

**THE POTENTIAL APPLICATION OF NANOTECHNOLOGY FOR THE
EX VIVO DIAGNOSIS OF PROSTATE CANCER**

BY

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PASS WITH DISTINCTION

**RESEARCH PAPER
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Abstract

This study investigates the possibility of three cancer assays: nanowires, nanopores and the biobarcode assay in the detection of protein biomarkers. Initially it focuses on the potential use of prostate-specific antigen (PSA) and describes in detail the practicalities and functionalities of the three assays, whilst comparing their advantages and disadvantages. It then deviates from PSA in search for other, more reliable biomarkers, such as interleukin-18 and it combines nanotechnology with these. To conclude, nanotechnology has significant potential for cancer diagnosis, with the biobarcode assay being the superior technique.

Introduction

The high prevalence of prostate cancer in men is increasingly reflected by the continually growing medical research in this field. With one in six men on average developing prostate cancer at some time in their lives, scientists are constantly searching for new ways to improve the highly polemic conventional assays that exist today. In 2009, over 75% of all men with invasive prostate cancer survived, which is a significant increase from 1970, where only 25% survived (Cancer Research UK). This is partly due to far more sensitive diagnostic tools but conventional techniques remain subordinate to their theoretical counterparts. Biomarkers are substances in the blood which can be measured to assess physiological abnormalities and many cancers release antigen precursors into the blood. Nanotechnology is the field of adapting and engineering nanoscale materials at an atomic level and this pertaining area of research presents a variety of applications for biomarker detection. In general, it is widely believed that nanotechnology assays have the capability to detect far lower concentrations of a particular biomarker than conventional assays and thus, cancer screenings could pick up cancer at an earlier stage. Mirkin, a prolific researcher of nanotechnology assays stated 'the first step towards a new cancer treatment and cure is a good diagnosis' (2007). Clearly if prostate cancer is caught too late, the subsequent development and spread of malignant cells is often the cause of death and thus, it is vital to shorten detection time if cancer therapies are to advance.

Prostate-specific antigen (PSA) is a precursor for prostate cancer that is currently used to assist the diagnosis of cancer. Ideally, a normal individual should have very little PSA in the blood and even a slightly raised level often results in a biopsy of the prostate tissue. Current assays have a low sensitivity and can only detect concentrations of above 0.1ng/ml of blood. Moreover, they are sometimes not qualitative and can only tell the doctor if PSA is present or not and the results are affected by pH and other proteins in the blood. Nanowires could detect PSA with a far higher precision than conventional assays as they work by detecting miniscule current disruptions. If a PSA molecule in the microfluidic channel docks onto the wire, it will cause a characteristic increase in resistance and this is registered by a computer to confirm a PSA binding has occurred. The frequency of these bindings is indicative of the concentration of the protein. The relative superiority of nanowires is creditable to their small size. However, nanopores offer a greater accuracy than nanowires as there is a very small chance of a false reading. Although nanopores were originally developed for DNA

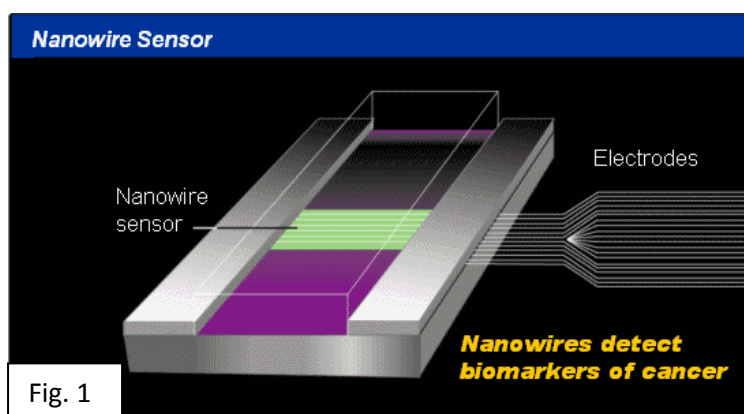
sequencing, they have proved useful in protein detection as the whole polypeptide is passed through a nanoscale pore and current disruptions occur for each amino acid. Although actuating the translocation of one polypeptide chain at a time through the pore is difficult, enzymes can be binded to the pore to assess protein inhibition. This would be useful if the protein actually caused the cancer. The biobarcode DNA assay offers: a far greater sensitivity, less chance of false readings and the possibility to detect more than one biomarker at once. It works by binding nanoparticles to a protein (such as PSA) and associating it with DNA, which subsequently bind to an optical chip. Light is then passed through the chip and the absorbance is related to the amount of DNA and this is related to the amount of protein present in the sample.

