Firdevs KOKKOKOĞLU

DEVELOPMENT OF AN ELECTROCHEMICAL DNA BIOSENSOR FOR DETECTION OF GENETIC MUTATIONS

FATİH UNIVERSITY

The Graduate School of Sciences and Engineering

Master of Science in
Genetics and Bioengineering

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APPROVAL PAGE

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February 2013

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M.S. Thesis – Genetics and Bioengineering
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Thesis Supervisor: Assist. Prof. Dr. Mehmet ŞENEL

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ABSTRACT

Breast cancer is the third most common cancer in women with about 22% of all cancer incidences arise from breast cancer. Recent several biomarkers have been associated with the disease and these include protein and genetic markers. Due to the absence of both rapid and sensitive diagnostic tools for breast cancer, it cannot be detected in its early stage which is the most crucial point in treatment.

The main goal of this thesis was construction of electrochemical based DNA biosensors. The breast cancer related genetic biomarkers were evaluated by electrochemical based biosensor surface and these cancer markers were investigated Two different immobilization techniques were investigated while developing the electrode surface. Both types of sensors were employed successfully in the current study for the detection of gene (BRCA1) biomarker related to breast cancer. In the first work pack, sensor surface development was studied in three different generations of ferrocene-cored PAMAM dendrimers to validate the effectiveness of various surface chemistry methods for DNA immobilisation and hybridization assays. In second work pack, the electrode surface was prepared by avidin -biotin interaction. The specificity of the developed surfaces for DNA immobilization was validated through performing DNA hybridization assay and non-specific interactions between control probe surface and detection probe. The obtained results in the main researches of the study have demonstrated a promising future through the sensor technology for the diagnosis of breast cancer at early stage without a difficulty tools due to the rapid and sensitive detection of genetic biomarkers.

Keywords: Breast Cancer, DNA Biomarkers, Ferrocene PAMAM Dendrimer.

GENETİK MUTASYONLARIN TESBİTİ İÇİN ELEKTROKİMYASAL DNA BİYOSENSORÜ GELİŞTİRİLMESİ

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ÖZ

Meme kanseri, tüm kanser vakalarında yaklaşık %22 lik oranda kadınlarda en sık görülen üçüncü kanser türüdür. Yakın zamanda meme kanseriyle ilişkili çeşitli protein ve genetik biyomarkerlar kullanılmaya başlanıldı. Meme kanseri için hızlı ve duyarlı bir tanı araçları hem yokluğu nedeniyle, hastalığın başlangıç sürecinde tesbiti yapılamamakta; ki erken tehşis kanser tedavisinde kritik bir noktadır.

Tezimin temel amacı elektrokimyasal tabanlı DNA biosensor dizaynı ve inşaasıdır. Meme kanseriyle bağlantılı genetic biyomarkerlar elektrokimyasal tabanlı biosensor yüzeylerinde, herhangi bir işaretleyi olmadan çalışıldı ve hızlı yanıt vermeye uygun olarak ve yüksek hassasiyete sahip sonuçlara ulaşıldı. Elektrot yüzeyi geliştirirken, iki farklı immobilizasyon yöntemi geliştirildi. Her iki yöntemde de meme kanseriyle bağlantılı BRCA1 geninde yer alan mutasyon tesbitine yönelik başarılı sonuçlar elde edildi. Çalışmanın ilk bölümünde, biyosensor yüzeyi geliştirilirken ferrocene çekirdekli PAMAM dendrimerinin üç farklı jenerasyonu kullanıldı ve bu geliştirilen yüzeyler DNA immobilizasyonu, hibridizasyon assayları ve mutasyon tesbiti amacıyla kullanıldı. Çalışmanın ikinci kısmında ise yüzey geliştirilirken biyotin ve avidinin birbirlerine karşı olan yüksek etkileşimi kullanıldı ve yine bu yüzeylerde mutasyon tesbitine yönetik çalışıldı. Geliştirilen biyosensörlerin hassasiyetin anlamak amacıyla, uyumlu olmayan DNA sekanslarıyla yüzeye muamele edildi. Elde edilen sonuçlara gore, meme kanserinin teşhisinde önemli rol oynayan BRCA1 genindeki mutasyon tesbitinin, geliştirilen biyosensörlerle hızlı ve hassas bir şekilde gerçekleştirilebileceği göstermektedir.

Anahtar Kelimeler: Meme Kanseri, DNA Biyosensör, Ferrocene PAMAM Dendrimer.

To my parents

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SYMBOL/ABBREVIATION

BRCA	BReast CAncer suppressor gene
b-BSA	Biotinylated - Bovine Serum Albumine
CEA	CarcinoEmbriyonic Antigen
CV	Cyclic Voltammetry
DNA	Deoxyribonucleid Acid
DPV	Differential Pulse Voltammetry
FT-IR	Fourier Transform Infrared Spectroscopy
Fc	Ferrocene
G	Generation of a Dendrimer
GCE	Glassy Carbon Electrode
HER2	Human Epidermal Growth Factor Receptor 2
NMR	Nuclear Magnetic Resonance
NC	Non-Complementary
MM	Mismatch
PAMAM	Poly (amidoamine) Dendrimer
PBS	Phosphate Buffered Solution
SAB	Sodium Acetate Buffer
SNP	Single Nucleotide Polymorphism
SPCE	Screen Printed Carbon Electrode
SPR	Surface Plasmon Resonance

CHAPTER 1

INTRODUCTION

1.1 BREAST CANCER

1.1.1 General Information about Breast Cancer

Breast cancer is the third most frequent cancer in the world (796,000 cases in 1990) and by far the most common malignancy of women (21% of all new cases). Worldwide, the ratio of mortality to incidence is about 61%. Despite the high incidence rates, in Western countries, 89% of women diagnosed with breast cancer are still alive 5 years after their diagnosis, which is due to detection and treatment. As a result, breast cancer ranks as the fifth cause of death from cancer overall, although it is still the leading cause of cancer mortality in women (the 314,000 annual deaths represent 14.1% of cancer deaths in females). The prevalence of carriers of the major susceptibility genes are BRCA1 and BRCA2 in the general population [1]. Approximately 10% of all cases of breast cancer exhibit a familial pattern of incidence. Efforts to identify the genetic basis of familial breast cancer reached fruition some five years ago, when the breast-cancer-susceptibility genes BRCA1 and BRCA2 were identified through positional cloning. Germline mutations in either of these genes account for 20-60% of breast cancer cases in families where multiple individuals are affected (~2-6% of all cases) [2]. Epidemiological studies sparked by the discovery of BRCA1 and BRCA2 have made clear several features of inherited mutations in the genes.

The term "genetic testing" covers an array of techniques including analysis of human DNA, RNA or protein. Genetic tests are used as a health care tool to detect gene variants associated with a specific disease or condition, as well as for non-clinical uses such as paternity testing and forensics. In the clinical setting, genetic tests can be performed to confirm a suspected diagnosis, to predict the possibility of future illness to

detect the presence of a carrier state in unaffected individuals (whose children may be at risk), and to predict response to therapy. Scientists are revealing ever more associations between particular gene mutations and disease, and over a thousand tests can now determine whether a person carries a particular disease-associated allele. As the number of tests continues to raise, their use in the health care setting is becoming more commonplace.

Early detection is a major factor contributing to the 3.2% annual decline in breast cancer death rates over the past 5 years. Unfortunately, currently available breast cancer screening tools such as mammography and breast examination miss up to 40% of early breast cancers and are least effective in detecting cancer in young women, whose tumors are often more aggressive. An invasive needle or surgical biopsy must be performed when an area of suspicion is identified to confirm, by cytologic or histologic evaluation, the presence of malignant disease. The development of noninvasive techniques that would distinguish between women with and women without breast cancer, as well as between different disease stages, is, therefore, of crucial importance [3].

1.1.2. Anatomy of Breast Cancer

The structure of the breast is very complex, because it is including fat and connective tissue; as well as lobes, lobules, ducts and lymph nodes.

Lobules and ducts; Each breast has a number of sections (lobules) that branch out from the nipple. Each lobules are arranged like the petals of a daisy. The lobes, lobules, and bulbs are all linked by thin tubes called ducts. These ducts lead to the nipple in the center of a dark area of skin called the areola.

Fat, ligaments and connective tissue; Spaces around the lobules and ducts are filled with fat, ligaments and connective tissue. The amount of fat in the breasts largely determines their size. The actual milk-producing structures are nearly the same in all women.

Muscles; The breast has no muscle tissue. Muscles lie underneath the breasts, however, separating them from the breast’s ribs.

Arteries and capillaries; Oxygen and nutrients travel to breast tissue through the blood in the arteries and capillaries.

Lymph nodes and lymph ducts; The lymphatic system is a network of lymph nodes and lymph ducts that helps fight infection [4].

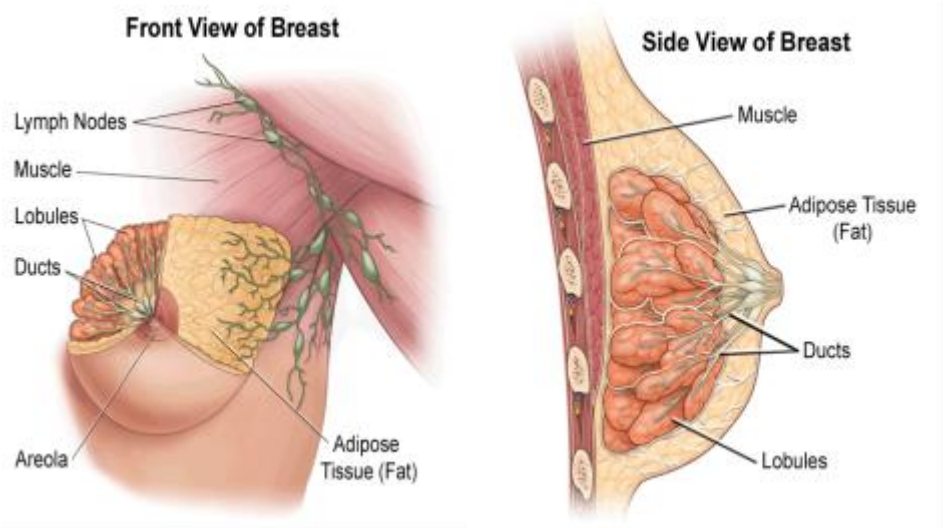


Figure 1.1 Anatomically front and side views of breast.

1.1.3 Genetics of Breast Cancer

Breast cancer, a disease in which malignant cells form in the tissues of the breast, is the most common type of cancer among women. Less than 1 percent of breast cancers occur in men. The risk of getting breast cancer increases with age, and inherited gene mutations or a family history of breast cancer may increase the risk.

Breast cancer is a cancer that starts in the tissues of the breast. There are two main types of breast cancer:

- Ductal carcinoma starts in the tubes (ducts) that move milk from the breast to the nipple. Most breast cancers are of this type.

- Lobular carcinoma starts in the parts of the breast, called lobules, that produce milk. In rare cases, breast cancer can start in other areas of the breast.

Breast cancer may be invasive or noninvasive. Invasive means it has spread from the milk duct or lobule to other tissues in the breast. Noninvasive means it has not yet invaded other breast tissue. Noninvasive breast cancer is called "in situ."

- Ductal carcinoma in situ (DCIS), or intra-ductal carcinoma, is breast cancer in the lining of the milk ducts that has not yet invaded nearby tissues. It may progress to invasive cancer if untreated.

- Lobular carcinoma in situ (LCIS) is a marker for an increased risk of invasive cancer in the same or both breasts [5].

According to the National Cancer Institute (NCI), the lifetime risk for a woman to develop breast cancer is 12.8 percent or one in eight, while the lifetime risk to develop ovarian cancer is 1.5 percent, or one in 67.

A gene is a basic unit of heredity that determines a person's traits. Genes are located on one of the 46 chromosomes housed within cells that make up all of the tissues of the body. They come in pairs, and work together to make proteins. One member of the gene pair is inherited from the mother, and one from the father. Cancers develop due to alterations (mutations) in genes. When an alteration or mutation in a gene is present in the eggs and sperm, also called germ cells, it is referred to as a "germ-line mutation." When a germ-line mutation is inherited it is present in all body cells. Only a small percentage of cancers involve inherited mutations that are passed from generation to generation. If a parent passes you a defective gene, you have an increased risk for breast cancer. The majority of cancers can be attributed to acquired mutations. "Acquired" means that the mutations occur only in the tissue that is affected by cancer and are not present in all cells of the body. Acquired mutations are not inherited and are not passed down to next generation. Some people have genes that make them more likely to develop breast cancer. There are the most common gene defects are found in the two genes, BRCA1 and BRCA2, that greatly increase the risk to the breast cancer and women who have family members with the breast cancer. These genes normally produce proteins that protect us from cancer [6].

1.1.4 Current Diagnosis Techniques

Breast cancer is an insidious disease that may develop over many years without signs and symptoms. If the disease is found and treated when localized to the breast the cure rate is improved. Women need to be motivated to accept and even demand screening for breast cancer. In the attemp to make screening more cost effective, emphasis has been placed on evaluation of high-risk factors for breast cancer such as positive family story, nulliparty etc. All women, at least those over 30, must be considered at risk for developing breast cancer [7].

1.1.4.1 Mass Screening

Screening is a procedure to detect disease in a presuambly well individual. In the case of breast cancer, screening involves primarily the detection of an abnormalities. So; make the differentiation between a benign and malignant process. A mass screening program for detection of early breast cancer differs in concept, approach and techniques from the breast examination in general medical practice. In mass screening for breast cancer, large number of woman are involved who presumably have no disease and would not be having the examination were it not for the availability of the screening program. Since the detection process involves many individuals without serious disease, it should be quick, safe, readily available, economical and acceptable to the woman being screened. It should be able to detect breast cancer at a stage when it can be treated effectively [8].

In Summary; the object of screening for breast cancer is to discover those among the apparently well who are in fact suffering from breast cancer. This may afford a better chance for diagnosis.

1.1.4.2 Mammography

A mammogram is an x-ray picture of the internal structure of the breast. It is used to examine the human breast and is used as a diagnostic and a screening tool. Additional angles and magnified views are taken of suspicious areas. Thus, to find tumors and to help tell the difference between benign and malignant disease.

The main role of mammography in the diagnosis of breast cancer; the increasing awareness that micrometastasis frequently occurs early in the course of breast carcinoma continues to alter the approach to early detection. The growth of a cancer to 1 cm size, the accepted threshold of palpability, already represent 30 doubling times; 75% of the tumor's life-span has already occurred and only three more doubling will be necessary to increase the size from 1 to 2 cm. It is suggested that a woman have a baseline mammogram at age 40, followed by a mammogram every couple of years until age 50. After 50, a woman should have a mammogram every year [9].

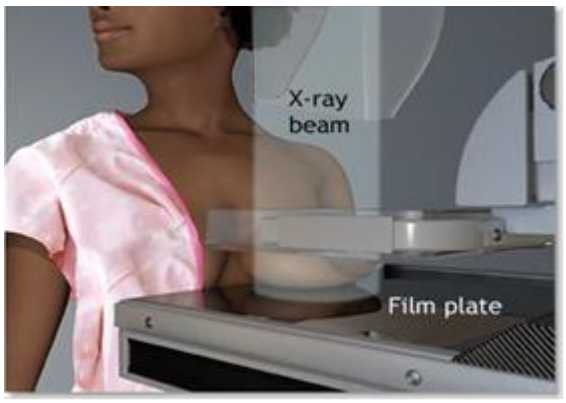


Figure 1.2 Picture of mammogram.

1.1.4.3 Biopsy

Breast biopsy is required when the benign or malignant nature of a breast abnormalities must be determined. Biopsy may be done by open or closed methods.

Open biopsy; removes the entire mass or just a part of the mass. Definitive surgical treatment may follow immediately after biopsy .

Closed biopsy; is done with a needle, and may be either an aspiration or core biopsy. In the needed aspiration technique, individual cells or cell clusters are obtained for cytologic examination.

A dominant mass of thickening in the breast is the most common reason for biopsy. One must not depend on mammogram or other diagnostic aids to determine the

being or malignant nature of a clinically dominant lesion. Only microscopic examination of tissue obtained by biopsy can conclusively indicate a benign situation. The various diagnostic aids may be used to add certain information, such as the presence of separate nonpalpable lesions in one or both breast. In addition, a positive mammogram can substantiate a clinical impression of malignancy, and by so doing aid in choosing a particular biopsy method [10].

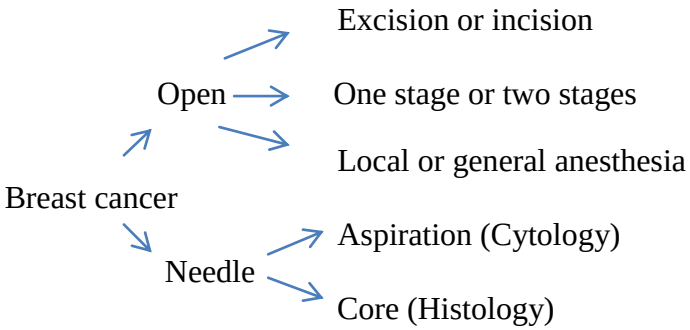


Figure 1.3 Options in diagnostic breast biopsy.

1.1.5 The Treatment Methods of Breast Cancer

Cancer treatment may be local or systemic. Local treatments involve only the area of disease. Radiation and surgery are forms of local treatment. Systemic treatments affect the entire body. Chemotherapy is a type of systemic treatment. In general, cancer treatments may include: chemotherapy medicines to kill cancer cells. Radiation therapy to destroy cancerous tissue. Surgery to remove cancerous tissue.

1.1.5.1 Chemotheraphy

Treatment is based on many factors, including: type and stage of the cancer, whether the cancer is sensitive to certain hormones, Whether the cancer overproduces (overexpresses) a gene called HER2/neu [11].

Chemotherapy refers to the use of anti-cancer drugs to kill breast cancer cells. The doctor who determines which drugs will be used, and in what dosage is known as an oncologist. Chemotherapy can be used for three major purposes:

Adjuvant therapy: The goal is to prevent or postpone cancer from coming back after the initial surgery and radiation. Even when the cancer seems to be confined to the breast and lymph glands under the arm, there is a chance that cells may have already spread to other areas that cannot be seen. Chemotherapy is given to try and kill these cells.

Neo-adjuvant therapy: Sometimes the cancer in the breast is so big that shrinking it first with chemotherapy may make it easier to do surgery [12].

To treat metastatic disease: If the cancer shows up in parts of the body other than the breast and lymph glands under the arm, it is called metastatic disease. Chemotherapy can be one of the main ways to kill cancer cells that have spread to other parts of the body. When to start chemotherapy, what drugs to use, and what side effects to expect varies from woman to woman.

1.1.5.2 Radiotheraphy

Radiation therapy is used to fight many types of cancer. Radiation targets rapidly dividing cells like cancer cells. Radiation prevents cell division and the replication of DNA (the genetic building blocks). So, we know that the cancer cells multiply faster than normal cells in the body. Because radiation is most harmful to quickly growing cells, radiation therapy damages cancer cells more than normal cells. This prevents the cancer cells from growing and dividing, and leads to cell death.

Radiation therapy is used to fight many types of cancer. Sometimes, radiation is the only treatment needed. It may also be used to:

- Shrink a tumor as much as possible before surgery

- Help prevent the cancer from coming back after surgery or chemotherapy

- Relieve symptoms caused by a tumor

- Treat cancers that cannot be removed with surgery [13].

