fluorescent Nanodiamonds (FNDs) hold great promise for biotechnological and biomedical applications. In this pure carbon material, a defect is applied, -two carbons are replaced by one empty spot and one Nitrogen. This is called the negatively charged nitrogen-vacancy center (NV^- center)[1]. The NV^- center absorbs green light, which is emitted after the electronic transition from the ground state to the excited state, $^3A \rightarrow {}^3E$ (as seen in Figure 1)[2]. The emission band peaks at 685 nm with a high fluorescence emission efficiency. The produced fluorescence emission is stable, which means that the fluorescence is undergoing neither photo bleaching nor photoblinking[1]. Bleaching is an irreversible process where the molecules change due to light exposure and will eventually be destroyed. While blinking is a reversible process in which the fluorophore alternates between a light and a dark state, so the fluorophore loses its intensity of fluorescent light in the dark state[3].These properties allow for the detection of single NV^- centers.

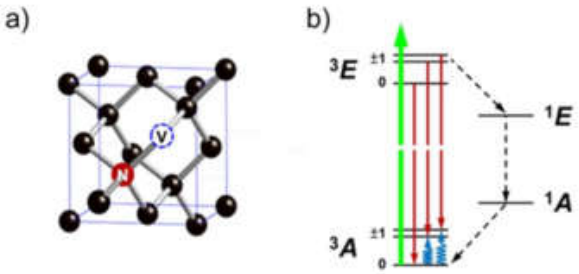

Figure 1. A. Structure of a NV^- center in a diamond. **B.** The electronic transition diagram (adapted from Hsiaou et al., 2016[2])

All of these features make FNDs a potential candidate for long term imaging *in vivo.* Several biocompatibility studies have been done *in vitro* with different types of cell lines and showed that FNDs are the least toxic of all tested carbon-based nanomaterials. In addition, FNDs after insertion do not cause any cytotoxic effects on the proliferation and differentiation of cells[4]. The past decade there have been a few studies on the biocompatibility and bio distribution- of FNDs *in vivo* in mice and nematodes[1,5]. In both studies, the results confirmed that FNDs are biocompatible and nontoxic and supporting the suggestion it could be used in whole organisms as a fluorescent tag or for delivering medicines[5].

Other fluorescent nanoparticles can be manufactured with wet chemistry methods, but FNDs can only be synthesized under extreme physical and chemical conditions. Due to the harsh conditions required to make FNDs, the process of fabrication is rather technically difficult but well established. Although, researchers in the field are still improving the diamonds by working toward using surface-functionalized Nanodiamonds for bio imaging, quantum sensing and drug delivery[2].

Because of these promising properties, FNDs are up for debate in different fields of medicine, including radiotherapy. Radiotherapy is an important component of anti-cancer treatment. Unfortunately, some patients do not respond to radiotherapy, and with current knowledge clinicians are unable to predict which patients have high risks at reappearance after radiotherapy. The ability to predict the outcome of radiotherapy would be a valuable aspect for various kind of reasons, now it is based on clinical parameters, for instance grade and tumor stage. Combining these parameters has led to a database with publicly available models for several cancer types (www.predictcancer.org). Despite that, there is a variation in response between patients with identical clinical characteristics so the models have to be improved. By adding blood-based, DNA-based or imaging biomarkers, the models can be upgraded and are able to guide clinicians at their decision- making[6].

For this review, breast carcinoma will be used as a model for introducing FNDs as a marker for bio-imaging. Breast cancer is the most common cancer affecting women from all over the world. Therefore early detection and treatment of this type of cancer will provide a brighter future for these women[7]. There has been considerable progress in the non-invasive functional and molecular imaging. Where the tools for treatment based not only on morphological criteria but also on biological information such as hypoxic status, metabolic and proliferative activity are becoming available. Development, validation and integration of imaging biomarkers using CT, MRI and PET to improve radiotherapy are therefore an important topic in clinical research[8].

This paper will discuss the current understanding of use of FNDs as a biomarker for cell tracking and nanoscale temperature sensing in mammary carcinoma. Also the current state of breast cancer research and the tumor's microenvironment will be described. Although much is known about mammary carcinoma and FNDs individually, the use of a FND as a biomarker *in vivo* is rather unacknowledged. Here, insight will be provided about the combination of the two and the future perspectives.