However, there is a great deal of controversy surrounding PSA as high levels also occur in non-cancerous conditions such as prostatitis and benign prostatic hypertrophy; therefore PSA screening is not reliable. Also if high PSA levels are found, the subsequent investigations performed can have harmful side effects – biopsies have the risk of infection. Therefore new biomarkers for prostate cancer are constantly being researched. One such study performed by Sang *et al* (2010) investigated the proteins involved in the break up of the basal layer around the prostate cells. These proteins diffuse into the blood and could indicate both the growth of cancer and the likelihood of metastasis. The problem is that these proteins occur in very small concentrations, but I believe that the aforementioned nanotechnology assays could detect these as a more reliable alternative to PSA. Without doubt, nanotechnology has a vast potential for cancer diagnosis in the future.

Discussion

Nanowires

Nanowires are *ex vivo* biosensors that can detect the presence of predetermined antigens and proteins in the blood. The NCI Alliance for Nanotechnology describes them as ‘nanoscale wires crossing a microfluidic channel’ (NCI Home). A current is passed through the wires, which are approximately 10nm in diameter and have resistances of up to 30 000 Ohms. Current is only allowed to flow in one direction due to the 1-dimensional nature of



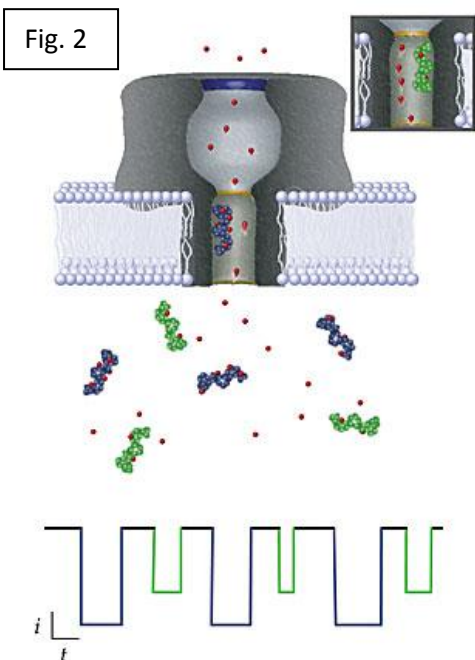
the wires and therefore, they exhibit ‘quantum confinement properties’. Electrons move along the length of the wires and not the width as it is too narrow. When a protein precursor collides with a corresponding protein (such as an antibody) on the wire, it momentarily alters its

resistance and this change is recorded by the computer systems that the wire is connected to (figure 1 shows this). The nanowires are typically built from silicon and they are conjugated from a 'bottom up approach' (Britannica), which means they are made from the constituent atoms rather than cut down from larger species. A study published in 'Nature Biotechnology' (Lieber *et al*, 2005) describes the potential applications of nanowires. Lieber and his team at Harvard are particularly interested in the detection of PSA and only one hundred-billionth of the protein that is usually found in a drop of blood is sufficient to activate the sensor. The method is effective as each precursor has characteristic resistance changes and attachment times. The wires can also be configured to bind to telomerase, an enzyme that adds non-coding DNA to the telomeres of the chromosomes. This means that during replication, only 100-200 nucleotides of non-coding DNA are lost, as opposed to a loss of coding DNA. Normally, in the absence of telomerase, cells reach the 'Hayflick constant' (Kaul, Wadhwa, 2003) and are unable to divide anymore as a critical chromosome length has been reached and essential nucleotides have been lost. However, with telomerase, cells can practically divide forever and their immortality is caused by a mutation in the oncogene. It is estimated that active telomerase is present in at least 80% of all cancers and thus, the ability to detect it early on with nanowires would be significantly beneficial. Bradt (2005) wrote that nanowires have the ability to 'immediately fill in details on exactly what type of cancer is present' and given the non-invasive nature of the procedure, the potential applications are endless.