1.1.5.3 Surgery

Surgery means that to remove cancerous tissue, there are 2 diffirent surgery options. Lumpectomy known as breast-conserving surgery, removes of only the tumor or lump; and a small amount of surrounding tissue. Mastectomy removes total removal of a breast or part of the breast and possible nearby structures.

1.1.5.4 Hormone Theraphy

Hormonal therapy is prescribed to women with ER-positive breast cancer to block certain hormones that fuel cancer growth. The most important hormonal therapy drug is tamoxifen. Its role blocks the effects of estrogen, which can help breast cancer cells survive and grow. Most women with estrogen-sensitive breast cancer benefit from this drug. Another drugs of hormonal therapy medicines called aromatase inhibitors, such as exemestane (Aromasin), have been shown to work just as well or even better than tamoxifen in postmenopausal women with breast cancer [14].

1.1.6 Breast Cancer Biomarkers

1.1.6.1 Protein Biomarkers

Table 1.1 Current promising biomarkers for the detection of breast cancer.

Name of biomarkers	Technology used for discovery	Type	Reference
RS/DJ-1	Humoral response	Autoantibody	[15]
p53	Humoral response	Autoantibody	[16]
HSP60	Humoral response	Autoantibody	[17]
HSP90	Humoral response	Autoantibody	[18]
Mucin-related	Humoral response	Autoantibody	[19]
CA 15-3	Serum profiling	Serum protein	[20]
RS/DJ-1	Serum profiling	Serum protein	[15]
HER-2/neu	Serum profiling	Serum protein	[21]
Lipophilin B	Nipple aspirate fluid profiling	Ductal protein	[22]
beta-globin	Nipple aspirate fluid profiling	Ductal protein	[22]
Hemopexin	Nipple aspirate fluid profiling	Ductal protein	[22]
Vitamin D-binding protein	Nipple Aspirate Fluid Profiling	Ductal protein	[22]

Protein biomarkers suitable for the prevention of breast cancer must be extremely sensitive, easily detectable and highly correlated with the disease. Nowadays lots of protein biomarkers have been investigated with different ways such as genetic, biochemical, or medical based methods for detection of breast cancer research including diagnosis, prognosis, treatment and protection. Although there are the most specific and studied protein biomarkers are selected in order to discuss. The widely investigated protein biomarkers are carcinoembriyonic antigen (CEA), cancer antigen 15-3 (CA 15-3) and human epidermal growth factor receptor 2 (HER2) which plays an important role in breast cancer [23].

1.1.6.1.1 CEA (Carcinoembryonic Antigen)

Carcinoembriyonic antigen was originally described by Gold in 1965.Carcinoembryonic antigen (CEA) is a protein normally found in many types of cells but associated with tumors and the developing fetus. Blood levels of this protein disappear or become very low after birth. In adults, an abnormal amount of CEA may be a sign of cancer. CEA is tested in blood. The normal range is 0 to 2.5 ng/ml in an adult non-smoker and 0 to 5.0 ng/ml in a smoker. It is a complex glycoprotein of molecular weight 20,000 that is associated with the plasma membrane of tumor cells, from which it may be released into the blood. Although CEA was first identified in colon cancer, an abnormal CEA blood level is specific neither for colon cancer nor for malignancy in general. Elevated CEA levels are found in a variety of cancers other than colonic, including pancreatic, gastric, lung, and breast. It is also detected in benign conditions including cirrhosis, inflamatory bowel disease, chronic lung disease, and pancreatitis. The CEA is often positive in malignancies other than colonic. In cancer of the breast, lung, pancreas, stomach, and ovary the CEA may be elevated and can be used to monitor the progress of disease or response to treatment [24].

1.1.6.1.2 CA 15-3 (Cancer Antigen 15-3)

Cancer Antigen 15-3 (CA 15-3) is a blood test that is given during or after treatment for breast cancer. It is most useful in monitoring advanced breast cancer and the response to treatment. CA 15-3 is a protein that is a normal product of your breast tissue, and it does not cause breast cancer. If a cancerous tumor is present in your breast, though, the levels of CA 15-3 may increase as the number of cancer cells increase.

Tumor cells will shed copies of the CA 15-3 protein, which can be measured by the blood test. After the completed treatment for breast cancer, the doctor may test your blood for CA 15-3 on a regular schedule to see if the levels of this antigen are rising or remaining steady. Rising levels of CA 15-3 may indicate a recurrence of breast cancer, but since other conditions can cause higher levels of this antigen, the test results must be taken in to consideration with the results of imaging studies and other tests for HER2/neu and BRCA genes [25].

1.1.6.1.3 HER-2 (Human Epidermal Growth Factor)

Human epidermal growth factor receptor 2 (HER-2), also known as c-erbB-2, is an oncogene located in chromosome 17 which encodes a transmembrane glycoprotein with a tyrosine kinase activity. It belongs to the epidermal growth factor receptor family. HER-2 gene is either amplified or overexpressed in 15–30% of invasive breast cancers, but not in normal adult or fetal breast. HER2 is a gene that sends control signals to your cells, telling them to grow, divide, and make repairs. A healthy breast cell has 2 copies of the HER2 gene. Some kinds of breast cancer get started when a breast cell has more than 2 copies of that gene, and those copies start over-producing the HER2 protein. As a result, the affected cells grow and divide much too quickly.Clinical applications for HER-2/neu tissue are mainly related to its prognostic or predictive value. The HER-2/neu oncoprotein is composed of three domains: the internal tyrosine portion responsible for intracellular signaling, the transmembrane and the external domain (ECD). This last portion is shed by proteolytic cleavage and can be determined by enzyme immunoassay in serum. Moderate increases in serum HER-2/neu may be found in the absence of malignancy, mainly in association with liver diseases. Likewise, the highest serum concentrations of this oncoprotein are found in patients with breast cancer, but lower concentrations may be found in other malignancies, particularly ovarian, prostate, and lung cancer (mainly adenocarcinomas) [26].

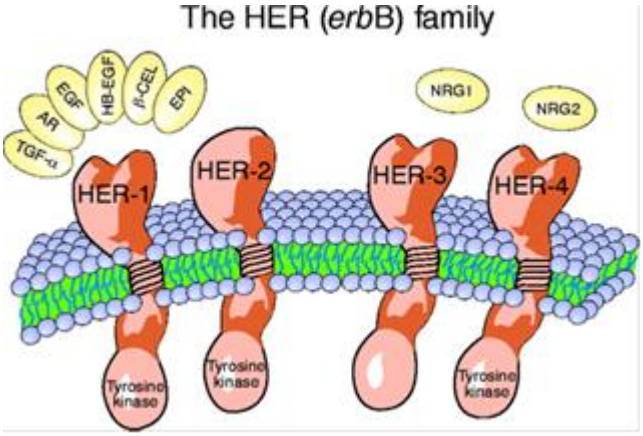


Figure 1.4 Picture of human epidermal growth factor receptor family.

1.1.6.2 Genetic Biomarkers

Genetic Biomarkers of Breast Cancer; a different kind of genes have been exposed to somatic mutation in human breast tumour cells or tissue. The mutated genes include that oncogenes and tumour suppressor genes like as genes encoding prteins that perform key functions in cell cycle regulation, DNA repair , apoptosis and telomerase activity.

1.1.6.2.1 BRCA1

In 1990, DNA linkage studies on large families with the above characteristics identified the first gene associated with breast cancer. Scientists named this gene “breast cancer 1” or BRCA1. It is located on chromosome 17. Mutations in the gene are transmitted in an autosomal dominant pattern in a family.The BRCA1 gene contains 22 coding exons and encodes a nuclear phosphoprotein of 1863 amino acid residues. In human breast cancer cell lines and spermatocytes, BRCA1 protein binds to Rad51, which functions in DNA recombination and repair, suggesting a role for BRCA1 specifically during mitosis and meiosis. Recent studies have suggested that BRCA1 contributes to cell cycle arrest and growth suppression through the induction of p21. BRCA1 and p53 cooperatively induce apoptosis of cancer cells, and BRCA1 may coordinately regulate gene expression together with p53 as tumor suppressors. The BRCA1 gene frequently is mutated in familial breast and/or ovarian cancers. To date, >500 distinct mutations, polymorphisms, and unclassified variants have been identified

by Breast Cancer Information Core database (BIC). Most small deletions, insertions, and point mutations lead to premature termination of translation and, thus, truncated proteins. Germline mutations in BRCA1 are thought to be responsible for; 45% of familial breast cancers and for >80% of the inherited breast cancer syndrome [27].

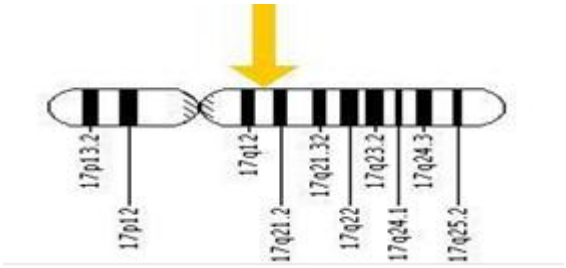


Figure 1.5 The BRCA1 gene is located on the long (q) arm of chromosome 17 at position 21.More precisely, the BRCA1 gene is located from base pair 41,196,311 to base pair 41,277,499 on chromosome 17.

1.1.6.2.2 BRCA2

The BRCA2 gene belongs to a class of genes known as tumor suppressor genes. Like many other tumor suppressors, the protein produced from the BRCA2 gene helps prevent cells from growing and dividing too rapidly or in an uncontrolled way. BRCA2 is located on chromosome 13. The BRCA2 gene provides instructions for making a protein that is directly involved in the repair of damaged DNA. In the nucleus of many types of normal cells, the BRCA2 protein interacts with several other proteins, including the proteins produced from the RAD51 and PALB2 genes, to mend breaks in DNA. These breaks can be caused by natural and medical radiation or other environmental exposures, and also occur when chromosomes exchange genetic material in preparation for cell division. By helping repair DNA, BRCA2 plays a role in maintaining the stability of a cell's genetic information. Researchers suspect that the BRCA2 protein may have additional functions within cells. For example, the protein may help regulate cytokinesis, which is the step in cell division when the fluid surrounding the nucleus (the cytoplasm) divides to form two separate cells. Researchers are investigating the protein's other potential activities. Both BRCA1 and BRCA2 are

tumor suppressor genes that usually have the job of controlling cell growth and cell death. Everyone has two BRCA1 (one on each chromosome #17) and two BRCA2 genes (one on each chromosome #13). When a person has one altered or mutated copy of either the BRCA1 or BRCA2 gene, their risk for various breast, ovarian, prostate and stomach cancers increases [28].

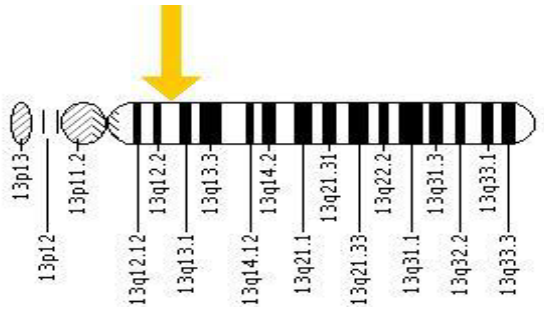


Figure 1.6 The BRCA2 gene's cytogenetic location: 13q12.3. Molecular Location on chromosome 13: base pairs 32,889,616 to 32,973,808.

1.1.6.2.3 p53

Tumor protein p53 is located in the nucleus of cells. When the DNA in a cell becomes damaged by agents such as, radiation or toxic chemicals from sunlight, this protein plays a critical role in determining the DNA repaired or the damaged cell will undergo apoptosis. This process prevents cells with mutated or damaged DNA from dividing, which helps prevent the development of tumors. If the DNA can be repaired, tumor protein p53 activates other genes to fix the damage. Because tumor protein p53 is essential for regulating cell division and preventing tumor formation, it has been nicknamed the "guardian of the genome."The increased breast cancer risk from the variations of the Tumor Protein 53 gene. The occurs some changes in this gene greatly increase the risk of developing the breast cancer.In some cases of breast cancer, one copy of the TP53 gene is lost and the remaining copy has a mutation that prevents the cell from producing any tumor protein p53. Without this protein, DNA damage accumulates and cells divide in an uncontrolled way, leading to a cancerous tumor [29].

1.2 BIOSENSORS

1.2.1 Principle of Biosensors

A biosensor is type of a biomedical instrument which is used for the detection of biologically important materials. This can create a response on the biosensor surface (bio-interface) and this biological response converted to an electrical signal by the biosensor. There are three main parts in a typical biosensor which are bio-recognition part, transducer and signal processor parts, as illustrated in Figure 1.7.

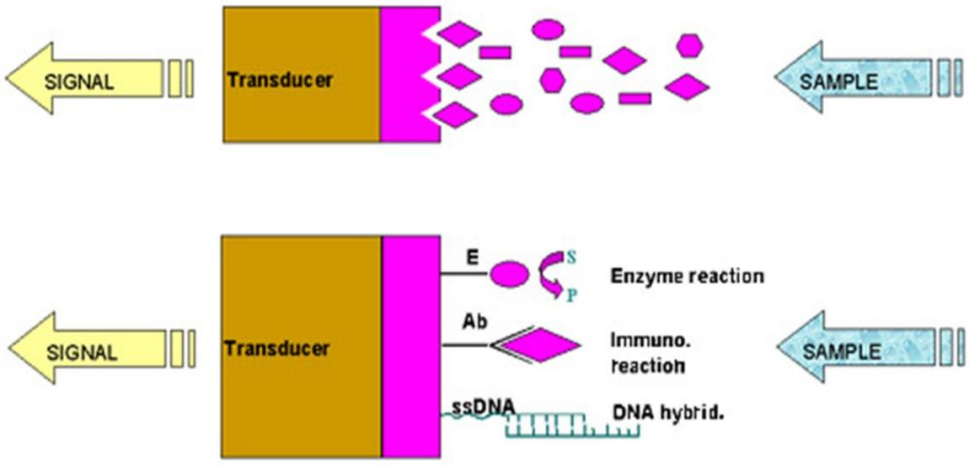


Figure 1.7 Schematic representation of main components of a biosensor.

The key part of a biosensor is bio-recognition part which used as a biochemical transducer which can be enzymes, tissues, bacteria, cells, antibodies/antigens, liposomes and organelles [30, 31]. The immobilization of the biocomponents on a suitable matrix is important fact for the development of the biosensor. Thus, most of the biological molecules such as enzymes, receptors, antibodies, cells etc. have very short lifetime in solution phase. The activity of immobilized molecules depends upon surface area, porosity, hydrophilic character of immobilizing matrix, reaction conditions and the methodology chosen for immobilization.

Biosensor development began with experience of biomolecules immobilization and stabilization on the one hand, and the miniaturization and functionalization of more

sophisticated transducers and signal processors, on the other [32]. The biochemical transducer or biocomponent converts the biochemical signal to an electrical signal. The nature of the biochemical interaction with analyte is a key factor for adaptation of suitable transducing system. The electronic transducer can frequently measure potential, current, light, temperature, mass, etc. Generally, biosensors can be classified according to the nature of the physical transducer: electrochemical, piezoelectric or optical sensors.

The electrical signal from the transducer is often low and superimposed upon a relatively high and noisy. The signal processing normally involves subtracting a 'reference' baseline signal, derived from a similar transducer without any biocatalytic membrane, from the sample signal, amplifying the resultant signal difference and electronically filtering out the unwanted signal noise. The relatively slow nature of the biosensor response considerably eases the problem of electrical noise filtration. The analogue signal produced at this stage may be output directly but is usually converted to a digital signal and passed to a signal processor stage where the data is processed, converted to concentration units and output to a display device or data store [33].

1.2.2 Classification of Biosensors Depending On The Type of Transducer

1.2.2.1 Electrochemical Biosensors

Electrochemical biosensors combine analytical power of electrochemical techniques with specificity of biological recognition processes. Generally the bioreaction produces an electrical signal that relates to the concentration of an analyte. For these purpose, a biospecific reagent is either immobilized or retained at a suitable electrode, which converts the biological recognition event into a quantitative amperometric or potentiometric response. The combination of the electrode with a biomolecule offers new and powerful analytical tools that are applicable to many challenging problems [34].

Also electrochemical biosensors have been the most widely used classes of biosensors due to their faster response, greater simplicity, high sensitivity and lower cost compared to other classes of the biosensors.

Amperometric biosensors measure the change of the current that produced during the oxidation or reduction of a product or reactant usually at a constant applied potential. The most important factor affecting on the amperometric biosensors is the electron transfer between catalytic molecule; for this aim generally using enzyme labelling DNA sequences for oxidase or dehydrogenase, such as; HRP (horseradish peroxidase) or AP (alkaline phosphatase) and the electrode surface most often involving a mediation or conducting polymer.

GU Tingting et al. reported an electrochemical measurements of the DNA-Cu(II)/chitosan membrane-modified GCE revealed that the copper ion embedded in the DNA/chitosan layer exhibited good electrochemical behaviors. The DNA-Cu(II)/chitosan/GC electrode showed an excellent electrocatalytic activity for the H_2O_2 reduction [35]. P. Abad-Valle et al. also used the enzyme reaction in a related DNA assay that is interaction with alkaline phosphatase-labelled streptavidin permits amplified indirect electrochemical detection. The analytical signal is constituted by an electrochemical process of indigo carmine, the soluble product of the enzymatic hydrolysis of 3-indoxyl phosphate. The use of a sensitive electrochemical technique such as square wave voltammetry [36].

Potentiometric biosensors measure the difference in the potential between two electrodes immersed in a solution. One of the electrodes is reference electrode and has a constant potential which is independent of its environment. The potential of the probe electrode is the potential at the interface between the solid and liquid phases, where oxidation and reduction reactions occur. Equilibrium is achieved when the rates of the oxidation and reduction reactions equal. The equilibrium potential is then given by Nernst equation which is defined in Eq. (1.1)

$$E = E_o - \left(\frac{RT}{zF}\right) \ln\left(\frac{[red]}{[ox]}\right) \tag{1.1}$$

Where [ox] and [red] represents the concentrations of the oxidized and reduced forms of the system, respectively. A biological component may be attached on the probe electrode by hydrophobic membrane to create a potentiometric biosensor [37]. Also, Potentiometric biosensors with conducting polymers can be produced using pH sensitivity of polymers [38]. Polypyrrole (PPY) sensitivity to NH_3 was used to produce such biosensors [39]. Conducting PPY molecular interfaces have also been implemented to modulate biological function of enzymes and living cells at the electrode surface by adjustment of electrode potentials. Ingebeandt et al. improved the general reliability of the label-free DNA hybridization detection based on silicon FET (Field Effect Transistor) microarrays. Enabling fast and fully electronic readout of ex situ hybridizations, such a method relied on the intrinsic charge of the DNA molecules and/or on changes of the interfacial impedance that followed binding of the target sequence. Channels modified with a fully non-complementary probe and three and two bases mismatched capture oligonucleotides provided reference signals that were subtracted from the outputs generated in the presence of single-base mismatched or perfectly matched probes. The resulting differential readout of the transfer-function cancelled out any signal change due to non-specific binding of the target DNA, thus allowing easy detection of the Single Nucleotide Polymorphism (SNP) in low ionic strength buffers when using low Alternating Current (AC) frequencies. The sensitivity of the method (μM range) required, however, further improvement [40].