Breast cancer research

The second most common type of cancer is breast cancer. It is the most frequent cancer affecting women with an estimated 1.67 million new cancer cases diagnosed in 2012, which is 25% off all cancers that year. Breast cancer is the most frequent cause of cancer death in women in less developed regions, it also is the second cause of cancer after lung cancer in well-developed regions[9]. Prediction of behavior of breast carcinoma by the use of prognostic indicator, such as tumor size and morphological features, is of high importance. Because this type of cancer is not a single disease entity and does not behave in standard fashion, there is a broad arrangement of treatment choices which include surgery and radiotherapy[10].

Current screening techniques

In 1968 the World Health Organization made the importance of breast cancer screening very clear. This formed the basis for the present breast screening program which detects a possible tumor before it becomes clinically apparent[10]. In the Netherlands it is a population based screening, in which women after the age of 50 until 75 receive an invitation every two years for a breast examination[11]. Mammography is the current standard for the breast screening internationally, although it is well tolerated by women, limitations exist. It has an overall sensitivity of 85%, only for women with dense breast tissue the sensitivity decreases to 68%. Dense breast tissue is a relevant problem for screening, because relatively the breasts consist of more glandular tissue than fat tissue. In the united states alone, 50% of the women fall into the category of having dense breasts[7,9].

Other techniques have been employed and are still used now to overcome the limitations of mammography and to improve breast cancer diagnosis. The most recent modality that allows for reconstruction of a 3D image of the breast is Digital Breast Tomosynthesis (DBT). Inherently, this overcomes the main limit of 2D imaging, overlapping of normal and pathologic breast tissue. DBT in combination with standard screening digital mammography increases cancer detection by a third while it reduces the amount of false positives by 15%. A downside of this technique is the additional breast compression time of an average of 10 seconds per view compared to digital mammography[7,9].

Automated whole-breast ultrasound is a method to provide supplemental screening to women with dense breasts. In comparison with mammography alone, ultrasound is able to find an increased number of smaller cancers. However, lack of uniformity, the shortage of qualified personnel, and time and skill necessary to detect these small cancers limits universal application[7,9].

Another method used as a screening technique is Magnetic Resonance Imaging (MRI). It has been a recommended technique for women with an improved lifetime risk of breast cancer, because of the fact that in comparison with the other methods it has the highest true positive rate and the patient's psychologic data can be included. Nevertheless, it requires an injection of intravenous gadolinium and is expensive, almost up to 5-10 the cost of screening mammography[9].

Biopsy of breast lesions

On the occasion that a form of breast cancer has been found it is essential to ensure the appropriate patient counseling and tailored treatment plans are performed. This is currently set in motion in the form of 'triple assessment', which includes clinical examination, imaging and needle biopsy. The most frequently used method is the Needle Core Biopsy (CB). It is a

fast, well tolerated, inexpensive and reliably manner to distinguish benign from malignant breast lesions in the majority of patients[10]. Another technique used to obtain diagnostic tissue is Fine Needle Aspiration Cytology (FNAC), here tissue is collected through a 20 gauge needle[12]. FNAC is a very simple procedure and can be performed in under 30 seconds, also there is a theoretically lower risk of local contamination. Downsides include the inaccessibility of some structures, the limited sample and the variable accuracy. CB has evolved as an addition to FNAC or as an alternative, because the procedure has an higher diagnostic accuracy and will provide additional information about the tumor type, grade and receptor status[10,12].

In the preliminary stages of patients with suspected breast cancer it has become a routine to perform ultrasound examination of the axilla, an area on the human body under the joint where the arm connects to the shoulder, and a needle biopsy of abnormal axillary nodes[10] (as seen in Figure 2). The axillary lymph nodes are the first place breast cancer is likely to spread. In the case of spreading, the lymph node status will be positive. Because of this, finding cancer in one of the lymph nodes affects the treatment[13]. The node-positive patients have to be handled with extra care, and an accurate diagnosis of malignancy has to be made while keeping the number of benign breast biopsies to a minimum[10].