Nanopores

The fundamental problems with nanowires revolve around the possibility of false readings and the inability to detect more than one protein at once. Nanopores on the other hand provide unprecedented potential for very accurate multi-protein detection, with virtually no false readings. A technique largely pioneered by Oxford University originally for DNA sequencing, nanopore detection could quickly and accurately identify a variety of protein

precursors in the blood, namely PSA and telomerase at the same time. Although nanopores have been used for many years to determine DNA base sequences, their oncological potential is yet to be exploited. Nanopores are small holes that consist of the protein alpha hemolysin and are inserted into an artificially created phospholipid membrane with a very high resistance (Oxford Nanopore Technologies). A potential difference exists across the pore and a current flows. When a given amino acid passes through the hole, a particular increase in resistance occurs and this is monitored by computer systems connected to the electrodes (figure 2 shows this). The frequency of these disruptions is indicative of the concentration of

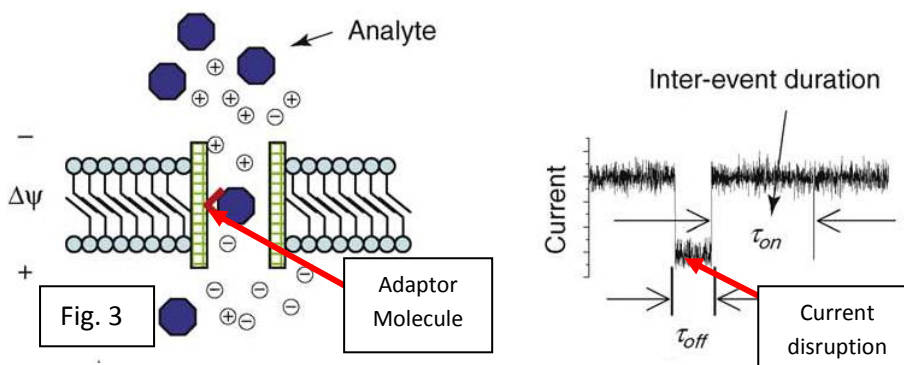


the specific protein. In 2007 Robertson *et al* from the National Institute of Standards and Technology (NIST) performed a study where they investigated protein detection using nanopores. They passed polyethylene glycol (PEG), a complex protein, through the nanopore and found that it could differentiate between ‘two nearly identical large molecules’. This accuracy is far superior to the aforementioned protein detection of nanowires. Notably, this was one of the first successful attempts to detect proteins using nanopores.

Despite the endless possibilities that nanopores could bring to the field of biocursor detection, the practicalities make this potential difficult to achieve. Primarily, polypeptide chains are not uniformly charged, unlike polynucleotide chains, and thus translocation through the pore is very difficult. Different parts of the same polypeptide chain are often positively and negatively charged so alternative approaches to electrostatic manipulation are required. A large amount of work was done by Movileanu *et al* (2009) in order to collate information from a variety of multidisciplinary fields on how to overcome this problem. It is universally agreed that alpha hemolysin is the most appropriate solution for the pore itself due to its robustness. It is the correct diameter and it forms with the hydrophobic section facing outwards to bind with the phospholipid bilayer. Polypeptide chains are often folded into shapes that would make detection impossible; a single string of amino acids needs to travel through the pore in order for the detection to be accurate. Auvray *et al* (2007) have used substances to facilitate the unfolding of protein chains, in particular ‘guanidinium hydrochloride’ in high concentrations. They found that maltose binding protein only triggers a disruptive resistance response in the presence of guanidinium hydrochloride. Moreover, it would be far more beneficial to conjugate this particle to the nanopore itself rather than it just being present in the surrounding solution. Lee *et al* (2009) have done just this and they found that it significantly increases the length of current disruptions.