1.2.2.2 Optical Biosensors

Optical biosensors are based on the measurement of light absorbed or emitted as consequence of a biochemical reaction. In such type of biosensors, light waves are guided by means of optical fibers to suitable detectors. The best example for optical studies, SPR (Surface Plasmon Resonance)- based genosensors. According to Tothill et.al developed of surface chemistry for SPR-based for the detection DNA molecules. They were costructed on SPR sensor chips to investigate DNA immobilization on Au surface. The surface chemistries are composition of the alkaethiols, dendrimers and self-assembled monolayer [41].

1.2.2.3 Piezoelectric Biosensors

These biosensors operate on the principle of generation of electric dipoles on subjecting an anisotropic natural crystal to mechanical stress. Due to the adsorption of an analyte, the mass of the crystal is increased resulting in altered frequency of oscillation. Such type of biosensors have been utilized for the measurement of DNA.

Mutlu et. al developed quartz crystal microbalance (QCM)-based DNA biosensor system. The developed QCM-based DNA biosensor represented promising results for a real-time, label-free, direct detection of DNA samples for the screening of GMOs [42]. Nicu et. al developed a new device based on 4 × 4 matrix micro-machined resonating piezoelectric membranes used as DNA–DNA hybridization biosensor is proposed. They used to measure the mass loading induced by the binding of streptavidin–conjugated gold nanoparticles to biotinylated target complementary DNA fixed onto the surface of the piezoelectric membranes. Obtained results indicate that micro-machined piezoelectric membranes have real potential as micro-mechanical biosensors [43].

1.2.3 Probe Immobilization Techniques

Immobilization is a technique to fix the recognition elements on the electrode surface. While developing a biosensor, one of the most important step is the method used to immobilize the recognition DNA on the transducer surface. A typical DNA biosensor designed by the immobilization of a single stranded (probe) DNA sequence on a transducer surface to recognize its complementary (target) DNA sequence via hybridization [44]. DNA has to be immobilized in a way that the bases remain available for further biorecognition of the complementary sequence. In this sense, the nature of the electrode plays a very important role. In effect, depending on the compromise of the bases in the interaction with the electrode surface could be or not to be accessible for the double helix formation [45]. There are various working electrode types such as gold, glassy carbon, platinum, carbon, paste and mercury. Various methods have been developed to attach the DNA probes to the solid surfaces of the DNA biosensors. The most common probe-immobilization approaches are: covalent bonging, adsorption, affinity interaction, entrapment and self-assembled monolayer.

1.2.3.1 Covalent bonding

The single stranded DNA sequences are immobilized via covalent chemical bonds between the transducer (working electrode) surface and a functional group of the DNA sequence, onto derivatized surface (glassy carbon or glassy carbon paste, platinum or gold surfaces) or crosslinking where a bifunctional agent is used to bond chemically the transducer to the single stranded DNA sequences or by means of spacer such as gluteraldehyde or carbodiimide.

1.2.3.2 Adsorption

This method based on the direct adsorption of the DNA on the substrate such as Nitrocellulose, nylone membranes or metal surface. Adsorption mechanism are generally categorized as either physical adsorption [46] which is carry out by soaking the surface with the solution that needs to be immobilised and leaving the surface to dry or electrochemical adsorption which uses the fact that the DNA backbone is negatively charged [47] so that a positive potential applied to an electrode attracts these biomolecules. Adsorption has the advantages of its easy of operation and it does not require other reagents or any special nucleic acid modification but its principal disadvantage is the variability of the nucleic acid layet due to distortion of the molecule by adsorption and consequently the poor hybridization efficiency.

1.2.3.3 Affinity interaction

Streptavidin and avidin are of the most stable proteins known. Its properties along with the ability of biotin to be incorporated easily into various biological materials, allows streptavidin to serve as versatile, powerpul affinity tag in a variety of biological applications. Due to strong binding between streptavidin/avidin and biotin, the both have been the most widely used affinity interaction in single stranded DNA immobilization [48].

Mir and Katakis proposed the use of competitive displacement of labelled probes and thet immobilized the biotin-capture probe through a biotin-streptavidin linkage [37] and another good example for attachment of biotinylated DNA probes through biotin-avidin interaction on the electrode surface [49,50].

1.2.3.4 Entrapment into a composite

In this method, the probe DNA sequences are immobilized by mixing mineral oil and graphite powder and with conductimetric paste the biosensor is constructed. This method does not require the modification of the probe sequences, however there is a limited accessibility to the capture probe for the hybridization of the complementary (target) sequences. Cho et al. [51] characterized the immobilization of thiol-modified oligomers on Au surfaces and subsequent hybridization with a perfect matched or single-base mismatched target using a QCM and fluorescence spectroscopy.

1.2.3.5 Self-assembling Monolayer

Self-assembling monolayer (SAM) of thiolated oligonucleotides is formed by spontaneous adsorption or chemical binding of molecules from a homogeneous solution onto a substrate. Gooding et.al study shows that immobilization of DNA in the form of a SAM onto gold surface using thiol chemistry [52]. SAM of therminally-thio-labeled oligonucleiotides onto gold surfaces offers a direct method of chemisorption of DNA probes onto transducer surfaces based on the formation of gold-thiol bonds [53]. The most widely used SAM in DNA immobilization is made by the adsorption of sulphur-based compounds such as thiols, disulphides or sulphide on glass or a metal surface such as gold, silver, and platinum. A mixed SAM consisting of single-stranded DNA and oligo (ethylene glycol)-terminated thiol, was reported by Boozer et al. [54] and another good example for self-assembly of organized monolayers of thiol-functionalized probes on gold electrodes [55].

1.3 DNA BIOSENSORS

Presently, there are two conventional ways used for detection and identification sequences or genes on the genome; by sequencing, i.e. reading DNA sequences base-by-base and by hybridization or binding of target sequences to pre-designed complementary probes, in which consequent stretches of bases or "words" of the genome are read at a time.

Genomic technologies currently in use are biosensors, whose operation relies on the specific binding between two complementary single-strands of DNA as described by the Watson-Crick base-pairing rules. Complementarity is managed by the Watson-Crick rules for base pairing such as occures double hydrogen bonds between tymine-adenine, also triple hydrogen bonds between cytosine and guanine bases. Basically, working principle of DNA-biosensors; hybridization process relies on the immobilization of a single-stranded DNA probe onto the electrode surface. The development of DNA-based biosensors on single-stranded nucleic acid, consists several steps. The first step, which is most important for sensitivity of the DNA-biosensor, a single-stranded DNA is containing the sequence of interested part of the genome, must be isolated from cells or synthesized on commercially. The design of genosensor is based on the hybridization dynamics of nucleic acid. Detection of DNA hybridization usually involves monitoring a current response, on the basis of a complementary matching recognition process, under controlled potential conditions [56]. The readout of the hybridization event in DNA-based biosensors occurs usually at a solid surface to which a recognition element (probe) is attached either physically or chemically or indirectly through a electrode surface, e.g. a polymer adsorbed or covalently attached. The target analyte in solution is selectively captured by a surface-bound probe for example in the case of single strand DNA (ssDNA) target binding to its tethered DNA probe complement according to Watson-Crick pairing rules; this biochemical interaction is then translated into a detectable signal by a given transduction mechanism and the signal is in turn processed by a computer for its final interpretation [57].

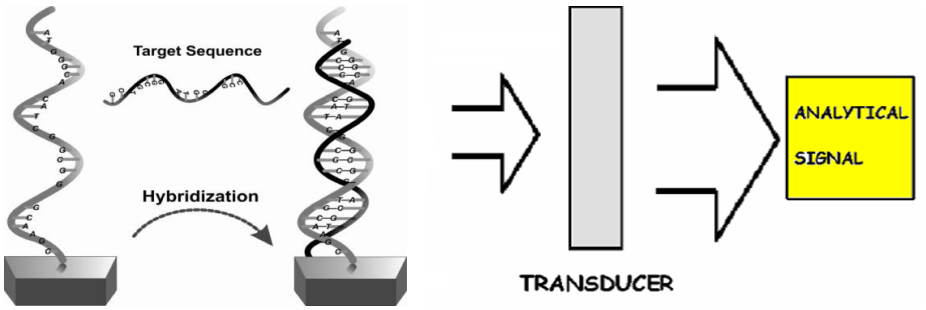


Figure 1.8 Schematic representation of DNA-biosensors.

1.3.1 DNA Structure

The nucleic acids (DNA and RNA) are polymers of nucleotides. They store and transmit genetic information. Both of them contain four major Purine and Pyrimidine bases; Adenine(A), Guanine (G), Cytosine (C) and Thymine (T) in DNA and Uracil (U) in RNA. The structures of the major bases are shown in Figure 1.9.

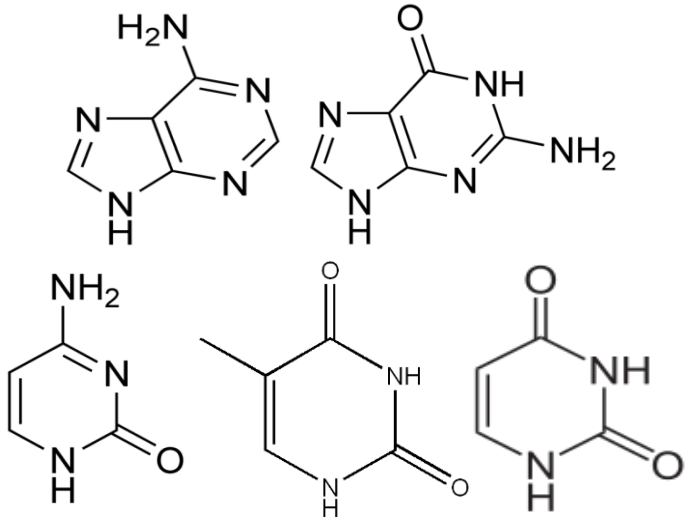


Figure 1.9 Major purines and pyrimidines from which nucleic acids are built [58].

Nucleic acids have two kinds of pentoses. The chemical structure of the pentose which contains five carbon atoms, labelled as C1' to C5'. The pentose is called ribose in RNA and deoxyribose in DNA because the DNA's pentose lacks an oxygen atom at C2'.

A nucleotide is composed of three main parts; Sugar (pentose), base (A, G, C, T or U) and phosphate group (see Figure 1.10). In DNA or RNA, a pentose sugar is interact with one phosphate group. If this phosphate group is removed, a nucleotide becomes a nucleoside.

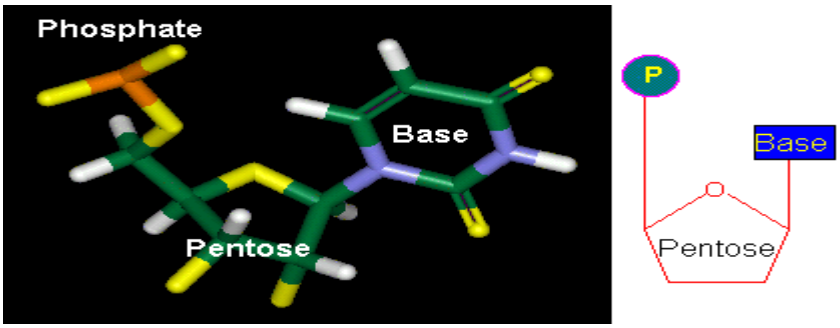


Figure 1.10 Structure of nucleotides. Right side, a simplified representation and left side is represents of computer model.

The consecutive nucleotides are covalently linked due to phosphate-group ‘bridges’, in which the 5’-hydroxyl group of one nucleotide unit is joined o the 3’-hydroxyl group of the next one by a phosphodiester linkage. Thus the covalent backbones of nucleic acids consist of alternating phosphate and pentose group, and the nitrogenous bases may be regarded as side groups joined to the backbone at regular intervals [59].

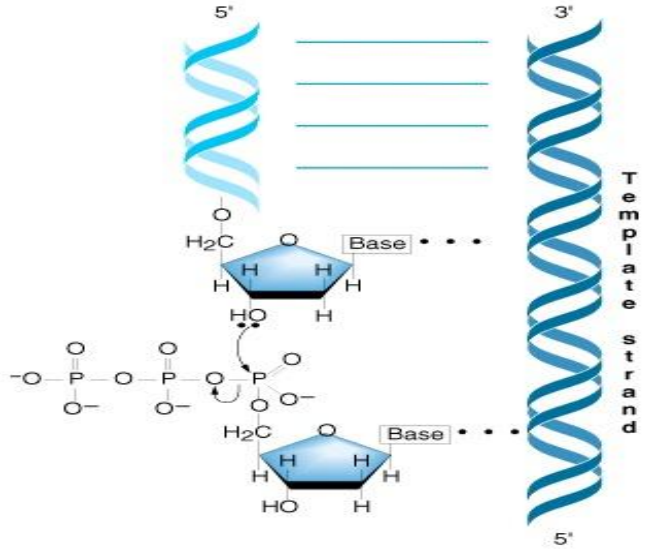


Figure 1.11 Formation of a 3'-5' phosphodiester bond in the covalent backbone of DNA synthesis. The phosphodiester bonds link successive nucleotide units. A nucleotide is about 0.34 nm long.

Since Watson and Crick proposed the double helix structure of deoxyribonucleic acid (DNA) in 1953, later in this process; identification and sequencing of DNA molecule have received much importance and popular [60]. The DNA is carrying genetic information are called genes. A gene is a molecular unit and the foundation material of heredity. Its basic role in the transmission of heredity information. It has great significance for healthy heritage. Thus DNA is the basic building of life. DNA sequences have structural purposes and each of them are involved in regulating the use of this genetic information. Also DNA, as the main component of gene chromosomes in the cells. All of the genetic information is determined precisely by the base sequences in the DNA. The DNA sequences which are distinct in any living organism, pathogen or virus and provide practical ways to identification and diagnosis for different kind of genetic diseases [61]. Any disorder in these base sequences can result in the degeneration of inheritance or the occurrence of diseases.

About DNA structure; it is a double-stranded molecule twisted into a helix. Each strand, comprises of a sugar-phosphate background and attached bases, is connected to a complementary strand by hydrogen bonding between paired bases. The bases are adenine (A), thymine (T), cytocine (C) and guanine (G).

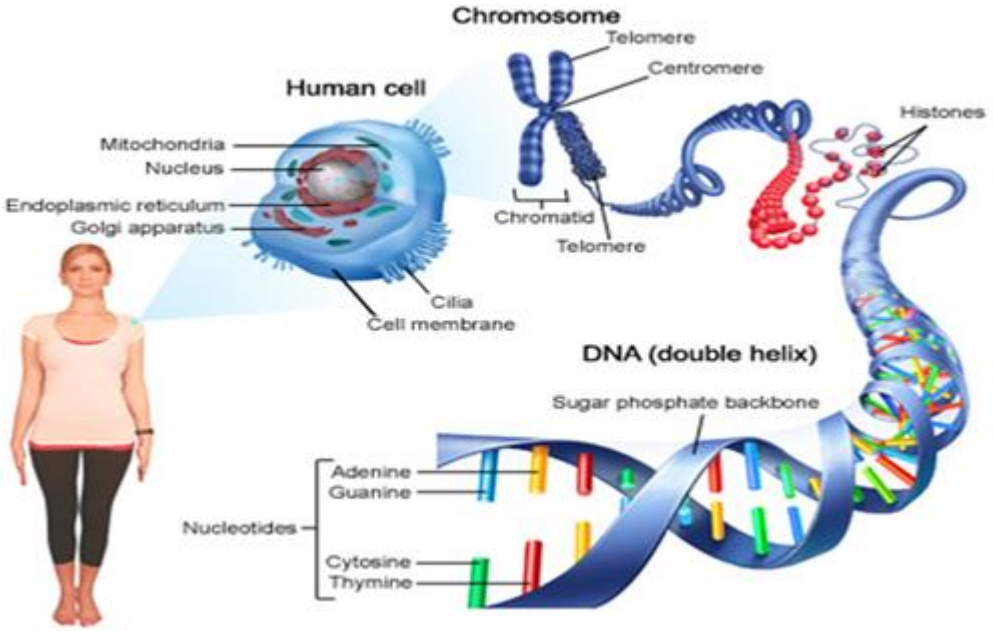


Figure 1.12 DNA that makes up genes is spooled within chromosomes inside the nucleus of a cell.

1.3.2 DNA Hybridization

The joining of the two complementary single strands of DNA through hydrogen bonding to form a double-stranded DNA is called hybridization [62]. As mentioned that to fulfill its main role (replication or expression of genetic information), the DNA double helix has the ability to separate the two strands without disrupting the covalent bonds that hold each strand intact. This temporary separation of the DNA strands and the subsequent reformation occurs under physiological conditions at rates needed for the maintenance of these genetic functions.

The noncovalent forces that stabilise the double-stranded DNA (dsDNA) structure may be disrupted by heated above a certain temperature, the two strands will start to dehybridize and eventually seperate into single strand, or exposure to low concentrations of salts. The process of strand separation is called denaturation. The single-stranded DNA (ssDNA) product is relatively stable, but removal of the denaturing conditions allows the DNA to re-form (re-anneal) the double-stranded helix. Such as the temperature is reduced, the two strands will eventually come together by diffusion and rehybridize to form double stranded structure. Similar hybridization process can be form between any single-stranded nucleic acid chains: DNA/DNA, RNA/RNA, and DNA/RNA (see Figure 1.13). These reaction process can be used to detect and characterize nucleotide sequences using a particular nucleotide sequence as a probe.

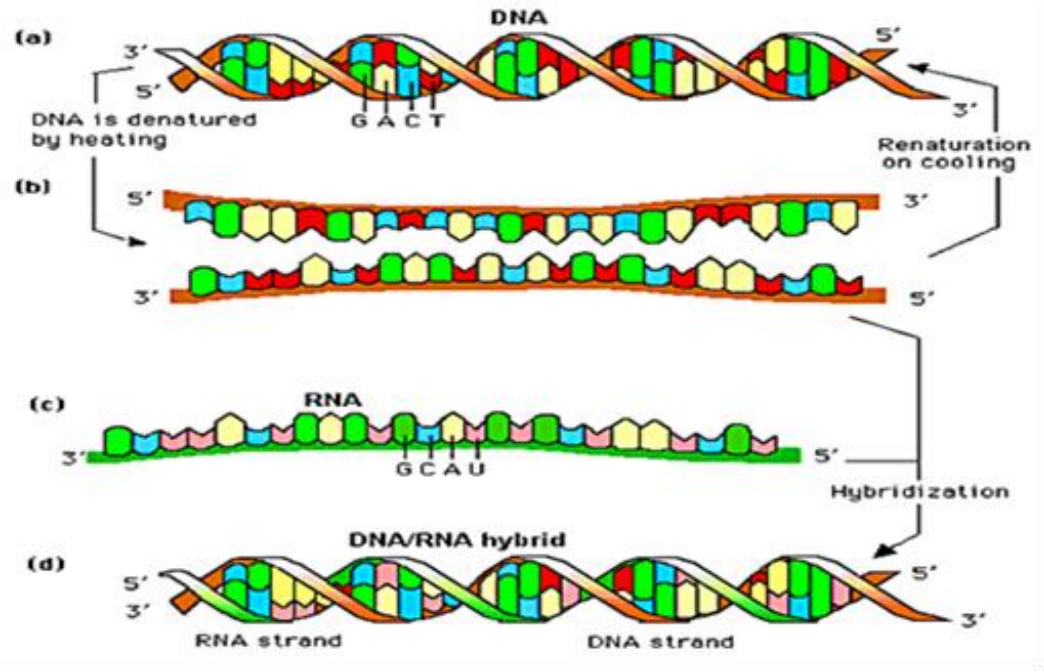


Figure 1.13 The process of DNA renaturation and hybridization. The two strands of a DNA molecule are denatured by heating to about 100°C (a to b). The DNA denaturation is reversible by keeping the two single stands of DNA for a prolonged period at 65°C (b to a). This process is called DNA renaturation or hybridization.The RNA competes with the coding DNA strand and forms double-stranded DNA/RNA hybrid molecule (c to d).