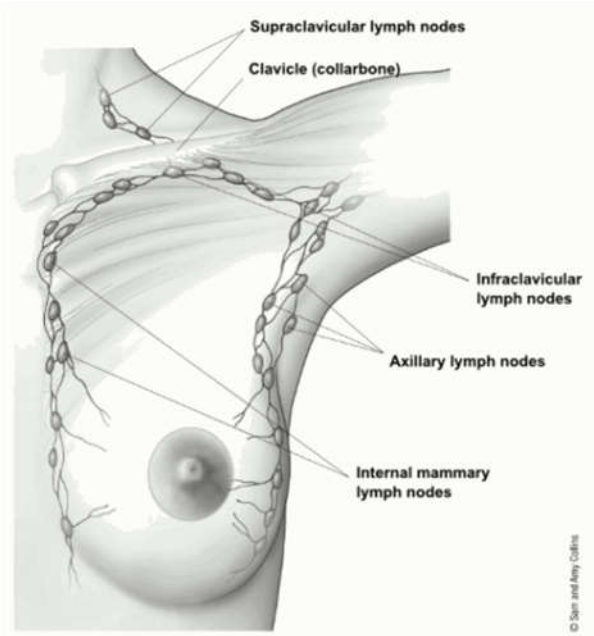

Figure 2. The lymph nodes in relation to the breast. (Adapted from American cancer society, 2016[13])

Breast cancer type and tumor size

The tissue removed during the biopsy is placed under a microscope and analyzed for cancer cells. Here, insight is provided whether it is a carcinoma or another type of cancer, such as sarcomas which are originated in fat, muscle or connective tissue instead of the epithelial cells. When enough tissue is available, the pathologist may be able to determine if the cancer is invasive or non-invasive (in situ)[13]. -In case of a FNAC biopsy, the pathologist may only be able to tell if the biopsy contains cancer cells[12]. For further treatment planning, an ultrasound measurement of the tumor size is useful. Although a clinical measurement is not sufficiently accurate, a correlation exists between the surgical and pathological assessment of tumor size. Breast carcinomas often have an asymmetric shape and the size is determined in terms of the largest diameter of the tumor[10]. Several studies have confirmed the significance of breast cancer size, as survival decreases with the increasing size of the tumor. Rosen and Groshen studied the relapse-free survival rate for patients with tumors for 18 years, 88% of the patients was free in 18 years with tumors less than 10 mm in diameter, 73% for the tumors with a diameter in between 11-13 mm, 65% for the tumors with a diameter between 14-16 mm and 59% for the tumors with a diameter between 17-22 mm. Also, a correlation exists between lymph node metastasis and tumor size, metastasis were identified in nearly 10% of tumors less than 10 mm in diameter. In comparison with the 35% of cancers measuring greater than 15 mm in diameter[14].

Cancer therapy

The initial preclinical phase in breast cancer research consists of the screening techniques, biopsies and the further determination of the tumor. It is certain that the steps in this stage do not yield any survival advantage when no effective treatments exist. So, the positive effects from screening are not derived from early detection but from early treatment[10]. Due to early treatment, breast cancer survival rates have improved by at least 30% for over the last 40 years. Along with these gains, the range of treatments options have also increased. Several complex treatment decisions may be offered to women with breast cancer in an early stage, about a range of different approaches including, radiotherapy, surgery and endocrine and chemotherapy[15]. In this review, our primary focus will be on radiotherapy, by reason of radiotherapy being the most important modality in the treatment of cancer, alone or in combination with other modes of treatment[8].

Radiotherapy

An important and effective element of current cancer treatment is radiotherapy. External beam radiotherapy contributes to relief or cure of many cancer patients and is used in approximately 40-50% of all cases. The aim of curative radiotherapy is to permanently control a tumor and its regional lymph node metastases without causing unacceptable damage to the normal tissue around it. All patients are individual and respond differently to the standard applied total radiation doses in 30-35 daily 2 Gray parts within 6-7 weeks. Up to now, most of the efforts to optimize radiotherapy have been in improving treatment from the physical and technological point of view. There has been less progress made to include the individual biological characteristics of each patient into treatment decisions. Multiple other factors may contribute either alone or in combination to the response of tumors to radiotherapy, such as re-oxygenation capacity during the course of radiotherapy, the number of cancer stem cells, repopulation, repair of radiation induced damage, and tumor hypoxia which have been investigated in experimental and clinical studies over the years. It appears to be very promising to integrate the previously mentioned information to further improve radiotherapy and thereby treatment outcome[8].

In order to improve, reliable predictive biologically rational markers are required which will optimize dose prescription and combined treatments for each individual patient. Some pre-treatment parameters, as tumor histology, stage, grade and performance are already taken into account in clinical practice to prescribe the best fit in available treatment, however parameters about the tumors biology are not taken into consideration. During treatment a tumor undergoes anatomical and biological changes, which could contribute to optimize treatment of an individual patient, if reliable tests to predict early tumor response are available. Not every patient will benefit from the same range of treatment interventions so such predictive tests are essential[8].