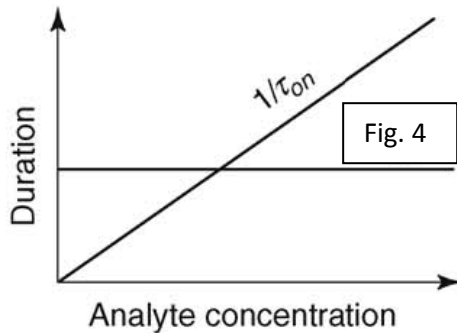
Another problem is the translocation of the protein at the correct rate to allow detection. This applies particularly in aqueous systems (which protein detection in the blood would be) and a great deal of research has been done on adaptor molecules. Howorka *et al* (2000) covalently bonded a polyethylene glycol ‘link’ molecule that bonds to both the nanopore and the adaptor molecule. Multiple adaptor molecules such as monoclonal antibodies,

receptors and glycoproteins could potentially all be bonded to the same link molecule, allowing the detection of many proteins with the same system. This increases reliability as more than



one raised protein level is unlikely to be idiopathic. Notably a set up such as the one in

figure 3 could be configured to allow characteristic binding durations to assess protein concentration as well as the frequency of interactions (figure 4). This would be particularly useful for detecting proteins in the blood as two separate pieces of evidence could reliably

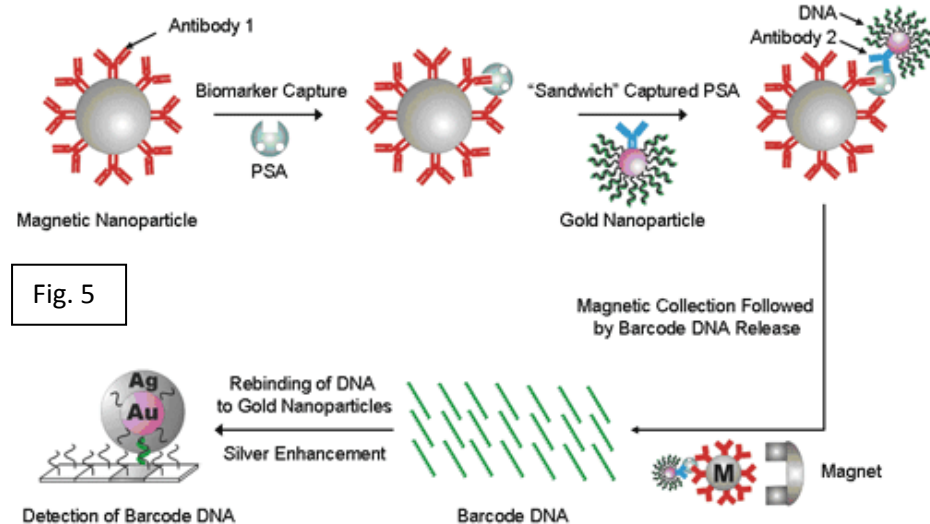


confirm the over expression of a precursor that should not normally occur in such high concentrations. Finally instead of conjugating a link molecule to the nanopore, it has been hypothesized that a protein inhibitor enzyme could be conjugated to the nanopore. Cheley *et al* (2006) used kinase to investigate the binding qualities of the polypeptide chains. If an interaction was made, a current disruption would occur. This information is

particularly useful in terms of finding inhibitors to cancer precursors in the body. If a certain precursor that signified cancer was detected, the inhibitor to that precursor could also be ascertained using this method, which creates therapeutic opportunities.

Biobarcode Assay

Despite nanopores being very accurate, like nanowires they are not sensitive enough as there is no amplification in the detected proteins. An assay that could potentially overcome this is the 'biobarcode assay' (Conrad, 2007). This technique was largely pioneered by Mirkin *et al* and is unique to all of the aforementioned assays in the way that it relies on an amplification system. The Centre for Cancer Nanotechnology Excellence (CCNE) has created a system capable of detecting extremely low levels of proteins (figure 5). There are two types of nanoparticles; one is magnetic and has many conjugated antibodies, while the second is much smaller and consists of gold atoms with many barcode DNA molecules attached. It also has antibodies capable of binding to the same target protein. In the study carried out by Mirkin in 2003, prostate-specific antibody was used to bind to prostate-specific antigen (PSA), the target protein. Both types of nanoparticles bind to the target proteins to form a 'sandwich' and the complex is then separated from solution by a magnetic field.