1.3.3 Transduction Mechanism

The transducer surface interaction between recognition elements is in most cases connected with a measurable change in the properties of the recognition elements themselves or of the surrounding local environment of electrode surface. The measurable change or transduction mechanism can consist of changes in electrical, optical or mass properties that are translated into a detectable signal that is processed electronically by a computer for its interpretation.

Briefly; for the transduction mechanism of DNA-based biosensor devices to be realised, three important steps need to be performed: the fabrication of an appropriate transducer, the selection of a recognition element able to ‘probe’ the analyte of interest and the immobilisation technique of a suitable probe onto the surface of the electrode. The use of nucleic acids has shown considerable potential in hybridisation studies for the detection of complementary oligonucleotides [63].

1.3.3.1 Non-Electrochemical Techniques

Depending on what physical property changes are sensed upon hybridization between nucleic acid molecules on the electrode surface, non-electrochemical transduction mechanisms can be classified as follows: optical [64] and gravimetric [65, 66].

First of all, optical methods of detection usually make use of fluorescently-labeled DNA targets by exciting the fluorescent labels with an intense light source, e.g. a laser and detecting the emitted photons. Total internal reflection fluorescence (TIRF) is an example of these methods [67, 68], consisting in the attachment of DNA probes on the surface of an optically dense solid medium such as glass on one end and are immersed in a less optically dense medium such as water.

The advantage of this optical method over traditional microarray scanning methods is that hybridization can be captured in real time owing to its high surface-specificity and therefore does not require disruptive washing steps that are necessary in traditional methods for the suppression of background noise. The sensitivity of TIRF has been claimed to go down even to the single molecule level. As an example of single molecule detection with a two-color detection scheme, Riittinger [69] labeled two alleles that differed only by one nucleotide with different fluorescent tags. The tags where incorporated by the use of a primer which facilitated the addition of a chain terminating fluorescently-labeled nucleotide at the SNP site of each allele. An equimolar solution containing both alleles was spotted onto polylysine-modified glass slides, after which two lasers of different wavelength were used to excite the corresponding labels. The emitted photons were sorted out by an appropriate emission filter allowing the number of adsorbed molecules to be counted. From the results it was concluded that the relative counts at the surface were reflective of the molar ratio of the molecules in solution and it was proposed that the method was reliable enough to be used as a genotyping assay.

Other optical methods rely on dielectric property changes at the interface of a metal and an organic biofilm, e.g. a nucleic acid monolayer. Such is the case of surface plasmon resonance (SPR) [70]. SPR has a number of configurations and designs, but in general it consists of a prism onto which a metal film (Au or Ag) is deposited; DNA

probes are then bound to the metal support, say through a metal/thiol bond and sit inside an electrolyte solution which contains DNA analytes. A laser (~600 nm or more generally, in the IR-visible wavelength region) is then passed through the prism and by striking the back end of the gold film excites surface plasmons at a specific resonance frequency. This resonance depends on the dielectric properties of the attached nucleic acid probe region; also, the angle of incident light at which the resonance occurs, the critical angle, changes upon variations in the dielectric properties of the biofilm and thus one has a means of tracking the development of hybridization in real time by exploiting the concomitant dielectric (or relative permittivity) changes. Lao and group did a recent study which compared DNA hybridization to both DNA and PNA probes by the use of SPR [71].

Gravimetric techniques track changes in mass on the electrode surface of piezoelectric substrates that occur with the creation of nucleic acid/DNA (or RNA) duplexes. Piezoelectric materials are able to transduce electric fields into mechanical stress. Therefore by applying an oscillating electric excitation one causes the piezoelectric material to vibrate and so generates mechanical waves of a desired frequency, where the sprawling waves can be made to oscillate perpendicularly- or in parallel to the underlying surface. An example is quartz crystal microbalance (QCM) where a quartz crystal slab is 'sandwiched' between two gold electrodes and an alternating electric field generates a standing wave within the crystal. For example, Okahata [72] made use of DNA probe molecules tethered to a QCM device to sense the hybridization with the complementary sequence within a circular M13 phage DNA molecule. A control non-complementary sequence was also used which did not cause a change in resonance frequency. An end-point detection of the hybridization event between PNA probes and DNA targets by the use of QCM was carried out by Ananthanawat and colleagues [73].

Another type of gravimetric sensor is that termed a surface acoustic wave (SAW) device. These sensors consist of two resonators: an input and output resonator, which commonly consist of interdigitated gold films that have been evaporated on a piezoelectric material. The two resonators are placed a distance apart from each other so that the waves generated at the input have a stretch of piezoelectric material to traverse before reaching the output resonator. Nucleic acid probes are therefore immobilized

onto this intermediate region and when binding DNA targets the increase in mass varies the mechanical properties of the piezoelectric device affecting thus the propagating mechanical wave. Differences in the input versus output signal characterize the medium crossed by the waves and serve as the transduction mechanism by which one detects the seizure of DNA targets. Recently, PNA probes were used in the diagnosis of human papilloma virus using a SAW sensor [74].

1.3.3.2 Electrochemical Techniques

In this section, to give some basis regarding techniques in detection by electrochemical biosensors. Basically electrochemical methods used to detect hybridization between nucleic acids are numerous and can be classified based on the techniques used: cyclic voltammetry [75, 76], differential pulse voltammetry [77], amperometry [78], chronopotentiometry [79] and electrochemical impedance spectroscopy (EIS).

Electrochemical biosensors are usually composed of three electrodes: a reference electrode, a counter electrode and a working electrode. The role of the reference electrode is maintaining a known and stable potential. The signal transduction occurs through the working electrode whereas the counter electrode serves as electric connection between the electrolytic solution and the working electrode [80].

Voltammetry consists in varying a potential and measuring the resulting current. There are many ways to vary the potential: differential pulse, polarography, differential staircase, linear sweep, etc. The most commonly used is cyclic voltammetry. The potential varies from two values V1 and V2 at a defined rate. When the potential reaches V2, the reaction is reversed and the potential comes back to V1. The results are plotted on a voltammogram: current against voltage. The current is measured between the working and counter electrodes and the voltage between the reference and working electrodes. Another example is a differential pulse voltammetry. Its wave form is composed of small pulses with constant amplitude that form a staircase. During chronoamperometry, the current is measured when a squarewave potential is applied to the working electrode. Thus a function of the current against time is obtained and changes in redox signals can be detected. Electrochemical Impedance Spectroscopy (EIS) consists in applying a sinusoidal potential and measuring the resulting current.

EIS is able to give information on the resistance and reactance of the system. Thus, it is used to detect changes in electrochemical characteristic of the biosensor surface. EIS can evaluate modifications in capacitance and, in this case, is refered to Faradic Impedance Spectroscopy. Field-Effect Transistor (FET) is a transitor that controls the conductivity of a channel by an electric field. A metal gate is laid between the source and the drain of the transistor. It is able to monitor changes in charges at the electrode surface. There are different types of FET. Among them, ion-selective field-effect transistor (ISFET) is widely used for biosensing application. In an ISFET, the metal gate is replaced by a ion sensing membrane.

1.3.3.2.1 Label-Based Electrochemical Detection

Electrochemical detection mode was developed for detection of labeled DNA targets. Early DNA based-biosensors made use of so called redox- or electroactive indicators as a way to detect the hybridization event. The first method allows a direct transduction translating the recognition behavior in a readable signal. These indicators interact with nucleic acids at the surface and their presence is determined by controlling the potential at the conductive support in such a way that indicators engage in oxidation/reduction reactions, yielding or receiving electrons respectively. After the application of target or complementary sequence on the probe immobilized surface, generally some specific materials are used for labeling process. Depending on the nature of their interaction with DNA molecules, indicators can be classified as intercalators (which intercalate between stacked bases in double-stranded DNA), groove-binders (which bind to the DNA major or minor grooves) and redox tags which are covalently bound to target molecules and/or probes. Indicators that bind to the sugar-phosphate backbone through electrostatic interaction have been used, but have the disadvantage of being less selective because they bind both single- and double stranded DNA molecules. However, electrostatically interacting indicators have been used to quantify number of surface-bound single-stranded and double-stranded DNA molecules [81, 82]. The labeling step enhances the sensitivity and the selectivity, but also increases the time, complexity and cost of measurement. Intercalators sit in-between the stack of bases formed upon hybridization and are thus specific to double-stranded DNA or PNA [83].

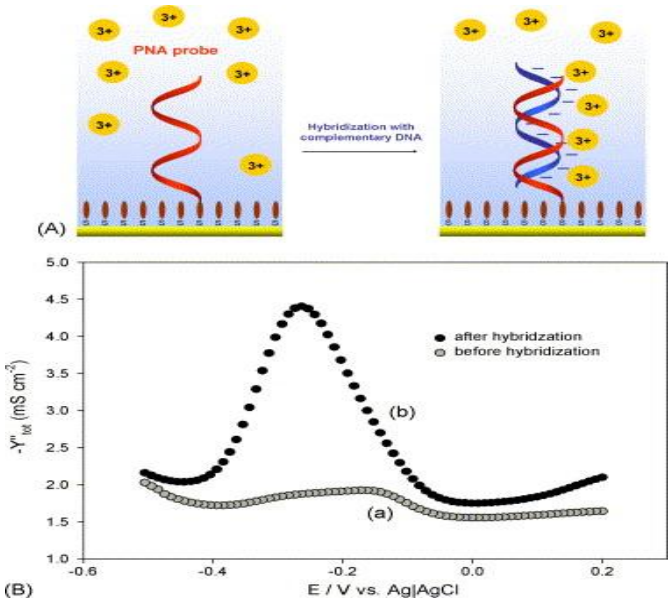


Figure 1.14 (A) Schematic representation of the electrostatic DNA hybridization detection method using $[Ru(NH3)_6]^{3+}$ as redox marker and PNA as capture probes on gold. (B) Imaginary admittances of the PNA sensor before (a) and after (b) hybridization with complementary DNA [80].

Some commonly used intercalators are: Methylene blue [84], organometallic compounds such as $Co(Phen)^{33+}$ (Cobalt phenantrolin complex) [85] or $Co(Bpy)^3$ (Cobalt bipyridine complex). An example of a groove-binder is Hoechst 33258 [86, 87]. Main disadvantages of using these types of indicators are that they tend to be toxic (e.g. daunomycin) and; complete knowledge of their interaction with DNA molecules is usually needed to interpret results quantitatively. For example, one talks of the number of binding sites occupied by the indicator, i.e. how many DNA nucleotide units are needed to accommodate it, a number which may depend on charge, size and solution concentration further confounding quantitative measurements [83].

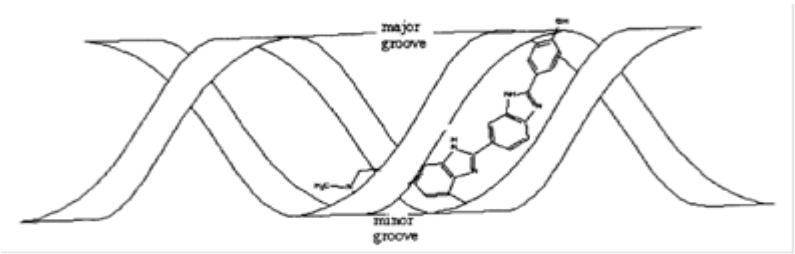


Figure 1.15: The binding of Hoechst 33258 to minor groove of DNA structure [86].

1.3.3.2.2 Label-Free Electrochemical Detection

In the last part of DNA hybridization detection techniqeus of electrochemically, to consider label-free DNA hybridization and genetic mutation detection technologies that have emerged where the transduction mechanism is based on either: 1) the intrinsic electrochemical properties of nucleic acid molecules or; 2) changes in interfacial properties as a result of the progress of hybridization. Examples of the former are the oxidation of nucleotide bases such as guanine and adenine [88, 89, 90]. As an example, two studies exploited the oxidation of guanine bases and used it as an indicator of DNA target capture [91, 92] where a decrease in redox activity of the bases was expected upon hybridization because the hydrogen bonds formed compromise the oxidation of the bases. These methods eliminate the need to label target or probe molecules. The second sets of methods are based on changes in surface charge (and thus surface potential) as an indicator of the occurrence of hybridization shown in Figure 1.16.

Impedimetric sensors measure changes in impedance of the nucleic acid layer at the interface, of the underlying support or both. Impedance in these instances is considered as a whole, in other words it involves a combination of resistance, capacitance and even inductance changes. Quite often an enzyme amplification strategy is used in impedimetric sensors in order to confer a higher sensitivity to the target capture event, where an enzyme catalyzes the transformation of reporter molecules in solution into electroactive or insoluble species thus enhancing or hindering the transfer of charge carriers at the surface respectively. Capacitive sensors, by contrast, often measure the ability of charge carriers to redistribute within the probe layer, the support (in the case of semiconductor substrates) or both as well as changes in dielectric properties of the interfacial region.

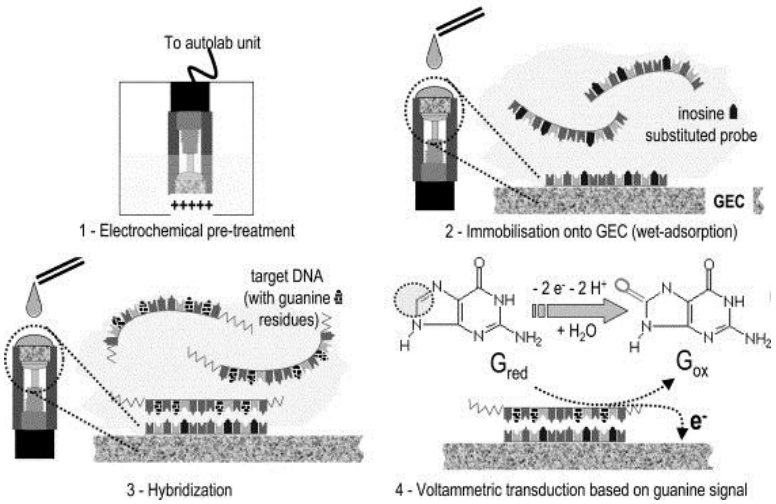


Figure 1.16 Schematic representation of the label-free electrochemical DNA biosensor related to the intrinsic electrochemical properties of guanine bases on Graphite–epoxy composites (GEC) (1) Electrochemical pre-treatment step. (2) Wet-adsorption immobilization step of an inosine substituted probe on the GEC electrode. (3) Hybridization step. (4) Voltammetric transduction step based on guanine oxidation signal [90].

To date there have been relatively few reports on label-free DNA hybridization assays which rely on changes in surface impedance or capacitance, perhaps the result of a poor understanding of the fundamental processes involved [93]. Hybridization events in impedimetric biosensors are in many instances characterized by a change in the charge transfer resistance Rct at the electrode surface in which the charge transfer to or from redox active reporter molecules in solution is enhanced as they are accumulated at the surface due to their affinity for DNA hybrids [94, 95] or; charge transfer may be mitigated when electroactive species in solution are hindered from reaching the surface as they are electrostatically repelled by the negative charge barrier erected by nucleic acid/DNA hybrids [96].

Alfonta et al. tethered oligonucleotide DNA probes onto a gold electrode immersed in a buffer solution containing the Fe $(CN)_6$ redox couple [97]. They showed that the electrostatic barrier that grows with formation of hybrids at the surface makes it more difficult for the redox molecules to reach the electrode and results in a diminished rate of electron transfer (i.e. current). The electrode response to the progress of hybridization is characterized by the enlarging radius of the semicircular trace in a Nyquist plot which is indicative of an increased charge transfer resistance {Rct}.

An interesting feature of this study is that probe ligation and its subsequent enzymatic scission was also detected by the use of this method. A similar scheme was used by the same group to detect the presence of a portion of the Tay-Sachs gene [96]. Peng and colleagues also used the rejection of the ferricyanide/ferrocyanide redox couple upon hybridization by tethering DNA probes to an electropolymer. [98]. Schematically probe immobilization shown in Figure 1.17.

Liu et al. [99] also used the above strategy in which mitigation of the current from the reduction of Fe (CN) $_6$ " indicates the evolution of hybridization. Due to the absence of both rapid and sensitive diagnostic tools for breast cancer, it cannot be detected in its early stage which is the most crucial point in treatment. Although the disease has available and widely investigated markers, the breast cancer has scarcely been studied with biosensing technology. In this study, genetic markers were evaluated by electrochemical-based biosensor platforms and the genetic cancer biomarkers were investigated with neither labelled probes nor time-consuming processes that lead to decrease sensitivity and accuracy of the results. With label-free, electrochemical-based biosensor technology we tried to achieved to detect different concentrations of the studied genetic biomarkers. Here, the development of a rapid and sensitive electrochemical electrode sensor assay for the detection of genetic markers for early diagnosis of breast cancer has presented.

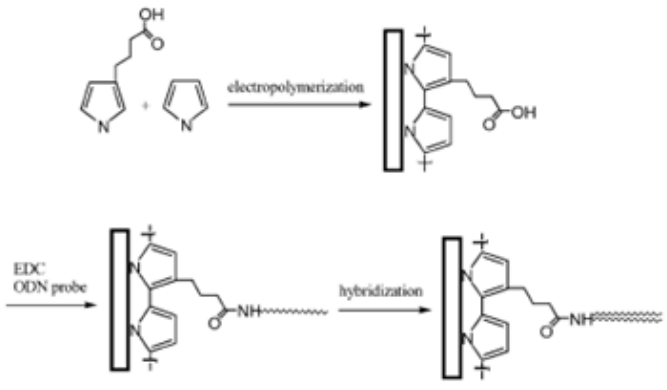


Figure 1.17 Schematic representation of the electrochemical DNA biosensor based on conducting polymers; related to changes in interfacial properties as a result of the progress of hybridization [98].

1.4 SUPPORT FOR BIOSENSORS CONSTRUCTION

1.4.1 Dendrimers

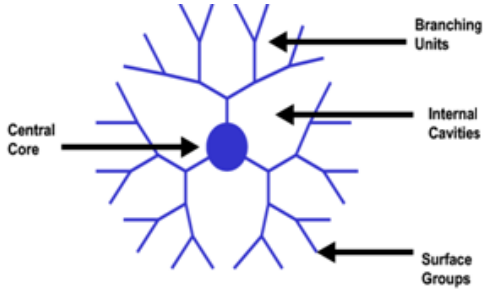


Figure 1.18 Structure of the dendrimers.

The name dendrimer is derived from Greek words dendron meaning "tree" and meros meaning "part." A major difference between linear polymers and dendrimers is that a linear polymer consists of long chains of molecules, like coils, criss-crossing each other. A dendrimer consists of molecular chains that branched out from a common center, and there is no entanglement between each dendrimer molecules. The first synthesis of these macromolecules is credited to Fritz Vögtle and coworkers in 1978 [100]. Dendrimers are a new class of polymeric materials which are new artificial macromolecules. They are globular, highly branched and monodisperse three-dimensional molecules with defined molecular weights, large numbers of functional groups on the surface. Due to their unique physical and chemical properties, dendrimers have wide ranges of potential applications. They are promise to be new, effective biomedical materials as surface modifications of development of biosensors. Dendrimer molecules can be basically described as the result of the sequential modification of a polyfunctional core with multifunctional monomers, also called dendrons. They are regular tree-like macromolecules, constituted of a series of branches linked to a central core and they composed of a large number of monomer units that were chemically linked together. In recent years, the dendrimers belong to a new class of synthetic, macromolecule possessing a regularly branched treelike structure. The dendrimers can be used to modify electrode surface due to their good biocompatibility and suıtable functional groups for chemical fixation.