Tumor microenvironment

It is well known that tumors are not a collection of homogeneous cancer cells, they act as very complex organs that may even be more complex than healthy tissues. Consequently, in order to understand the biology of a tumor both the microenvironment and the cell types within a tumor need to be studied[16].

Tumor hypoxia

The importance of oxygen in radiotherapy was already suggested in the early 1900s. In 1909 it was demonstrated that the radiation response of skin was decreased if the blood flow in the irradiated area was reduced by compression. Eventually this led to the speculation that oxygen deficiency (hypoxia) was an important source of radiation resistance[17]. Regardless the tissue type, studies have demonstrated that hypoxia is a common feature in most solid tumors, such as invasive breast cancer[18].

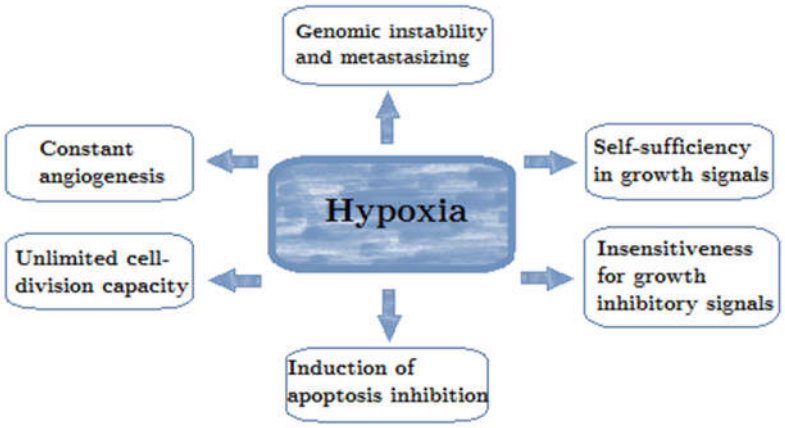

Figure 3. The influence of the hypoxic microenvironment what gives expression to the characteristics of cancer cells which distinguish them from healthy cells. (Adapted from Wouters et al. 2010[18])

The oxygen deficiency is caused by the disruption of balance between the consumption and the supply of oxygen. Depending on the duration and cause of the deficiency, hypoxia can be either chronic (diffusion-limited) or intermittent (perfusion-limited). Chronic hypoxia is not only dependent on a low vascular density. The newly formed vessels in tumors are often irregularly orientated, whereby not every region is as adequately provided by oxygen. Also the high proliferation rate of tumor cells will cause the oxygen consumption to increase, which makes the usage even higher than the supply. The diffusion limit will thereby be restrained and thus the intravascular oxygen pressure will drop, which will result in a flexibility decrease of red blood cells. Due to this decrease, the viscosity of blood will increase, which leads to a slow flow rate and inefficient oxygen transport[18].

Besides genetic and epigenetic changes which are associated with the development of cancer, also the tumor microenvironment is considered as an noteworthy factor in the malignant progression of tumors (as seen in Figure 3). On one hand the oxygen deficiency is cytotoxic which provokes a stress response in the cancer cells. On the other hand, hypoxia induces a wide range of adaptive mechanisms, largely coordinated by the transcription factor hypoxia-inducible factor 1 (HIF1)[18]. In structure HIF1 consist of an oxygen tension regulated α-subunit heterodimer and a constitutive expressed β-subunit. The HIF family contains 3 α-isoforms and 3 β-isoforms. In breast cancer, HIF-1α can be used as an endogenous marker for hypoxia. In

literature, accumulation of HIF-1α is associated with a worse prognosis for women with breast cancer in an early stage[18].

The influence of hypoxia on different tumor biological processes has many therapeutic implications. Next to the previously mentioned consequences, -low tumoral oxygen levels are known to decrease the effectiveness to radiotherapy, however the precise mechanisms which cause this resistance are not yet entirely understood. Radiotherapy causes DNA damage either by direct ionization of DNA or indirectly by interaction with the radicals formed by ionization of water surrounding DNA. This results in single- or double-strand DNA breaks. Afterwards, oxygen molecules react with these free radicals, thereby changing the chemical composition of DNA strand breaks (as seen in Figure 4). As a consequence of this change, they are recognized by enzymes of the DNA Damage Repair (DDR) pathways[6]. In the presence of oxygen the free radicals undergo a chemical reaction which fixates the DNA damage (DNA-OO·)[18]. In contradiction with the radicals under hypoxic conditions where the free radicals, in the absence of oxygen, restore their original form (DNA-H). This phenomenon counteracts the fixation of DNA damage and is therefore a major cause of chemotherapy and radiorestistance[6].