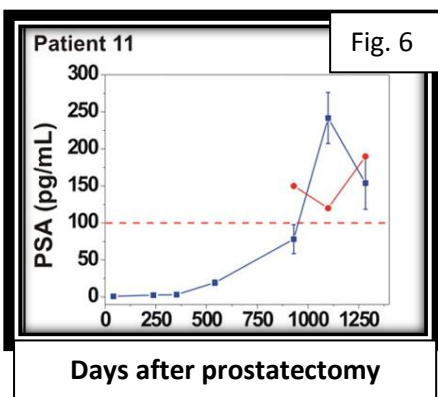


Subsequent release of the barcode DNA is rendered with chemical treatment and the amount of protein present in the solution determines the amount of barcode

DNA released. Since there are many DNA molecules for every target protein detected, the procedure amplifies the results, allowing very small concentrations to be detected. The barcode DNA molecules bind to corresponding DNA sequences on a chip and the gold nanoparticles (now with a silver coating) rebind to the exposed parts of the DNA. Light is then transmitted through the chip and the amount of absorption is related to the concentration of DNA-gold nanoparticle complexes on the chip's surface. A greater light absorption is indicative of a higher protein concentration.

This procedure has many advantages that elevate it above other techniques. Firstly the likelihood of false readings due to other proteins and changing pH is negligible due to the way the complexes are separated from solution with a magnetic field. Moreover, the assay is far quicker as a high concentration of magnetic and gold nanoparticles can be present allowing the target proteins to be 'found' faster than in the electrical based assays. Zhang *et al* (2004) performed a study where the biobarcode procedure was used to detect multiple proteins at once; this concept is mainly hypothetical in nanopore detection demonstrating the superiority of the biobarcode assay. Three different proteins associated with ovarian cancer were correctly identified using three types of barcode DNA and a unique optical procedure to differentiate between them. Being able to detect relative levels of more than one biomarker for prostate cancer would increase reliability and thus validity of the diagnosis.

Finally the technique's unprecedented sensitivity allows it to detect very low PSA levels in the blood after a radical prostatectomy. Conventional techniques are only able to detect levels of greater than 0.1ng/ml, with a 'high' reading being any value greater than 4ng/ml (National Cancer Institute). The biobarcode assay can detect picogram quantities making it at least 300 times more precise. Another study by Mirkin *et al* (2009) has demonstrated this



ability. Normally after the prostate is removed, the level of PSA decreases below detectable levels but if it starts to increase again there is no way of knowing until it rises above 0.1ng/ml. This time may be too late and the ability to detect rising levels early may allow therapeutic intervention before recurrence and metastasis occur. Figure 6 shows how the levels rise significantly, but they are only detectable after about day 1000. Therefore the biobarcode assay adds a new dimension to PSA detection

as a method for cancer recurrence diagnosis.

An alternative Approach

Despite this, I do not believe that PSA concentration is a reliable diagnostic tool due to the way a high concentration can also occur in benign conditions such as prostatitis and benign prostatic hyperplasia. Bearing this in mind, the aforementioned nanotechnology assays could utilise the information gathered in a seemingly unrelated study on protein precursors.