1.4.1.1 PAMAM Dendrimers

Among the dendrimers, Poly(amidoamine) (PAMAM) have been widely studied because it allows the precise control of size, shape, placement of functional group, minimum toxicity and wide availability [101]. Polyamidoamine (PAMAM) dendrimers are a unique class of highly branched polymeric macromolecules with numerous arms extending from a center [102]. PAMAM dendrimers having NH_2 terminal groups were then covalently linked on the precise places where the aldehyde groups were placed. PAMAM dendrimers, initially developed by Tomalia et. al in the early 1980s. [103]. Analytical chemists have paid attention to the significant application potential on biosensor of the polyamidoamine (PAMAM) dendrimers because of their high geometric symmetry, easily controlled nano size, controllable surface functionality, and chemical stability [104, 105]. Dendrimers are capable of increasing the concentration of hydrophobic molecules at the electrode-solution interface, improving the sensitivity as well as the selectivity of certain specific electrochemical reactions [106]. The dendrimers can be used to modify electrode surface due to their good biocompatibility and suıtable functional groups for chemical fixation. Interest in dendrimers has grown steadily over the past decade due to use of these molecules in numerous industrial and biomedical applications [107, 108]

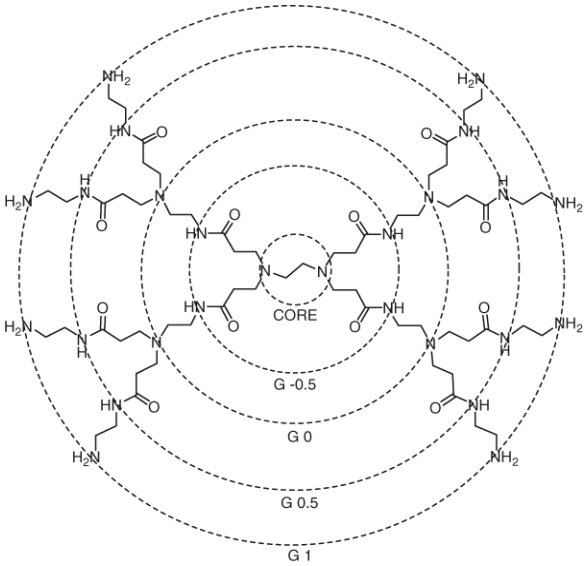


Figure 1.19 Structure of the PAMAM Dendrimers.

The synthesis of dendrimers can be carried out in two major ways: by a divergent approach where the molecule grows from the center to the periphery, and a convergent approach (see Figure 1.20) where the dendrimer molecule is built starting from the periphery fragments. The choice of the divergent or convergent synthetic methods is determined by the available chemical reactions, requirements toward the dendrimer molecules, or the type of the "building blocks" used in the dendrimer construction [109]. The concept of repetitive growth with branching was first reported [100] in 1978 by Vögtle who applied it to the construction of low molecular weight amines. This was followed [110] closely by the parallel and independent development of devergent, macromolecular synthesis of true dendrimers in Tomalia Group.

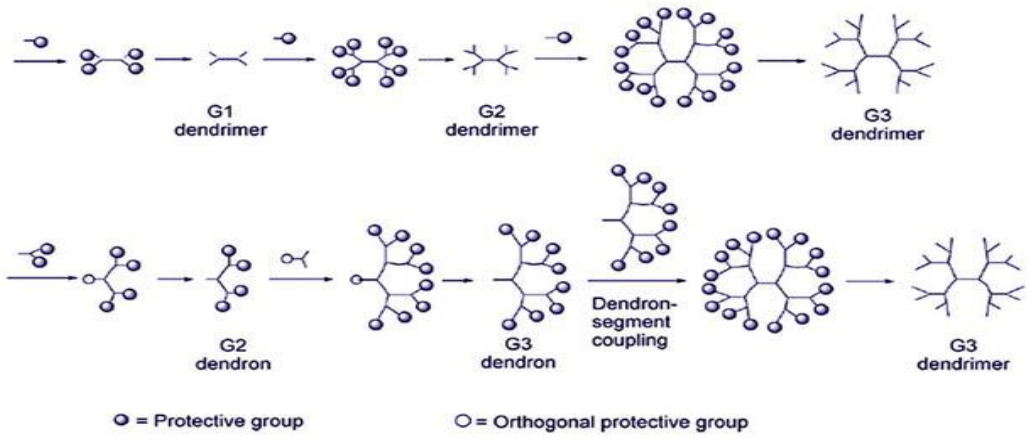


Figure 1.20 Schematic of dendrimer growth by the divergent and the convergent methods.

Recently, increasing attention has been received in sensing applications. The amine or carboxyl-terminated dendrimers on their periphery have the advantages of being able to be conjugated to other molecules via an amide linkage, which is one of the most fundamental and widespread chemical bonds in nature. For instance, the amine-terminated PAMAM dendrimers were attached to an activated mercaptoundecanoic acid (MUDA) self-assembled monolayer (SAM) via covalent amide linkages [111].

The fourth-generation polyamidoamine (G4 PAMAM) polymers, is of particular interest because of its nanoscopic spherical structure and good biocompatibility.

G4-PAMAM dendrimer possesses 64 primary amine groups on the surface and has a globular shape with a diameter about 4.5 nm. According to articles when using G4 PAMAM to immbilize DNA showed high sensitivity and selectivity for DNA hybridization assay [112]. Aixue et al prepared self-assembled gold electrode with G4 PAMAM dendrimers and aminoethanethiol to immobilize DNA, and used electrochemical impedance spectroscopy (EIS) to monitor the DNA hybridization analysis. It showed high sensitivity and selectivity for DNA hybridization assay [113].

Due to the presence of spherical G4 PAMAM on the electrode, the surface area and the density of amino groups on the electrode increased obviously, and this would result in the increase of the immobilized DNA probe. Thus, a higher sensitivity could be expected for the electrochemical detection of DNA hybridization compared with that of only RSH-DNA-modified Au electrode without PAMAM. Tsukruk et al systematically studied assembled films of dendrimers in monolayers or multilayers on a solid surface [114, 115]. Eunkyung et al used a partially ferrocenyl-tethered poly (amidoamine) dendrimer (Fc-D) to enhance the electronic signals of DNA detection as well as a building block to immobilize capture probe, which provided a new idea for the immobilization of DNA probe on the electrode surface. In this study, a novel DNA electrochemical biosensor has been prepared. First, the GCE surface with carboxyl was prepared via electrochemical oxidation. Then, G1 PAMAM, whose terminal unit has four amino groups, was chemi-absorbed onto the surface of GCE by forming the stable amide linkages. Thus, one DNA monolayer was immobilized on the GCE surface by covalent binding amino groups and 5′-end phosphate groups between PAMAM and DNA [116].

Mao et al. [117] demonstrated a new impedimetric DNA biosensor with second-generation poly (amidoamine) dendrimer (G2-PAMAM) covalently functionalized onto multi-walled carbon nanotube (MWNT) electronic transducers as the tether for surface confinement of probe DNA. G2-PAMAM dendrimer was covalently functionalized onto purified MWNTs and the as-formed G2-PAMAM-functionalized MWNT composite (i.e., G2-PAMAM/MWNT) was used both as the support to confine the single-stranded DNA (ssDNA) probe and as the electronic transducer to form the DNA biosensors. In this context, NH_2-riched second-generation poly (amidoamine) (G2-PAMAM) dendrimer is covalently attached onto carbon nanotube electronic

transducers to serve as the tether for surface confinement of a large amount of probe DNA, as shown in (Figure 1.21). Upon the occurrence of hybridization events between surface-confined ssDNA probe with target DNA in solution to form a double-stranded DNA (dsDNA) at electrode surface, the negative charge in the electrode/electrolyte interface and, as such, the interfacial charge-transfer resistance of the electrodes towards the $Fe(CN)_6^{3-/4-}$ redox couple were changed. Such a change was used for the impedimetric DNA biosensing. The use of G2-PAMAM dendrimer attached onto MWNT electronic transducer as the tether for probe DNA provides a large number of amino groups to increase the surface binding of probe DNA, results in the increase the sensitivity of the impedimetric biosensor for the target DNA.

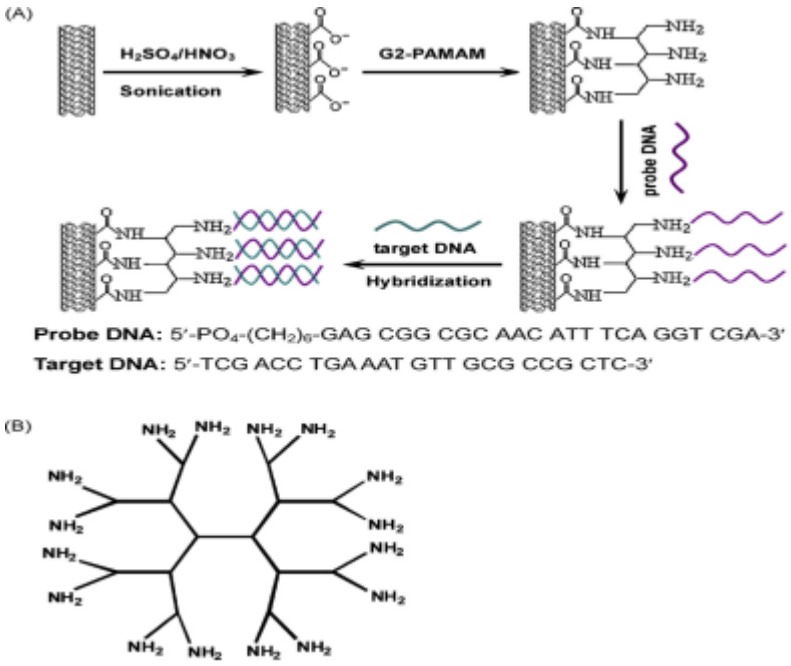


Figure 1.21 (A) Second-generation PAMAM dendrimer is covalently attached onto carbon nanotube electronic transducers to serve as the tether for surface immobilization of a large amount of probe DNA and then hybridization process with target sequences (B) amino end-dendrimer structure [117].

Yang et al. [118] reported bioactive films with the incorporation of amino-terminated G4 PAMAM dendrimers have been prepared via layer-by-layer self-assembly methods on a gold electrode and used for the DNA hybridization analysis. They were applied a multilayered organic thin film with the incorporation of amino-

terminated G4 PAMAM dendrimers was fabricated on gold substrate. The amino-terminated G4 PAMAM dendrimers were first covalently attached to the aminoethanethiol modified gold electrode through glutaraldehyde coupling. Subsequently, the dendrimers were modified with glutaraldehyde again. This three-dimensional, activated PAMAM dendrimer films was used for the immobilization of amino-modified probe DNA. Due to the unique chemical and structural properties of dendrimers and the high sensitivity of EIS technique, this PAMAM film modified electrode exhibited high sensitivity and selectivity for DNA hybridization analysis.

1.4.2 Ferrocene

Elucidation of the structure of ferrocene, $Fe(\eta5\text{-}C_5H_5)2$, in the early 1950's provided a leap forward in understanding the structure of unsaturated organic ligands coordinated with metals through π-orbitals, Many of ferrocene and its derivatives have been synthesized and characterized. They are considerable interest in various areas of research and application, like asymmetric catalysis, functional biomaterials, non-linear optics and electrochemistry due to the quasi-reversible oxidation of iron II [119]. The redox behavior of ferrocene is sensitive to its covalent or noncovalent binding to other molecules.

Cyclic voltammetry (CV) is the tool most commonly used in the electrochemical characterization of redox-active compounds. A unique property of metallocenes is the possibility of introducing substituents on one or both of the cyclopentadienyl rings while retaining the properties of a simple one-electron redox couple. The electrochemical oxidation potential is tunable by changing the nature of the substituents. Therefore, ferrocenes allow the use of a large variety of electrochemical detection (ECD) techniques, including amperometry or voltammetry [120]. We will begin by considering the redox couple formed by the oxidation of ferrocene to the ferrocenium cation. Both the neutral ferrocene molecule and the cation are stable in solution, and the redox couple is said to be reversible. Irreversible couples arise when one of the species formed by oxidation (or reduction) undergoes a chemical change. The species that is then re-reduced is chemically different from the species oxidized and reduction takes place at a voltage different from the oxidation voltage. In principle, the oxidation and reduction of ferrocene take place at the same potential. However, there is typically a finite solution resistance between the working and reference electrodes resulting in a

slight difference in potential between the oxidation and reduction steps (ΔE). A typical CV for ferrocene is shown below.

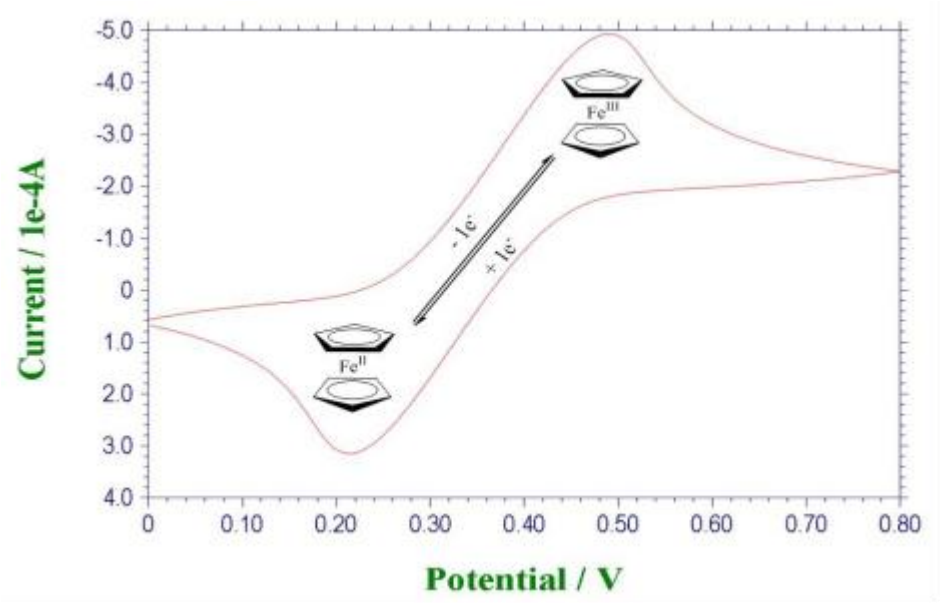


Figure 1.22 Structure and redox behavior of the ferrocene [121].

The horizontal coordinate of the plot is electrochemical potential presented in units of volts or millivolts. The more negative the reduction potential, the more difficult the species is to reduce, and the higher the energy of the electronic orbital receiving the electron. Species that are reduced at high negative potentials are typically strong reducing agents in their reduced form. The vertical coordinate is current in units of microamps. Current is a measure of the number of electrons transferred in the process, and it is typically quite small since the number of electroactive species absorbed on the electrode surface is small. CV plots are conventionally presented with voltage increasing negatively to the right. The current increase above the horizontal is associated with a reduction process as the scan in potential is made in a negative direction and it is said to be cathodic. Once all the species on the electrode has been reduced the scan may be reversed in direction so as to oxidize the species that was just reduced. The electrons produced by the oxidation step appear as an anodic current below the horizontal. If the couple, consisting of oxidation and reduction steps, is reversible the cathodic and anodic currents will be the same. The potential at the

working electrode is monitored and controlled precisely with respect to the reference electrode via the potentiostat (P). The potentiostat is typically interfaced to a computer which controls the scan in potential at the working electrode and records current transfer. The counter electrode is used to compensate for the resistance of the solution.

Bin Fang et al. [122] have designed a new hybridization detection system in order to build an electrochemical based; between a ferrocene-functionalized polythiophene transducer and single-stranded PNA probes on the nanogold modified electrode is investigated (see Figure 1.23). PNA can be used as a coupling substrate for DNA immobilization with good sensitivity, selectivity, and reversibility. The approach does not require the labeling of any nucleic acids probes or targets prior to the analysis, making the method advantageous in terms of speed and low costs. The potentialities of a simple and specific DNA detection method based on the CV and DPV responses of adsorbed ferrocene-containing cationic polythiophene on the PNA–DNA duplex after hybridization has been evaluated. Moreover The level of nonspecific adsorption on these PNA surfaces is sufficiently low so we can clearly distinguish mismatches of only four base pair using CV or DPV measurements.

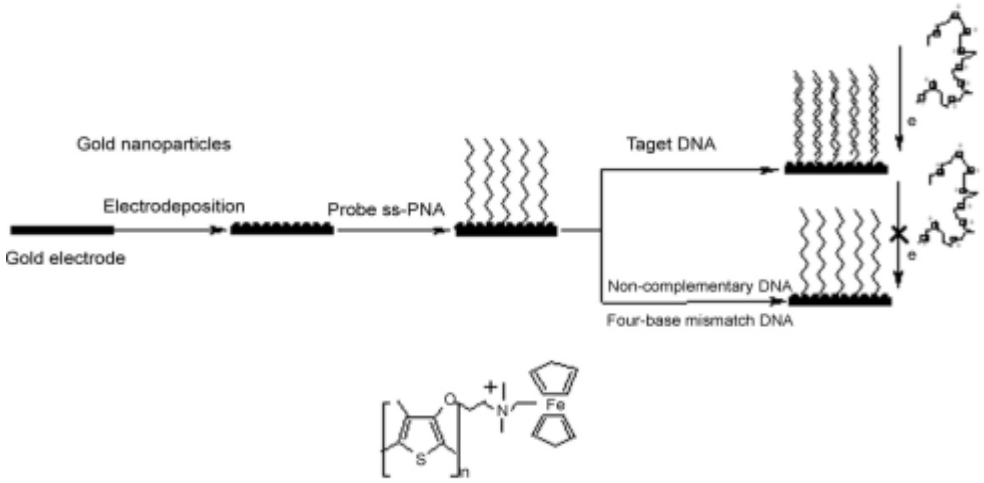


Figure 1.23 Chemical structure of polymer, schematic illustration of the steps involved in the fabrication of the ss-PNA probe modified nanogold electrode and hybridization with different DNA [122].

H. Korri-Youssoufi et.al [123] also used the ferrocene in a related DNA assay that reported a type of biosensor for DNA hybridization based on a copolymer formed with pyrrole substituted with ferrocenyl groups acting as electrochemical probes (see Figure 1.24), and N-hydroxyphthalimide as a leaving group to allow covalent attachment of the DNA probe onto small microelectrodes arranged in a matrix array format. The electrochemical response of the sensors was evaluated and compared to those deposited on a macroelectrode. Results show that an enhancement of the sensitivity of the detection was obtained by using a well-defined electrode (or cell) architecture in a chip array format. The detection limit calculated in the case of the chip format is evaluated to 0.05 fmol. Thus, by combining an electrochemical relay, the ferrocene and the conducting polymer as transducer, we have demonstrated that such system was promising in the design of high-density microelectrode arrays based on multiple probes for simultaneous detection of various DNA targets.