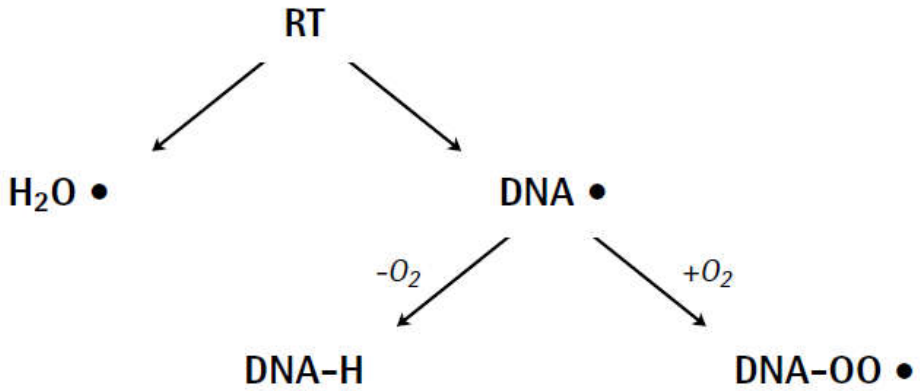

Figure 4. When cells are exposed to ionized radiation (RT), free radicals come into existence in DNA· or H_2O·. In the absence of O_2, DNA will be restored in its original form, in the presence of O_2, DNA damage will be fixated. (Adapted from Wouters et al. 2010[18])

Targeting the DNA damage

Detection of hypoxia, preferably in a non-invasive manner, could predict treatment outcome and serve as a tool to support treatment decisions[16]. Although there is no good or easy way to predict the presence of hypoxia, detection in the form of imaging might be a good way to select cancer patients who would benefit from treatments that overcome or take advantage of the presence of hypoxia. There are a couple of ways to assess the tissue oxygenation status *in vivo* (both invasive and non-invasive) or *in vitro*, using material from previously mentioned biopsies[19].

At first, it is important to be able to target the DNA damage caused by hypoxia, which could be done with help of epigenetics. Epigenetic mechanisms, which involve DNA methylation and histone modification, have been shown to have a crucial function in carcinogenesis and tumor progression. They are considered as potential targets for anticancer therapy and several other major pathologies. So, great potential lies in the development of 'epigenetic therapies'.

Especially, DNA methyltransferases and histone deacetylases have shown promising anti-tumorigenic effects[20,21].

DNA methylation consists of the introduction of a methyl-group at the C^5 position of the cytosine base in the DNA. Global hypo methylation is the most frequently observed in cancer, the best characterized epigenetic modification in malignant cells is the methylation of CpG sites in the human genome. This methylation is maintained by DNA methyltransferases (DNMTs). CpG sites are genomic regions containing high amounts of cytosine bases followed by guanine bases, which are present in 70% of all mammalian promotors. When methylated they cause stable heritable transcriptional silencing[6,20].

Histone modifications can alter the electrostatic charges and recruit binding proteins that are frequently part of the chromatin remodeling complex. Thereby, it makes the chromatin less accessible for other proteins such as transcription factors. At the moment, 16 types of histone modifications have been detected, including acetylation and methylation. In general, the acetylation of histones marks active, transcriptionally competent regions. On the other hand, histone methylation can be a marker for both active and inactive regions of chromatin. Both histone acetylation and methylation will influence transcription and other processes based on DNA[6,20].

The importance of epigenetic alterations in DNA is widely accepted, though their application as a biomarker in radiotherapy has not been studied very thorough. Recent literature does describe four views on the extensive interaction between epigenetics and hypoxia. First, the expression of two genes, *Von-Hippel Lindau* (VHL) and *prolyl 4-hydroxylase domain 3* (PHD3), which are responsible for the degradation of HIF in presence of oxygen can be epigenetically regulated. Second, the epigenetic mechanisms maintain a transcriptionally active chromatin confirmation around HIF binding site regions in which they can regulate HIF binding. During the initial hypoxic response, epigenetic modifying enzymes are in direct contact with HIF-1α, which participates in the co-activation of hypoxia inducible genes. Third, a few of the histone demethylase genes are direct HIF-1 target genes. As a result, the possibility exists of direct hypoxic regulation of histone demethylases. Finally, some global changes in DNA methylation and histone modifications are observed in response to hypoxia. Which results in repression or transcriptional activation.[6]

Because of the fact that hypoxia is noted as an important component of determining the response to radiotherapy, more knowledge on the influence of epigenetics in the hypoxic responses emphasizes the overall importance of epigenetics in radiotherapy.