Sang *et al* (2010) investigated the presence of a variety of unknown prostate cancer precursors that cause the formation of metastatic cells. Clearly prostate cancer cells are not harmful unless they break away and form secondary cancers and for this to occur, the breakdown of the basement membrane and basal layer must happen. Although a great deal of information is known about basement membrane degradation, before 2010, very little was known about the role of proteases in the breakdown of the basal cells. Detecting these biomarkers is superior to PSA as high levels in the blood are virtually unique to cancer. In the study, a variety of frozen prostate cells including basal, endothelial, stromal and leukocytes; both cancerous and non-cancerous were isolated and incubated in a 37°C *in vitro* environment. Subsequent proteomic analysis of the cells, including densitometric analysis, and mass spectroscopy allowed the detection of a variety of precursors. Originally it was thought that protease enzymes were the primary cause of the breakdown of the basal layer but only 10-30% of prostate cancers with high enzyme levels metastasised. This led scientists like Man *et al* to believe that the cytokines in the stromal cells led to autoimmune responses and due to this, leukocyte analysis presents an area of growing interest. Primarily, the enzyme caspase-1, which causes the activation of the cytokine interleukin-18, was grossly over expressed in the leukocytes around the cancer cells. It is believed that active interleukin-18 is heavily involved in the break-up of the basal layer and it was therefore unsurprising that it was also over expressed in the tumour microenvironment. Furthermore, cellular retinoic anti-binding protein was under expressed in malignant basal cells. This protein normally causes the apoptosis (programmed cell death) of malignant cells but the stromal cells limit its production, causing the growth of cancerous cells. Therefore I believe that the abundance of interleukin-18 could be tested in the blood, bearing in mind the increased or decreased levels in a cancerous individual compared to the norm. Two concentration thresholds should be determined; the first would indicate that cancer is growing and the second would indicate that it has spread (or is about to spread). These values would be predetermined in a laboratory.

Conventional protein assays could be used but nanowires would quantitatively detect smaller increases in interleukin-18 levels. This could be actuated by combining an interleukin-18 receptor 1 molecule as a complementary binding site on the wire. However there is a possibility of false readings as more than one type of target protein could cause characteristic changes in the current of the wires. Nanopores have a much lower chance of false readings and various enzymes could be used to assess the possibilities of inhibition of interleukin-18. However, by far the most sensitive assay would be the biobarcode technique as it could detect very slightly raised levels of interleukin-18. Therefore any level above the norm could be detected very early on with minimal false readings. Furthermore the assay's ability to detect multiple proteins could be exploited by simultaneously registering an abnormally low level of cellular retinoic anti-binding protein as well. This confirmation would provide a greater validity in the diagnosis.

Conclusion

Although most nanotechnology assays remain in the lab, the possibility of trials and commercial usage is increasingly likely. The hypothetical ability of a physician to perform a blood test on a patient, where the results are available in minutes as apposed to a long wait for lab results, provides a new dimension to prostate cancer diagnosis. Using a machine no bigger than a small laptop containing any of the aforementioned nanotechnology assays, would produce more accurate and sensitive results than conventional techniques could. Nanowires and nanopores would use electrical resistance disruptions to utilise this potential, however they are incapable of detecting miniscule concentrations. The biobarcode assay is far more precise as it amplifies the result using many DNA molecules for every protein molecule. It has the added benefit of making the results more reliable as the nanoparticle complexes are removed from solution by a magnetic field, so pH does not affect the result. The problem the assays is that they have only largely undergone *in vitro* testing. More resources need to be put into trials to assess realistic potential. Like nanowires, nanopores need to have conjugated docking molecules on their surface for the protein and these are often hard to bind to the assay. As suggested, one solution would be to use selective adaptor molecules such as poly-ethylene glycol to act as an intermediate between the dock and the assay itself. However these techniques are all limited by the poor diagnostic performance of PSA. Increased levels in the blood do not necessarily denote prostate cancer. Making the readings more precise is irrelevant while the inherent reliability of the method is low. This can be overcome by detecting other proteins such as interleukin-18, which have diffused into the blood from the malignant prostate cells. As demonstrated by Sang *et al* (2010), high levels are unique to prostate cancer, if thyroiditis, uterine adenomyosis and severe sepsis are excluded. The concentration gives an indication of tumour load; from the primary stage to the metastatic advance. Furthermore, the possibility of the simultaneous detection of low cellular retinoic anti-binding protein could confirm prostate cancer. However, the study performed is only *in vitro* and many microenvironments behave differently *in vivo*. Many large-scale human trials need to be performed before a correlation with prostate cancer can be confirmed. Despite this, the vast amount of economic and scientific support that nanotechnology receives makes it a strong candidate for future prostate cancer diagnosis. It offers the potential for very early detection, which allows tailored therapy to take place earlier. The chances of metastasis and death could be significantly reduced. If research into nanotechnology continues, the efficacy of the potential assays will increase, meaning the scientific community is one step closer to beating cancer.

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