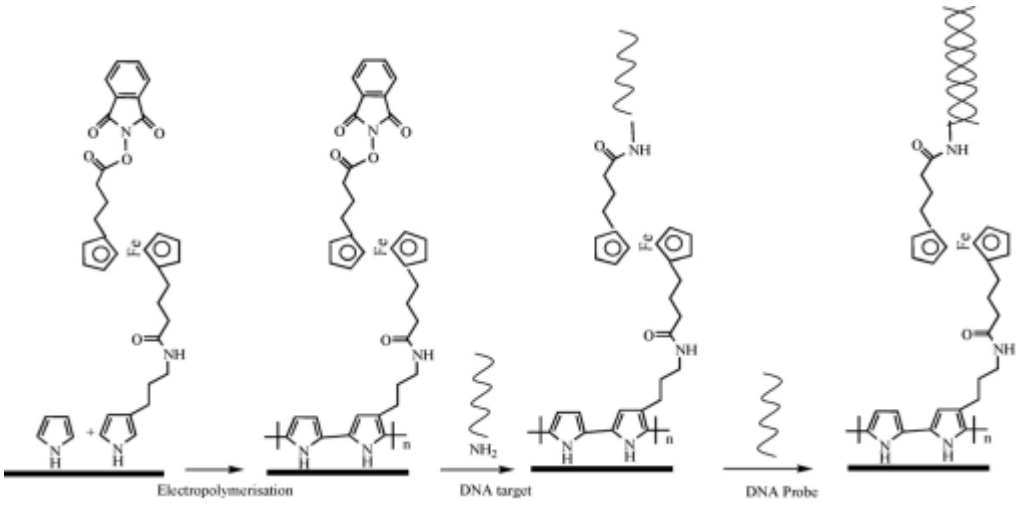


Figure 1.24 Synthetic strategy for the construction of the biosensors by electrochemical copolymerization reaction followed by covalent attachment of DNA probe and hybridization of the DNA target [123].

1.5 OBJECTIVE OF THE STUDY

Regarding the technological issues, many detection methods for genetically cancer diagnostic rely on labeling of DNA targets, a process which can be time-consuming, labor-intensive and even expensive. This thesis thus proposes a method for

the label-free electrochemical detection of DNA targets and genetic mutations, as capture DNA-probes. Single stranded DNA sequences are attached to a gold and screen printed carbon electrode surface where the mechanism by which hybridization is detected consists in measuring the change in electron movement upon binding of targets. The general objective of this study is to design, construct and development affinity biosensors for the electrochemical detection of DNA hybridization and genetic mutation anaysis by label-free analytical measurement techniques. In order to carry out this general objective, the following particular objectives were established.

- To design and construct by surface chemistry applications built in three electrode s in one compartment cell system use of ferrocene-cored PAMAM Dendrimers to enhance capacity of the sensor surface
- To determine DNA immobilization on the modified gold electrode surface and then detection of DNA hybridization with the complementary sequences, as an important step to be studied based on ferrocene-oxidation.
- To design and develop a DNA-based biosensor by adsorption method for probe immobilization on the screen printed carbon electrode. The working electrode surface integrated with neutravidin for able to catch modified DNA sequences labeled with biotin.
- To develop another strategies for DNA hybridization detection by using guanine-oxidation signal as label-free easy to be directly detected by differential pulse voltammetry technique.
- To demonstrate the possibility of using conventional method is three electrode system and using screen printed electrodes as a working electrode for a DNA-based biosensor based on covalent or adsorbtion immobilization methods respectvely. These electrodes composite and integrated with modified single stranded DNA probe sequences that can detect DNA hybridization and genetic mutations related to BRCA1 gene as a model biomarkers of breast cancer.
- Different spectroscopic, microscopic and electrochemical techniques were used to characterize the prepared immobilization matrix and working electrode surfaces.

CHAPTER 2

EXPERIMENTAL PART

The special emphasis was given to the development of rapid and sensitive electrochemical based biosensors by two different immobilization techniques. The first part focuses on the covalent immobilization of probe sequences on the modified electrode which was treated with Fc-cored PAMAM dendrimers and profit by the electrochemical properties of ferrocene. In this part three electrodes in one compartment cell system was used. In the second part also about of probe modification over the screen printed carbon electrode surface by neutravidin-biotion affinity interaction and take advantage of guanine oxidation. In that part utilized intinsic electrochemical properties of of nucleic acid molecules as an oxidation of guanine bases. Both of these techniques developed for detection of hybridization and mutation detection on the sequences.

2.1 WORK PACK #1: COVALENT IMMOBILIZATION WITH Fc-CORED PAMAM DENDRIMER LAYER

2.1.1 Materials

Cystaminiumdichloride, gluteraldehyde, ethylenediamine and methylacrylate was purchased from Merck. Methanol was purchased from Sigma Aldrich and Acetone was purchased from Emboy.

Table 2.1 The used oligonucleotide sequences in work pack #1.

Probe Sequence	:	(5'-3') [NH2] ATGGCTGCGTGTGACGCGCTCAG
Target Sequence	:	(5'-3') CTGAGCGCGTCACACGCAGCCAT
One Point Mutation Sequence	:	(5'-3') CTGGGCGCGTCACACGCAGCCAT
NonComplementary Sequence	:	(5'-3') AATACCTGTATTCCTCGCCTGTC

2.1.2 Instrumentations

The FTIR-ATR spectra (4000–400 cm^{-1}) were recorded with a Bruker spectrometer. NMR spectra were recorded in $CDCl_3$ using a Bruker 400 MHz spectrometer.

Distilled water machine model was Arium 61316 (Sartorius Stedim Biotech System). Incubation model was Electro-Mag, hot air sterilizer (Lab oven). Stirrer model was MSH-20A (WiseStir Lab Instruments). Autoclave model was CLG-40M (Automatic Steam Sterilizer).

For all the electrochemical experimental measurements were performed using the CHI Model 842B analyzer device and a gold plate working modified electrodes (1 cm^2), a platinum plate counter electrode (1 cm^2), an Ag/AgCl-saturated KCl reference electrode and a conventional three electrode electrochemical cell were used (Purchased from CH Instruments Inc.).

An electrochemical analyser was used for CV and DPV analysis, working gold electrodes, counter electrode, reference electrode and Gamma Alumina Powders (0.1-0.3-1.15 Micron) were obtained from CH Instruments, Inc. All oligonucleotides were bought from Alpha-DNA (USA/Canada). Tris-EDTA Buffer (1.0 M Tris, HCI, pH ~8.0 containing 0.1 M EDTA) and Phosphate buffer solution (PBS, 0.05 M pH 7.4) was prepared. Cyclic voltammetry was used to characterize the electrochemical properties of formation on the bare gold electrodes and the modified electrodes surface with different layers. The experiments were performed on the CHI Model with 842B software. Potential was swept from 0.0V to +0.6 V with scan rate of 0.1V/s. Phosphate Buffer Solution (50mM pH 7.4) was used for the CV measurements.

Differential pulse voltammetry wave form is composed of small pulses with constant amplitude that form a staircase. The CHI model with 842B software was used for this aim. The working buffer for DPV measurement was PBS with 50mM pH 7.4. For DPV studies, the following parameters have been used; Initial E(V); 0.2, Final E(V); 0.6, Increase E (V); 0.004, Amplitude(V); 0.5, Pulse width(s); 0.05, Pulse period(s); 0.2; Quiet time (s); 2, Sensitivity (A/V); $1e^{-5}$.

The gold electrode as working electrode, the Ag/AgCl as reference electrode and platinum electrode as counter electrodes dipped into ~ 10ml of Phosphate Buffer 0.05 M as supporting electrolyte. The DPV peak height at the potential around of +0.45 V as the analytical signal was used in all of the measurements. All CV and DPV measurements were carried out at room temperature.

2.1.3 Synthesis of ferrocene-cored PAMAM Dendrimers (G1, G2, G3)

The Fc-cored PAMAM dendrimer and its generations were synthesized by Dr. Şenel PhD study [121]. A solution of ethylenediamine and formly ferrocene in toluene was heated to reflux for 6h. After removing the solvent in vacuo the residue was solved in ethanol. $NaBH_4$ was added to the mixture which was heated reflux for 4 h. the solvent was removed in vacuo and the residue was suspended in CH_2Cl_2. The organic layer was washed three times with water and dried over Na_2SO_4.

The amine-terminated ferrocene cored PAMAM dendrimers was synthesized divergently by initial Michael addition of methanolic solution of ferrocene amine with excess methyl acrylate (1:10 molar ratio) (see Figure 2.1). The reaction mixture was stirred for three days at room temperature. The excess methylacrylate was removed under vacuum at 40–50 °C temperature to afford the ester-functionalized derivative G0.5. The reaction mixture was next submitted to the reaction sequence leading to the next generation Fc-PAMAM dendrimer G1.5, consisting of the exhaustive amidation of the ester functionalized G 0.5 to ethylenediamine (1:30 molar ratio), followed by Michael addition of the resulting amine with methylacrylate (20 equiv of G0.5). Excess reagents were removed under vacuum at 60-70 °C temperature. Repetition of this two-step procedure ultimately leads to the next generation Fc-PAMAM dendrimer G3.

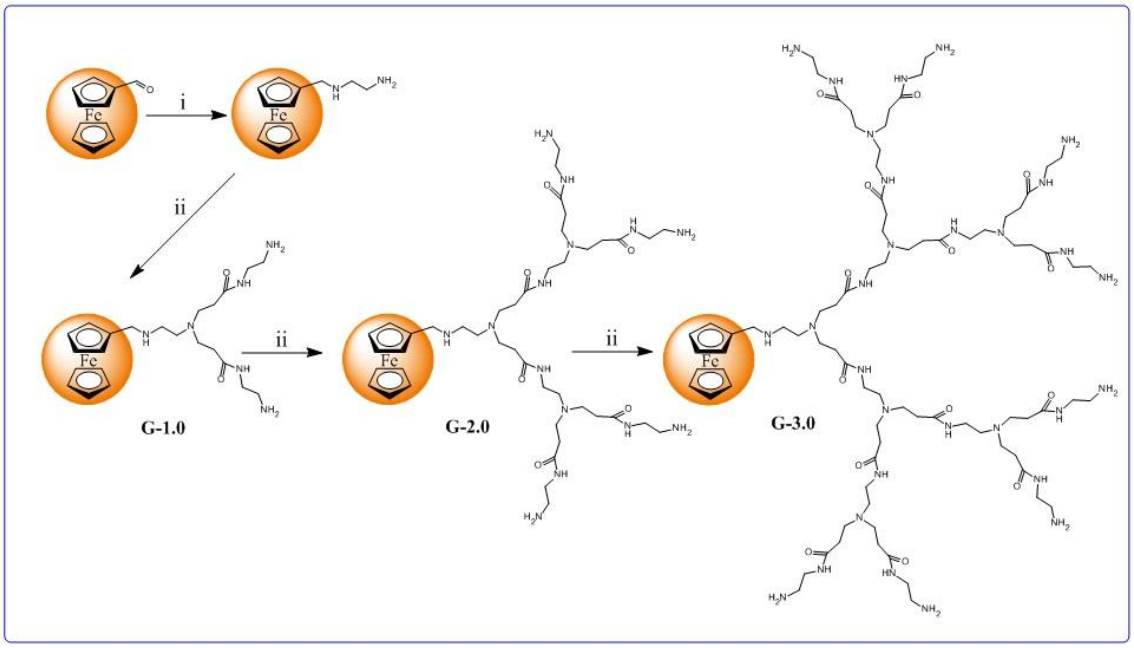


Figure 2.1 Synthetic pathway of Fc-PAMAM dendrimers i) ethylenediamine and NaBH4 reduction, ii) methyl acrylate in methanol and ethylenediamine in methanol.

2.1.4 Preparation of Dendrimer Modified Electrode

Before each new measurements, prior to use the surfaces of the Au electrodes were polished with 1.0, 0.5, 0.3µm Al_2O_3 powder and then washed with distilled water and absolute ethanol and acetone for 5 min to remove alumina particles. And then electrochemically cleaning procedure was applied. The electrodes were etched in 0.5 mol L^{-1} H_2SO_4 solution by cyclic-potential scanning between −0.3 and + 1.7 V until a reproducible voltammetric response was obtained. This polishing and cleaning procedure was repeated before every electrode modification step.

Before each new measurements, prior to use the surfaces of the Au electrodes were polished with 1.0, 0.5, 0.3µm Al_2O_3 powder and then washed with distilled water and absolute ethanol and acetone for 5 min to remove alumina particles. And then electrochemically cleaning procedure was applied. The electrodes were etched in 0.5 mol L^{-1} H_2SO_4 solution by cyclic-potential scanning between − 0.3 and + 1.7 V until a reproducible voltammetric response was obtained. This polishing and cleaning procedure was repeated before every electrode modification step.

The clean gold electrodes were immediately immersed into cystamine solution (0.2M, in pure methanol) aqueous solution for overnight. After these electrodes were thoroughly rinsed with distilled water to remove physically absorbed cystamine. For the next step was preparation of the cystamine/gluteraldehyde layers; the surface of the electrodes were immersed %1 gluteraldehyde solution for 30 mins at room temperature. After that electrodes were rinsed with distilled water. For the next step was preparation of the cystamine/gluteraldehyde/Fc-PAMAM dendrimer layers; Fc-PAMAM dendrimers were linked chemically to the functionalized gold electrodes by promoting the creation of amine bonds CHO(NH_2) between the CHO ends of the gluteraldehyde and amine peripheral groups (NH_2) of the Fc-PAMAM dendrimers. In the last step, after the this was achieved by immersing the gold electrodes in a solutions containing gluteraldehyde (1%, coupling reagent to create carboxly bonds) and then 20 µL Fc-PAMAM dendrimers of generation 1.0 (G1), 2.0 (G2) and 3.0 (G3) was dropped on the electrode surface for 1,30 h at room temperature. And then these electrodes were immersed again %1 gluteraldehyde solution again. Subsequently, the Fc-cored PAMAM dendrimer-functionalized gold surfaces were ready for immobilization of probe DNA sequences.

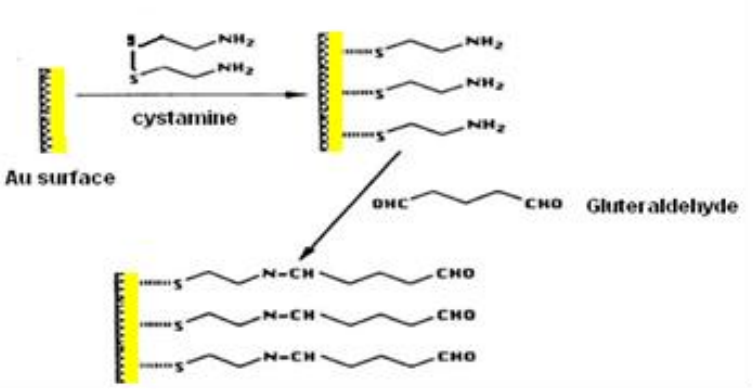


Figure 2.2 Bare gold electrode as working electrode modifications with cystamine and gluteraldehyde (amine-reactive homobifunctional crosslinker) for coating Fc-cored PAMAM dendrimers.

2.1.5 Capture Probe Immobilization

In this part, four different single-stranded DNA sequences were used. The used DNA sequences were Capture Probe, Target Sequence (complementary), One Point Mutant type and Non-Complementary (for control) sequences. The developed DNA-biosensor provided that a reliable discrimination against Non-Complementary DNA as well against one - base mismatch sequences. The covalent immobilization of single stranded DNA sequences on Fc-cored PAMAM dendrimers layer; gluteraldehyde was used as a bifunctional crosslinker agent for the single stranded probe DNA sequence immobilization. After that these gold electrodes were dipped into a glass beaker containing gluteraldehyde solution (%1) for 30 min at room temperature. Later, the gold electrodes were washed with ultrapure water gently. In the last step, $5x10^{-8}$ M of single stranded probe sequences was applied to the active electrode surface by a pipette.

These electrodes were allowed to incubate for an hour. Finally the electrodes were washed with ultrapure water and PBS solution to remove physically adsorbed DNA probe molecules. The bare gold electrodes and the modified electrodes were denoted as Au, Au/cys, Au/cys/GA, Au/cys/GA/Fc-PAMAM (G1, G2 or G3), Au/cys/GA/Fc-PAMAM/GA, Au/cys/GA/Fc-PAMAM/GA/ss-DNA capture probes.

2.1.6 Hybridization Assay for Point Mutations

After each of the single stranded DNA probes were assembled, the electrode surface was used to interact with complementary sequence solution. The target solution of complementary sequence was injected onto the electrode surface by a micro-pipette. The concentrations of complementary sequence solutions were 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13} and 0 M, respectively. For each time measurement, the injection volume was 20µL. The response value was read after 40 minutes of hybridization time of the incubation in a moisture medium at 42˚C. After the incubation period, the biosensor was gently immersed into the ultrapure water ten times to remove physically adsorbed target sequences. Finally, the biosensor was again put into the cell containing Phosphate Buffer solution (50 mM, pH 7.4) as a electrolyte solution, and the electrochemical measurements were taken with same parameters as described in the previous part. The difference in electron movements between the biosensor unbounded and bounded target sequences was used for preparing of target calibration curves.

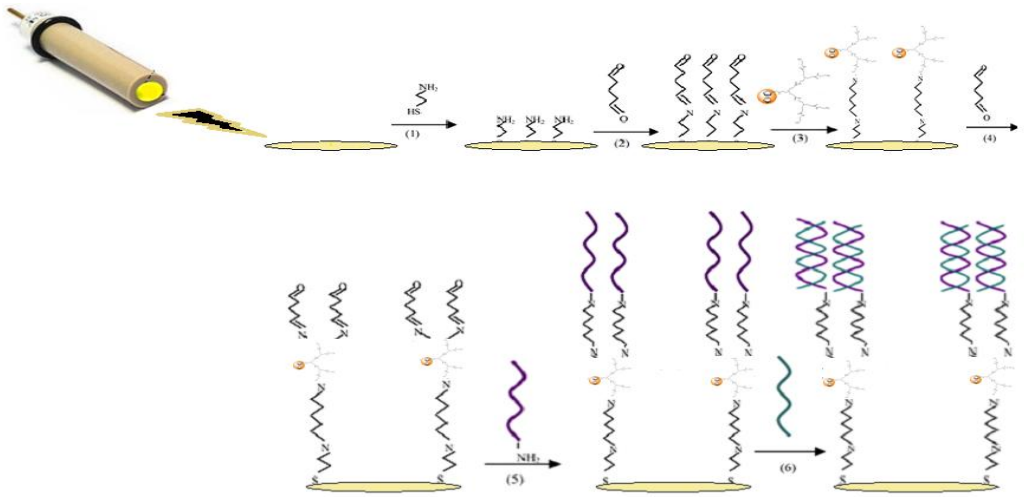


Figure 2.3 Summarise of the electrode surface preparation for detection of the hybridization and mutation detection procedure for work pack #1.

2.2 WORK PACK #2: BASED ON AFFINITY IMMOBILIZATION BY NEUTRAVIDIN-BIOTIN INTERACTION

2.2.1 Materials

Neutravidin was purchased from Thermo Scientific, Biotinylated-Bovine Serum Albumin and Tris-EDTA Buffer (1.0 M Tris, HCI, pH;~8.0 containing 0,1 M EDTA) was purchased from Sigma Aldrich. Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4). Ultrapure water (18 MΩ cm^{-1}) was obtained from a Milli-Q water system (Millipore Corp., Tokyo, Japan).