Cell tracking and nanoscale sensing with fluorescent Nanodiamonds

Despite the growing interest and the increasing amount of evidence of an actual link between epigenetic alterations and radiotherapy response, at present time there are no epigenetic biomarkers for the radiotherapy-response ready to use in a day-to-day practice. -When looking at the interaction between hypoxia and epigenetics, the microenvironment might be critical in determining the success of epigenetic drugs[6]. Because of their promising optical properties in combination with its excellent biocompatibility, as mentioned in the introduction, Fluorescent Nanodiamonds (FNDs) tend to be ideal for long-term cell tracking applications and as a suitable candidate to function as a thermometer in living cells[2]. Which is exactly what is needed to discover more about the microenvironment around a breast cancer tumor. First the FNDs have to be taken up by the cells before any further progress can be made, including long term cell tracking and the nanoscale temperature sensing.

Insertion of Fluorescent Nanodiamonds

A successful targeted FND for cancer chemotherapy or radiotherapy should ideally be able to avoid non-specific uptake by nonmalignant cells, such as the Mononuclear Phagocyte System (MPS), and get efficiently into tumor cells. The MPS consists of specialized phagocytic cells, including the macrophages and monocytes in the connective tissue. Recognition and elimination of particles that enter the body is a primary function of the MPS. A immune response can be initiated when the MPS is activated by an exogenous particle, such as a FND[22].

The non-specific uptake of particles is mainly dependent on the surface charge and chemistry of the particles. Therefore, the particles are often coated with an electrically neutral hydrophilic surface layer, a coating that protects it against non-specific cellular uptake[22]. The most commonly used coating material up to 2013, is polyethylene glycol (PEG). Which is a non-ionic hydrophilic polymer that prevents the adsorption of proteins and subsequently, the cellular uptake[23]. In spite of the promising characteristics, a significant amount of drawbacks has been found of PEG coating, such as limiting endosomal escape and inducing the immune response. An alternative coating material has been proposed, the hyper branched Polyglycerol (PG). PG should be more pliable for further functionalization than PEG, due to numerous surface hydroxyl groups[22].

A drug carrier based on FNDs with a surface coating of PG (FND-PG) has been developed by Zhao et al[24]. To achieve the tumor cell targeting, the FND-PG was conjugated with cyclic Arg-Gly-Asp (RGD) peptide (as seen in Figure 5). The RGD peptide binds specifically to the integrin receptor $\alpha_v\beta_3$[22]. Integrins are dimeric adhesion receptors which modulate cellular attachment to the extracellular matrix or to neighboring cells. The interaction of integrins with their substrates regulates various cellular functions associated with tumor development, metastatic progression, including cell adhesion, migration, invasion, proliferation and survival. This specific integrin, $\alpha_v\beta_3$, is overexpressed in multiple types of malignant tumor and tumor tissue cells. It has been strongly suggested that

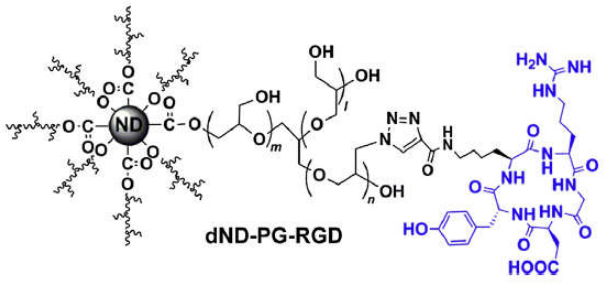

Figure 5. The FND surface coated with Polyglycerol (PG) and conjugated with cyclic Arg-Gly-Asp (RGD) peptide.(Adapted from Zhao et al. 2014[22])

$\alpha_v\beta_3$ plays a potential role in tumor progression, especially in invasive tumors such as breast cancer[25].

With the FND-PG-RGD it is possible to have them not be taken up by the non-phagocytic cells but selectively taken up by tumor cells which are over-expressing the integrin receptor $\alpha_v\beta_3$, such as breast cancer.[22]

Long term cell tracking

After the coated FNDs have been inserted in the tumor cells, they can be used for tracking. The internalization of FNDs do not cause any obvious cytotoxic and disadvantageous effects on the proliferation of human cancer cells[2]. Particularly in stem cell and cancer research FNDs can serve as a nanoprobe for long term labeling and tracking of cell division, proliferation and differentiation[2].

In HeLa cells, human cervical cancer cells, - Fang et al.[26] have been able to insert FNDs *in vitro* and track these cells continuously for over a week (as seen in Figure 6). This confirms the previous theoretical findings that FNDs can serve as a useful nanoprobe for long term labeling[26]. Also tracking and finding slow-proliferating cancer stem-cells with FNDs is now a possibility. Many malignant tumors contain a small sub-population of cells called cancer stem cells (CSCs). These cells are also relatively resistant to radiotherapy treatments and are responsible for tumor initiation, growth and recurrence. Some of these CSCs are preserved in a quiescent state, which preserves their self-renewal capacity after treatment. Up to now, researches have not been able to completely understand the mechanism of this stem cell quiescence, although it is of high importance to track and identify the fate of these cells[27]. With this skill, the cell division can be examined by time lapse imaging with differential interference contrast and epifluorescence microscopy[26].

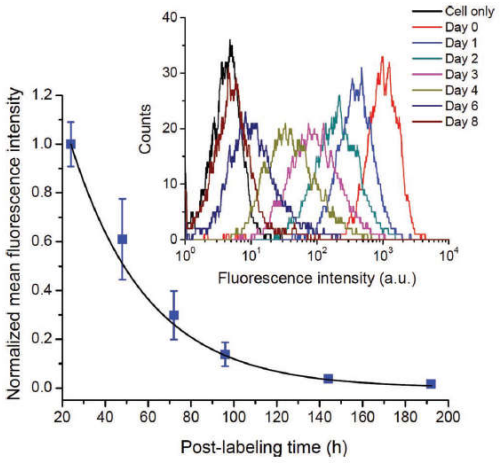

Figure 6. A graph of FND-labeled HeLa cells which are tracked over 8 days by flow cytometry. The fluorescence decreases exponentially with time due to cell proliferation. (Adapted from Fang et al. 2011[26])

Nanoscale temperature sensing

Visualizing thermal changes at subcellular resolution can reveal the heat production processes in depth and how they are correlated with biological activities. Recent developments in luminescence nanothermometry have made it practical to measure temperature changes in intracellular environment with high precision[2]. In 2013, the first application of FNDs as a thermometer in living cells was made by Kucksko et al.[28]. Several achievements have been made afterwards but the field of nanoscale temperature sensing is still at the start and has still many obstructions to overcome.

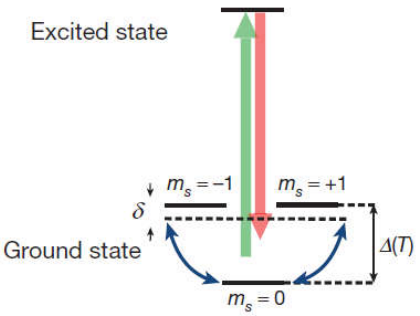

Figure 7. A simplified NV center level diagram showing a ground-state spin triplet and an excited state. At zero magnetic field, the sublevels are split by a temperature dependent zero field splitting ΔT. (Adapted from Kucsko et al. 2013[28])

The NV^- center in diamond has ground and excited states, which are spin triplet (S=1) and are split into three spin sublevels, with a total spin of 1[29] (as seen in Figure 7). These spin states can be coherently manipulated by using microwave pulses and then initialized and detected with laser illumination[28]. The energy difference between spin sublevels is D=2,87 GHz for the ground state and D=1.42 GHz for the excited state ,where D is also known as the zero-field splitting[29]. This optical measurement can be used to perform Electron Spin Resonance spectroscopy (ESR) which is called the Optically Detected Magnetic Resonance effect (ODMR). ODMR is a double resonance technique and the effect is a characteristic of NV centers[28,29].