Table 2.2 The used oligonucleotides sequences in work pack #2.

Capture Probe Sequence	:	(5'-3') [Biotin] GATTTTCTTCCTTTTGTTC
Target Sequence	:	(5'-3') [Biotin] GAACAAAAGGAAGAAAATC
One Point Mutation (1PM)	:	(5'-3') [Biotin] GAACAAAAGGAATAAAATC
Three Point Mutation (3PM)	:	(5'-3') [Biotin] CAACAAAAGCAACAAAATC
Non-Complementary Sequence	:	(5'-3') [Biotin] GGTCAGGTGGGGGGTACGCCAGG

2.2.2 Instrumentations

In this part of the study, a sensitive label-free electrochemical DNA biosensor was constructed by using a commercial screen-printing carbon ink electrode (namely Disposable Electrochemical Printed chip) as a basis.

A screen printed carbon working electrode, a platinum counter electrode, an Ag/AgCl-saturated reference electrode were used. Electrochemical measurements were performed using a AUTOLAB ECO CHEMIE PGESTAT Electrochemical Analyzer. All measurements were carried out at room temperature. They were performed with sodium acetate buffer solutions by applying the desired potential and allowing the steady state current to be reached.

Oxygen free nitrogen was purchased from BOC (Manchester, UK). Incubation model was iEMS 1415 (Labsystem, Helsinki, Finland). Heat oven (Carbolite, model PN 120, Hope, UK). An electrochemical analyzer Autolab PGESTAT (ECO CHEMIE, the Netherlands) was used for CV and DPV analysis and screen printed carbon electrodes obtained from the printing facilities from DuPontLtd (UK) and all oligonucleotides were bought from Sigma-Aldrich (Poole, UK).

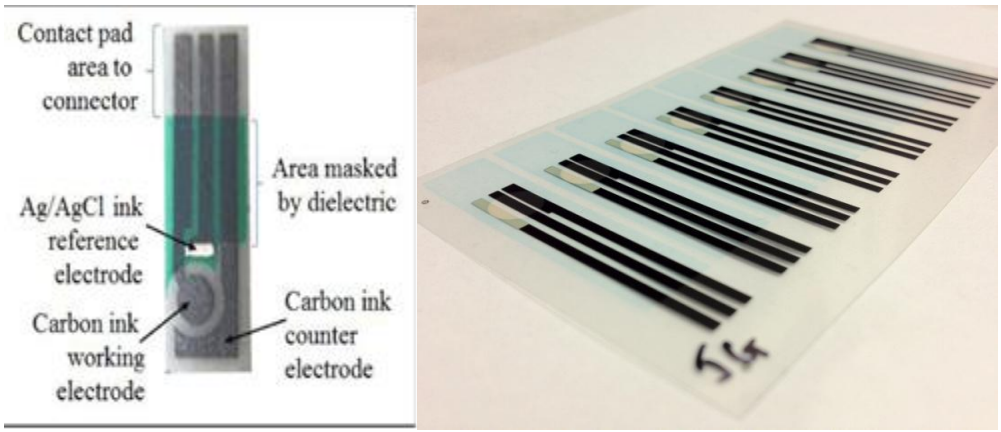


Figure 2.4 Screen printed carbon electrode.

Buffer and solution preparation;

All buffers and stock solutions used in this study were prepared using deionized and autoclaved water and stored in refrigerator (4˚C) until its use. These are outlined below.

- Neutravidin solution was prepared from Neutravidin powder with the distilled water.

- Milk Blocking Solution was prepared with distilled water (1:10 volume ratio)

- Biotinylated-Bovine Serum Albumin was prepared with distilled water

- TE buffer was diluted with distilled water (1:100 volume ratio)

- Supporting Electrolyte; the mixture of 0.1 M Sodium Acetate Buffer (SAB) pH 4.8, 0.1 M Potassium Chloride (KCI) (1:1 volume ratio)

2.2.3 Electrode Surface Modifications

The avidin-biotin interaction is one of the strongest non-covalent binding events. This interaction has unique characteristics that make it ideal as a bridge system in DNA hybridisation assay. In this study we applied that the immobilization of single stranded capture probe DNA sequences onto disposable screen printed carbon electrode using an neutravidin-biotin based procedure. This procedure involved the controlled formation of neutravidin layers (by adsorption) onto the electrode surface and the subsequent binding of a DNA probe biotinylated at its 5’ end. Prior the immobilization of the single stranded DNA sequences, the electrode surface was heat treated in an oven for 15 min at 120°C to clean the surface. The surface of screen printed carbon electrode was coated with 100µM/ml Neutravidin by adsorption prior to DNA capture. All electrodes were performed under controlled environment provided by an incubator at 37 °C for 2 hours. The next step we applied that the immobilization of single stranded capture probe DNA sequences onto disposable screen printed carbon electrode surface using an avidin-biotin based procedure. The subsequent binding of a DNA probe modified with biotin at its 5’ end. After put Capture probe DNA incubation at room temperature during 1 hour. By this way the biotinylated-capture DNA sequences could be simply attached over the electrode surface.

The b-BSA blocking solution was dropped onto Capture probe immobilized electrode surface. The electrodes were performed under controlled environment provided by an incubator at 37 °C for 90 min.

After washing process, the complementary, NC or MM sequences were treated. About hybridization procedure; the resulting step was the desired concentration (5, 10, 20, 40μM) DNA sequences were added on the electrode surface. Finally electrodes were performed under controlled environment provided by an incubator at 37 °C for 40 min for hybridization reaction.

All of assay steps finished, the electrochemical measurement methods (DPV and CV) were applied. Screen printed carbon electrode as working electrode, the Ag/AgCl as reference electrode and platinum electrode as auxiliary on the surface containing 100μl of sodium acetate buffer 0.1 M as supporting electrolyte.

At all the assays developed in this study, the target and mutation related to BRCA1 breast cancer gene was determined by DPV and CV. The DPV peak height at the potential around of +1.0 V as the analytical signal was used in all of the measurements. Parameters of DPV have been used; modulation time; 0.05, interval time; 0.5, initial potential; 0.7, end potential; 1.4, step potential; 0.005, modulation amplitude; 0.025, number of scan; 1. Also the CV responses were saved. The CV parameters were; start potential; 1.5 (V), first vertex potential; 0.5 (V), secondary vortex potential; 1.5 (V), step potential; 0.01 (V), scan rate number of data points; 0.05 (V/s), number of data points; 200.

2.2.4 DNA Capture and Hybridization Assays for Mutation Detection

In this thesis, five different single-stranded DNA sequences were used. The used DNA sequences were Capture probe, wild-type (complementary or target), one point mutant and three point mutant type and Non-Complementary sequences. The developed biosensors provide a reliable discrimination against Non-Complementary DNA as well against one and three-base mismatches. All the application procedure was summarized in the Figure 2.5.

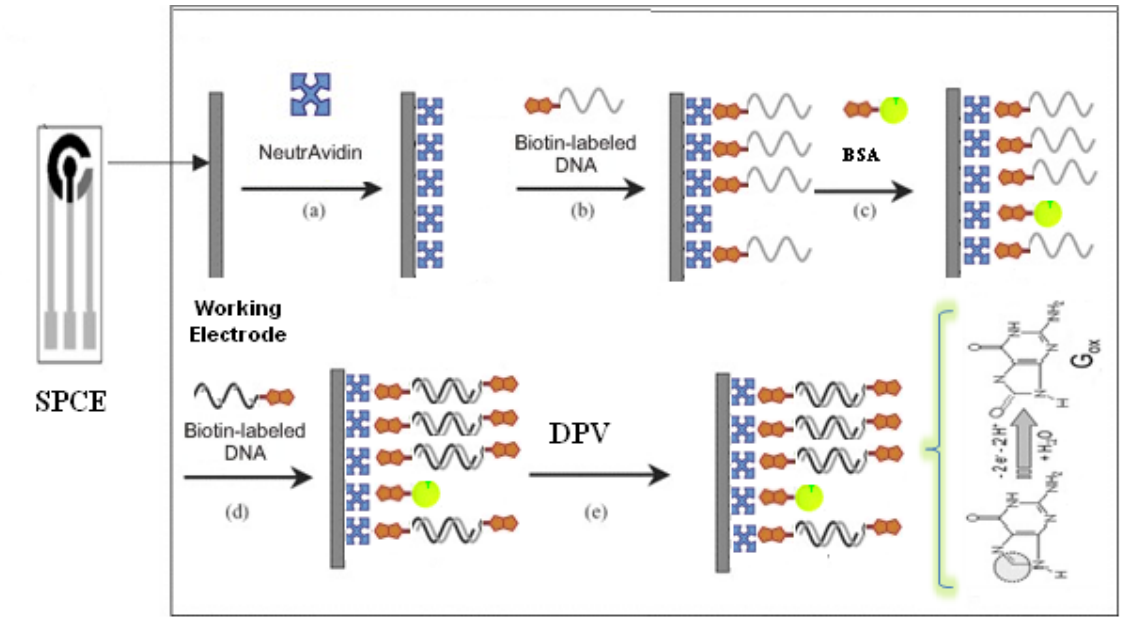


Figure 2.5 Schematic representation of work pack 2, the electrode surface preparation for detection of the hybridization and mutation detection procedure.

CHAPTER 3

RESULT AND DISCUSSIONS

Electrochemical DNA based biosensors for potential detection and diagnosis applications of breast cancer have recently been reported. According to these reports; early detection of breast cancer does allow for increased treatment options. In the past, ‘’early diagnosis ‘’meant the finding of a small lump in the breast. Based on the pioneer work of Gershon-Cohen and associates in their classic paper on ‘’biological predetermism‘’ in 1963, regarding the doubling time of breast cancers, it was found that the average breast cancer doubles at about the rate of one every 110 days, so that it probably has been present for about 9 years before it reaches the size of 1 cm. This is the size that is usually needed for clinical palpation. Truly earyl diagnosis, where the prognosis for diagnosis is excellent with appropriate theraphy, means the fiding of cancer in this non-palpable stage. In the past there was no way of finding these non-palpable cancers, however nowadays, using the biosensor technologies and genetic tests with BRCA1 and BRCA2 genes may also show that susceptibility to breast cancer high risk.

In the first work pack, the developed DNA-biosensor surfaces with different generations of PAMAM dendrimers were showed great efficiency for DNA immobilization by covalent binding technique. The all generations of the Fc-cored PAMAM allows the direct detection of DNA hybridization and genetic mutation thanks to ferrocene into the core part of the dendrimer structure. In the second work pack, the capture probe DNAs modified with biotin were immobilized onto screen printed electrode surface by means of the neutrAvidin-biotin linkage. At assay model system the capture DNA was hybridized with the complementary target, non-complementary target and mutant sequences which were one and three point.

3.1 WORK PACK #1: COVALENT IMMOBILIZATION WITH Fc-CORED PAMAM DENDRIMER LAYERS

3.1.1 Characterization of Fc-PAMAM Dendrimers

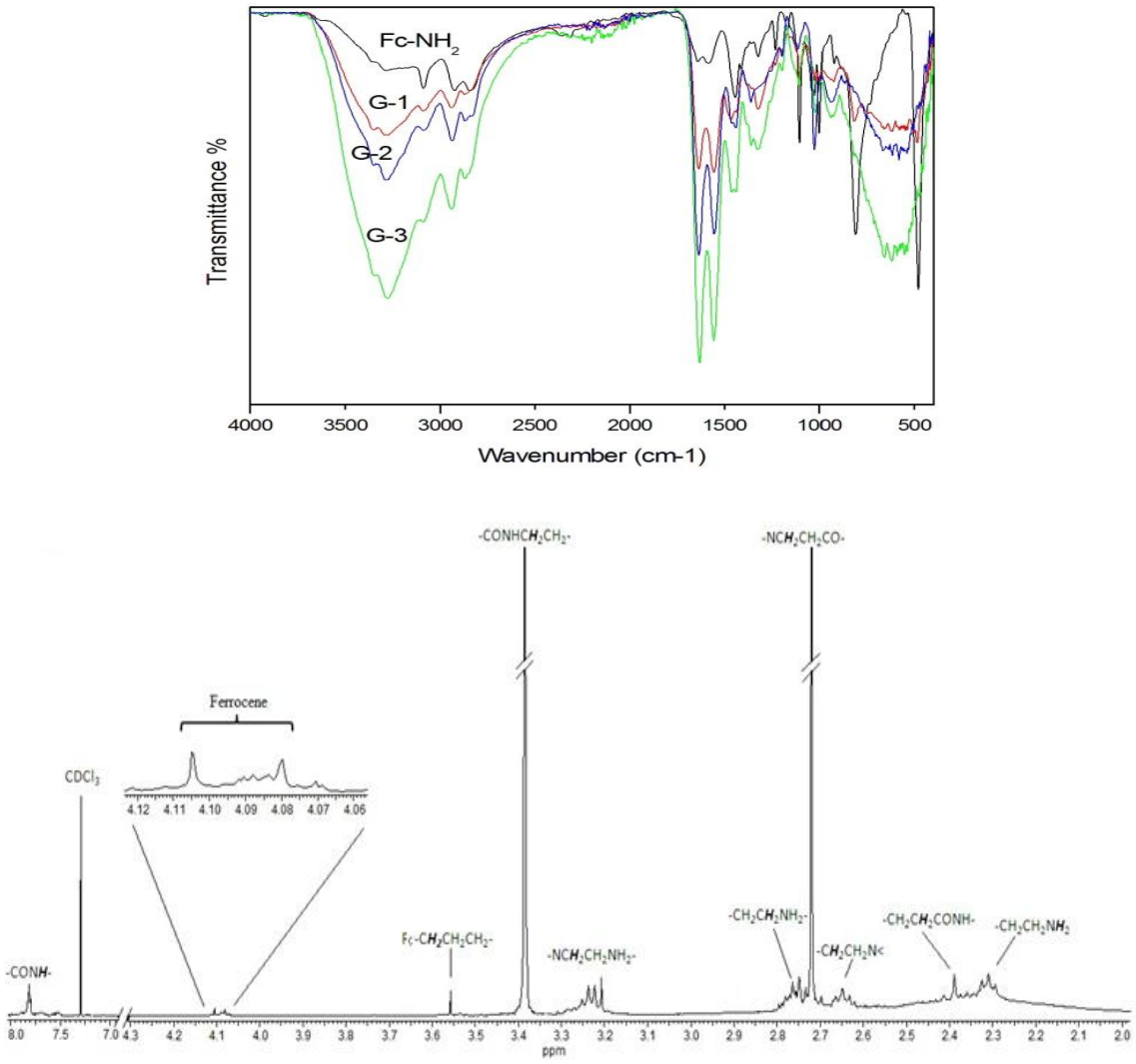


Figure 3.1 A) FT-IR spectra of Fc-PAMAM dendrimers with different generations. B) 1H NMR spectrum of Fc-PAMAM dendritic wedge G3 [124].

Figure 3.1 A shows a comparison of the FT-IR spectra, between 4000 - 400 cm^{-1} of the Fc-PAMAM dendrimers from G-0 to G-3. The peaks around at 3280 cm^{-1} can be attributed to the stretching vibration of N-H, indicating the growing of the PAMAM

dendrimers on the Fc monomer, other characteristic bands at around 1640 cm^{-1} are assigned to the vibration of the amide group in PAMAM dendrimers. Moreover, the intensity of these bands at 3280 and 1640 cm^{-1} obviously increased with increasing the generation of the dendrimers. The structure of Fc-PAMAM dendrimer (G3) can be determined from the detailed 1H NMR spectrum (Figure 3.1 B). In the spectrum, the peaks between 4.08 and 4.11 ppm are specific resonance signals of ferrocene protons.

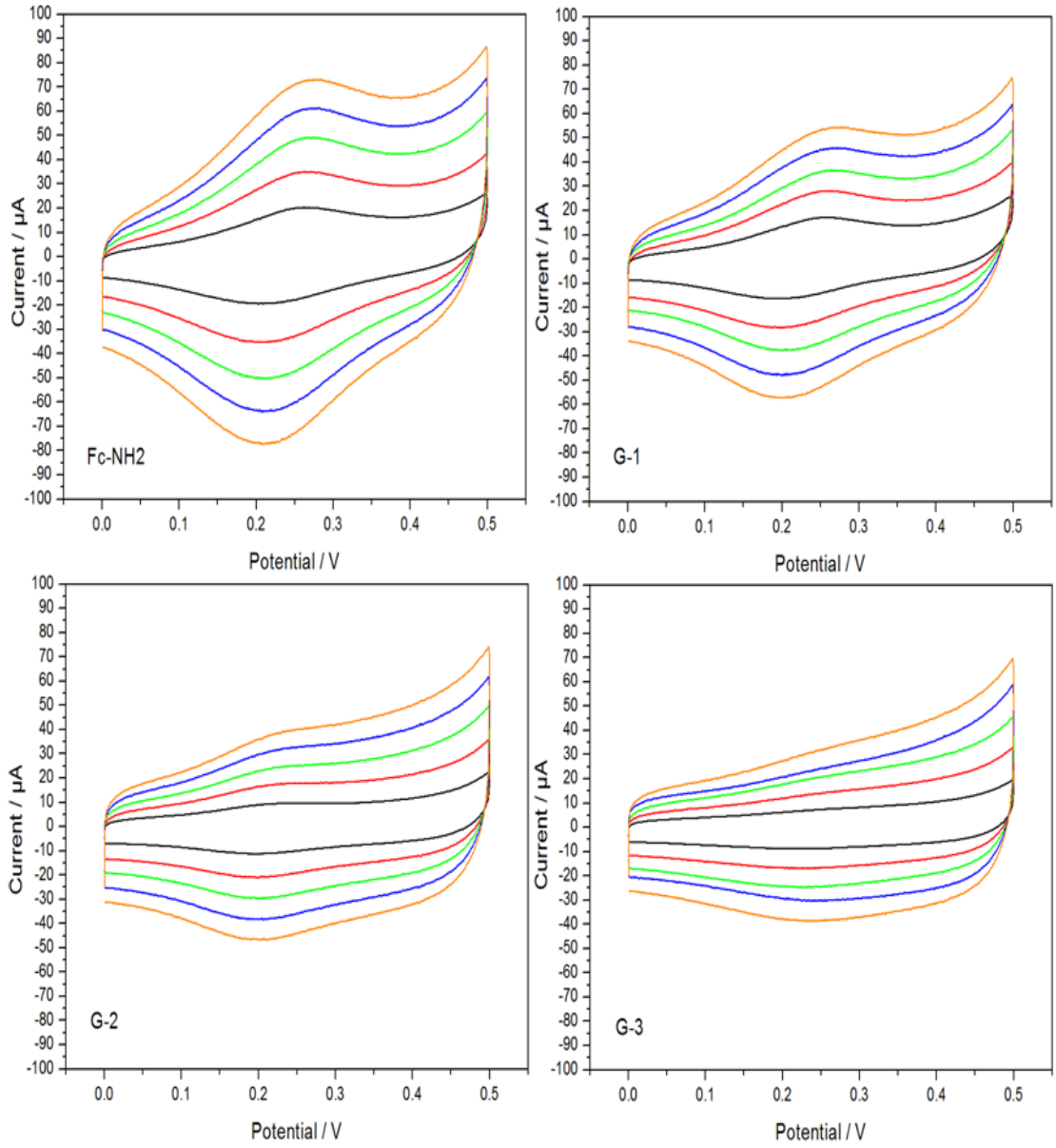


Figure 3.2 Cyclic voltammograms of Fc-NH_2, Fc-PAMAM-G1 (G-1), Fc-PAMAM-G2 (G-2) and Fc-PAMAM-G3 (G-3) electrodes in solution 10 mM PBS solution at different scan rates: 100, 200, 300, 400 and 500 mV/s.