In absence of an external magnetic field, the transition frequency is dependent on temperature due to thermally induced lattice strains. The transition frequency is the principle that NV center based thermometry relies on, which can be optically detected with high spatial resolution. The change in temperature can induce crystal strain, which in turn shifts the magnetic resonance, which aids a precise measurement of the thermal shift ($\Delta D/\Delta T$). The thermal shift, which varies from -75 kHz/K at 300 K to -150 kHz/K at 600 K, can expose the temperature change[2,28]. The local temperature change was deduced with a precision of 0.05-5 mK and a spatial resolution of 200 nm^2. Apart from high sensitivity, NV^- center based thermometry offers multiple advantages. First, as a result of a diamond's inertness, it is robust to changes in the local chemical environment. Second, it can be used in a wide range of temperatures which is of interest in the study of nanoscale chemical reactions.[28]

In the experiments of Kucksko et al.[28] it was demonstrated that the nitrogen vacancy centers in nanodiamonds can be used as robust temperature sensors that combine the virtues of submicrometre spatial resolution, biocompatibility and thermal sensitivity. Further improvements of sensitivity may allow for real-time observations of non-equilibrium subcellular processes in medical and biological applications. Additionally, when the technique is combined with two-photon microscopy identification of local tumor activity may be possible *in vivo*, by mapping atypical thermogenesis at a single-cell level[28]. Although, due to the non-transparency of the human body and of the fact that NV^- centers are not very bright, this might be an enormous challenge. Conclusively, the combination of thermal ablation therapy and the temperature sensor could be a possible tool for selective identification and killing of malignant cells without causing damage to the surrounding tissue[28].

Conclusion and future perspectives

Findings in the recent years have led to better understanding of breast cancer and FNDs on their own. Individually on each subject a lot of information has been gathered, but yet not all of it is known. The application of nanodiamonds in biomedicine in general has been a breakthrough. After initial discovery in 2005[2] that NV centers are brightly fluorescent and highly biocompatible, a lot of attention has been paid to the potential use of FNDs in life sciences. Since then FNDs have developed into a key element which is capable to form a bridge over the information gap between physics and biology. Although there is still no actual clinical application of FNDs, in future there definitely will be one.

-This paper showed an overview of a possible application of FNDs as a marker for bio-imaging, using breast carcinomas as a model. This type of cancer is chosen as a model, because it is the most common type of cancer affecting women from all over the world and due to its shape and size a biopsy is taken relatively easy. At the moment clinicians are unable to predict a fitting treatment plan for each patient individually. Therefore, a lot of the patients are treated in the same manner, in which the most frequently used method in the treatment of cancer is radiotherapy.

Due to hypoxia, the response to radiotherapy decreases, so unfortunately some patients do not respond to the prescribed treatment. Therefore detection of hypoxia, could help and predict treatment outcome and serve as a tool to support tailored treatment decisions. In hypoxia, the major cause of radioresistance, is the fixation of DNA damage. Which is a result of a reaction of oxygen and free radicals. Epigenetics could help to target the DNA damage, which involve histone modifications and DNA methylation. Great potential lies in the development of 'epigenetic therapies' because these aberrations could influence treatment outcome. Even though the importance of the epigenetic alterations in DNA have been widely accepted, their application as a biomarker in radiotherapy have not been studied very thorough. A growing number of studies has focused on the clinical applicability if epigenetic drugs as anti-cancer treatment but none of these drugs have been approved for their application yet.

Therefore, it might be interesting to look into the microenvironment around the tumor in more detail. The microenvironment might be critical in determining the success of epigenetic drugs. FNDs are a suitable candidate for gathering more information about this problem, due to their ability of long-term cell tracking and visualizing thermal changes at a subcellular level. At the moment these applications cannot be done *in vivo* but *in vitro* a great deal of research is already done. Researchers are trying to adequately perform deep tissue imaging of FNDs at sufficient levels of sensitivity and resolution[2]. Moreover, the characteristics of FNDs give rise to an exceptional variety of other possible applications, including strain, pH, pressure and magnetic and electric fields.

A prospect in the future is that researchers will be able to visualize, track and quantify molecules which is needed if one wishes to entirely understand complex biological systems both *in vitro* and *in vivo* with help of FNDs. Suggestions exist to bind cytostatic drugs to FNDs and integrate them in tumors through tumor-specific coatings, or guide them to the tumor with help of an ABEL trap[30]. Also, the free-radicals, which are a main source of fixating the DNA damage, still remain a topic of interest due to the fact that some of the exact mechanisms are still unclear[31]. In addition, the amount of Reactive Oxygen Species (ROS) could be a possible indication for hypoxia, although it is still not certain if ROS increases or decreases in hypoxia[32].

Detection of ROS is already one of the potential options using diamond particles and, in combination with the knowledge of hypoxia, would be a very interesting subject for future studies[1]. Because FNDs have shown to be a capable and versatile tool, the surface-functionalized FNDs are expected to become one of the most favorable optical nanoprobes for bio-imaging, diagnostics and treatment. By using these, we can greatly improve the outcome after radiotherapy treatment and make treatment plans for each patient individually.

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