The voltammetric behavior of the ferrocene-containing dendrimers between 0.0 and 0.5 V shown in Figure 3.2 were obtained at different modified electrodes; a) Fc-NH2, b) Fc-PAMAM-G-1, c) Fc-PAMAM-G-2 and d) Fc-PAMAM-G-3, respectively. As anticipated, the electrochemistry of the dendrimers was dominated by the one-electron, reversible oxidation of the ferrocene nucleus. Initially, the ferric ion in the core Fc exists in both the reduced form (Fe (II)) and oxidized form (Fe (III)). During the forward scan, Fe (II) is oxidized to Fe (III), and subsequently an oxidation current peak is observed. During the reverse scan, Fe (III) is reduced. The difference of the redox peaks was increased with increasing a scan rate. The voltammetric behavior also indicates that ferrocene has been immobilized on the surface of the gold electrode. Qualitatively, these findings agree with other reports on the electrochemical behavior of dendrimers having an electroactive core [125-127]. The diffusion coefficients decrease with generation due to the anticipated increase in the dendrimer molecular weights and radii. The higher the generation of the dendrimer, the further away the dendritic bulk keeps the ferrocene subunit from the electrode surface. Overall, our results clearly indicate that dendrimer growth extending from one of the cyclopentadienyl rings of the electroactive ferrocene core tends to hinder kinetically the heterogeneous electron-transfer reactions of these dendrimers with the electrode.

3.1.2 Hybridization Time Optimization

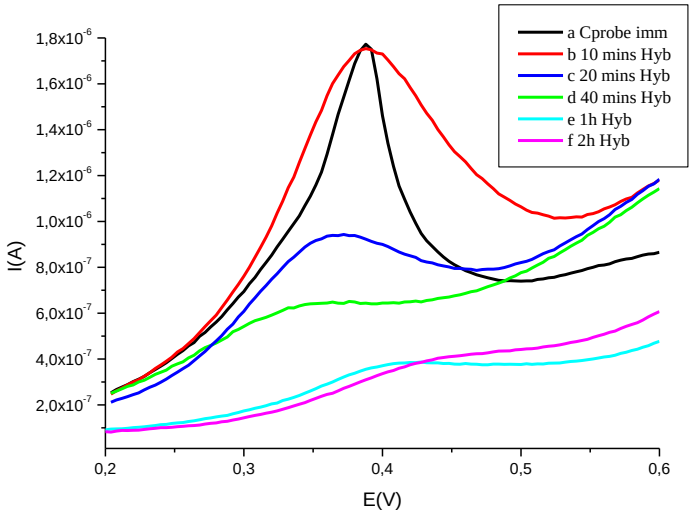


Figure 3.3 Optimization of hybridization time with amine-modified probes and target sequences; (a-black line) Capture probe immobilization, (b-red line) 10 mins,(c-blue line) 20 mins, (d-green line) 40 mins, (e-pink line) 1 h and (f-bright blue line) 2h respectively. The immobilization time, $5x10^{-8}$ M for each capture probe and same ratio of target sequence, the peaks are all presented as the change of reduction currents by differential pulse voltammetry.

The electrochemical behavior of Fc-cored-PAMAM modified electrodes depends on hybridization time with amine-modified probes immobilized polymer modified electrodes and treated with target sequences was also investigated by using DPV technique (see Figure 3.3). There is a well-developed peak at 0.37 V by using hybridized DNA sequences on the Fc-cored PAMAM-G1 modified electrode. After the target DNA solution was dropped onto modified electrode during 10, 20, 40, 60 and 120 min, the DPVs of these peaks were recorded. After progressing the DNA hybridization time, a significant decrease at the peak currents was observed by using DPV. The best hybridization result of obtained after 40 min, because this time is enough for take place hydrogen bond between DNA sequences and occurs double stranded DNA structure. After 40 min, begins to damage hybridized DNA fragmentations. There were additional signals coming from possible adduct(s), besides to the polymer signal in the different hybridization time, such as, 1 h and 2 h. There was also a significant shift at the oxidation peak potential of polymer in 2 h hybridization time. This result may be attributed due to the specific interaction between DNA molecule onto Fc-cored-PAMAM modified surface. Additional signals coming from possible adduct(s), besides to the polymer signal may be also attributed due to the specific interaction between DNA and the fc-cored PAMAM dendrimer structure. In the earlier literature, it has been shown that there are electrocatalytic oxidations of guanine and adenine in the presence of Fe (II/III) couple [128].

3.1.3 Electrochemical Detection of Capture Probe Immobilization and Hybridization with Target Sequence on the Gold Electrode

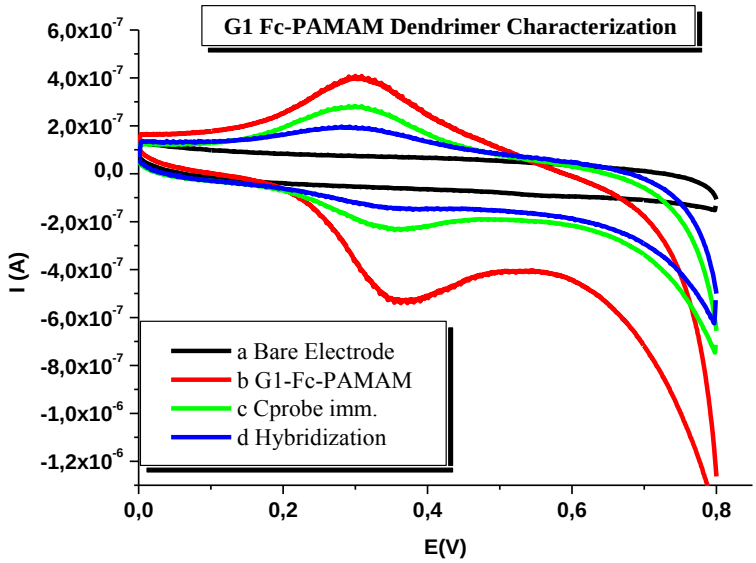


Figure 3.4 Electrochemical characterization of the G1 Fc-PAMAM coated biosensor. Cyclic voltammograms of different electrodes in PBS. (a) The bare Au electrode; (b) Au/Cys/Fc-PAMAM-G1 modified electrode; (c) Au/Cys/Fc-PAMAM-G1/Capture Probe(ssDNA) modified electrode; (d)Au/Cys/Fc-PAMAM-G1/Capture Probe (ssDNA)/Complementary DNA electrode. Scan rate, 0.1 Vs^{-1}.

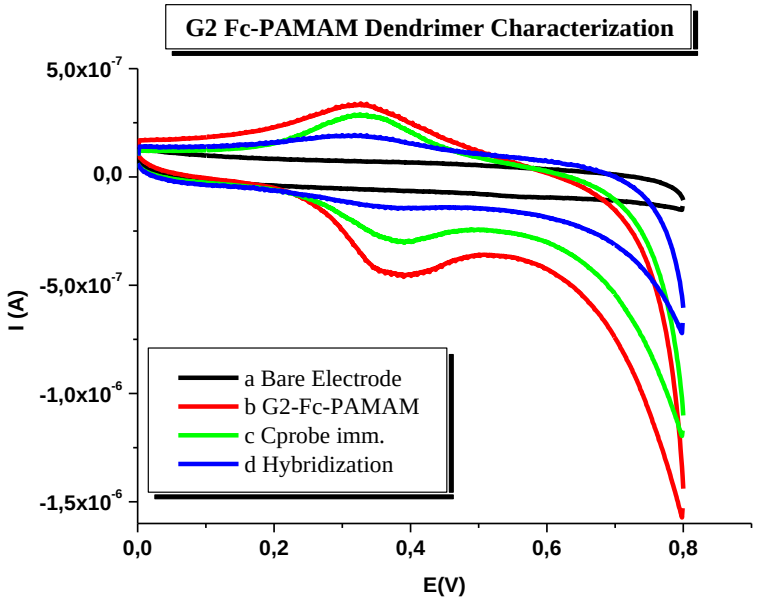


Figure 3.5 Electrochemical characterization of the G2 Fc-PAMAM coated biosensor. Cyclic voltammograms of different electrodes in PBS. (a) The bare Au electrode; (b) Au/Cys/Fc-PAMAM-G2 modified electrode; (c) Au/Cys/Fc-PAMAM-G2/Capture Probe(ssDNA) modified electrode; (d)Au/Cys/Fc-PAMAM-G2/Capture Probe (ssDNA)/Complementary DNA electrode. Scan rate, 0.1 Vs^{-1}.

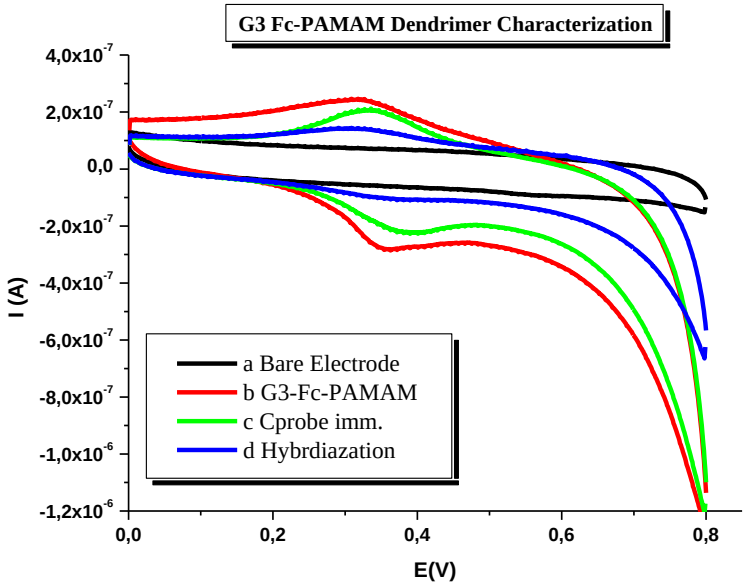


Figure 3.6 Electrochemical characterization of the G3 Fc-PAMAM coated biosensor. Cyclic voltammograms of different electrodes in PBS. (a) The bare Au electrode; (b)Au/Cys/Fc-PAMAM-G3 modified electrode; (c) Au/Cys/Fc-PAMAM-G3/Capture Probe(ssDNA) modified electrode; (d) Au/Cys/Fc-PAMAM-G3/Capture Probe (ssDNA)/complementary DNA electrode. Scan rate, 0.1 Vs^{-1}.

The stepwise modification process of different materials on the electrode surface was characterized by the properties of the Au/cys/Fc-PAMAM/Capture probe and Complementary (target) DNA sequences the characteristics of the immobilization and hybridization of DNA were studied by cyclic voltammetry (CV) (see Figure 3.4, 3.5 and 3.6). From the CV results, it could be clearly found that no reversible redox peaks corresponding to ferrocene electrone movement at bare Au electrode surface (black lines on each graphs). When the Au electrode was coated with a generations of Fc-cored PAMAM dendrimers , a pair of well-defined redox peaks and a sharp increase in peak to peak separation were found (red curve), suggesting that the Fc-cored PAMAM dendrimers significantly encouraced the kinetic of electron transfer process of the dendrimer due to the high level electron movements of the ferrocene. That is the increased values of the peak current indicate that Au/Fc-PAMAM can attract more ferrocene ion in the core of the dendrimer structure to participate in electrode reaction on the modified electrode surface. Compared with a Au/Fc-PAMAM electrode, the currents response of electrone transfer on the Au/Fc-PAMAM/capture probe decreased, and ΔIp increased obviously (green curve). The reason may be the fact that ssDNA is negative charge , and the electrostatic repulsion resists to access movement of electron to the electrode surface to block its electron transfer on the electrode surface. It fully confirmed that ss-DNA had already immobilized on the Au electrode. After treated with complementary sequence, occured hybridization between DNA sequences (blue curve). Compared with a capture probe immobilization on the Au electrode, the currents response of ferrocene on the Au/Fc-PAMAM/capture probe DNA/complementary DNA decreased, and also ΔIp increased obviously (blue curve). The electron transfer of ferrocene at the electrode surface was blocked because of the increase of surface thickness. The reason is that the ionized ferrocene molecules on Au electrode was charged negatively at pH 7.4, so the peak currents of the ferrocene ion into the PAMAM structure (curve a) increased as compared to the response obtained using the bare gold electrodes (curve b). It can be explained in terms of electrostatic repulsion between the negatively charged capture probe and the modified electrode surface, this also operates when the Fc-PAMAM compounds are further incorporated on the corresponding electrode surface.

Comparing Au/cys/Fc-PAMAM (curve b) with Au/cys/Fc-PAMAM/capture probe DNA (curve c), the peak currents of ferrocene ion on the Au/cys/Fc-PAMAM/capture probe electrode decreased obviously as compared to those obtained with the just Fc-PAMAM modified Au electrode, this also reflect the electrostatic adsorption between the positively charged surface confined Fc-PAMAM molecule and the negatively charged capture probe DNA sequence. The results show that the generations of Fc-PAMAM dendrimer (G-1, G-2, G-3) was successfully attached to the cysteamine modified gold electrode surface, and the Au/cys/Fc-PAMAM electrode has a good electrochemical response with ferrocene cored dendrimer.

When G-3 Fc-PAMAM dendrimer were immobilized on the electrode, owning to their ferrocene cored structure, the surface area and the density of amino groups on the electrode increased, this would result in the increase of immobilized capture probe (ssDNA). Thus, a higher sensitivity could be expected as compared with that of the other generations. Then the capture probe (ssDNA) was immobilized on the Au/cys/Fc-PAMAM (curve b), and the currents response of ferrocene oxidation on the electrode surface decreased significantly (curve c). The decrease of the current response is attributed to the negative charge of the ssDNA capture probe, which led to the electrostatic repulsion blocking the access of ferrocene ions to the electrode surface and thus the electron transfer on the electrode surface. This indicates that capture probe (ssDNA) has immobilized on the Fc-PAMAM/Au electrode (curve c). And then the currents response of ferrocene movements on the Au/cys/Fc-PAMAM/Capture probe/Complementary sequence electrode (curve d) decreased compared with the Au/cys/Fc-PAMAM/Capture probe on the electrode (curve c), so the ΔIp decreased obviously.

3.1.4 Voltammetric Responses of Fc-cored PAMAM Dendrimers' Generations on the Gold Electrode Surface

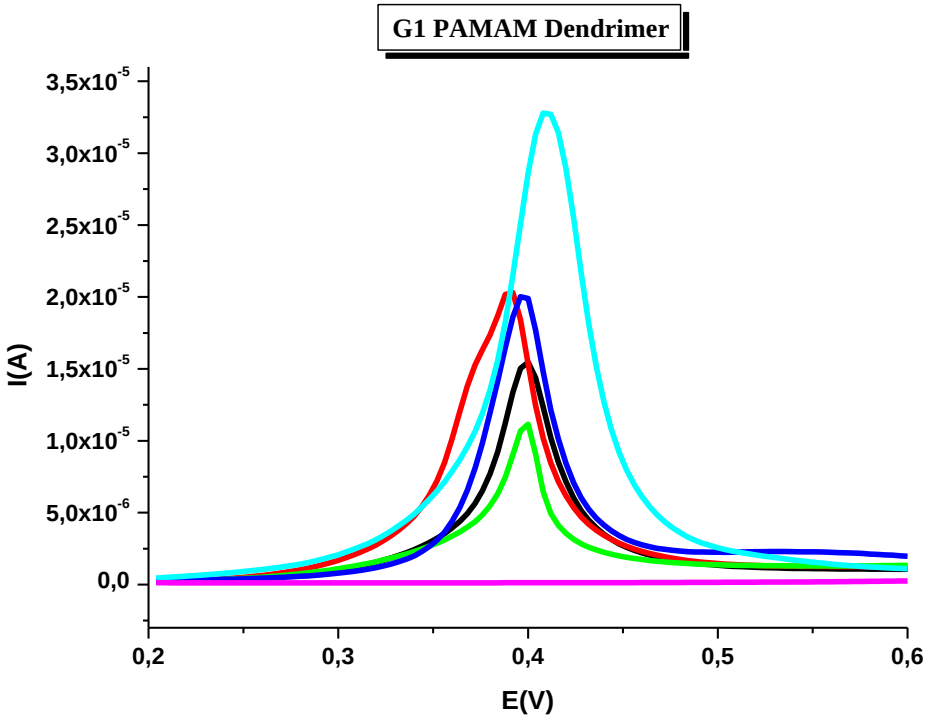


Figure 3.7 The DPV voltammograms for the oxidation signals of ferrocene during hybridization, point mutation and control measurements; (black solid line) bare Au electrode, (bright blue line) the first generation (G1) of Fc-PAMAM dendrimer, (blue line) capture probe immobilized, (green line) hybridization with complementary target sequence, (black line) MM sequence which is one-point mutant sequence, (red line) NC sequence for control, respectively. The conditions are; hybridization time; 40 mins, hybridization temperature; 42˚C. DPV scan is from +0.20 to +0.60, non-stirred solution.

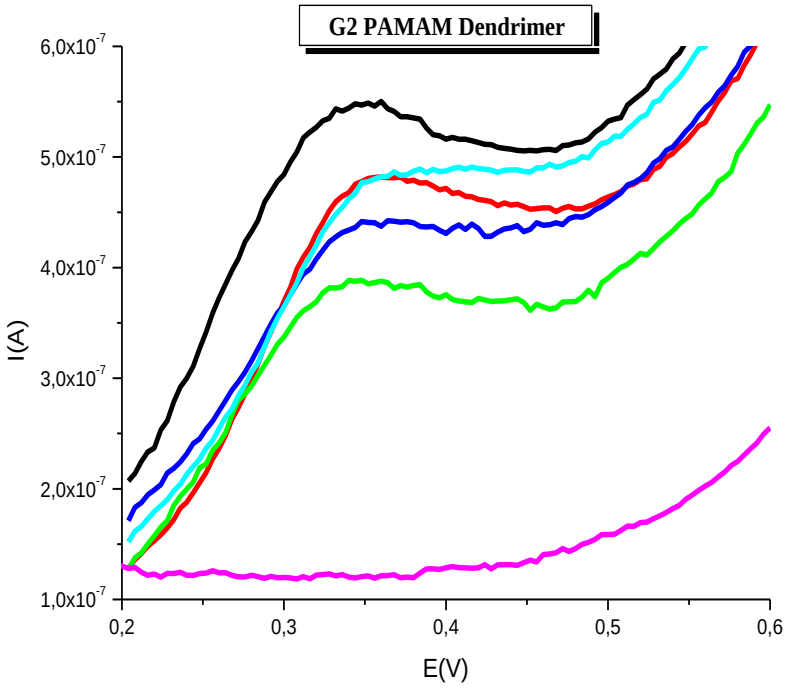


Figure 3.8 The DPV voltammograms for the oxidation signals of ferrocene during hybridization, point mutation and control measurements; (pink line) bare Au electrode, (black line) the second generation (G2) of Fc-PAMAM dendrimer, (bright blue line) capture probe immobilized, (green line) hybridization with complementary target sequence, (blue line) MM sequence which is one-point mutant sequence, (red line) NC sequence for control, respectively. The conditions are; hybridization time; 40 mins, hybridization temperature; 42˚C. DPV scan is from +0.20 to +0.60, nonstirred solution.

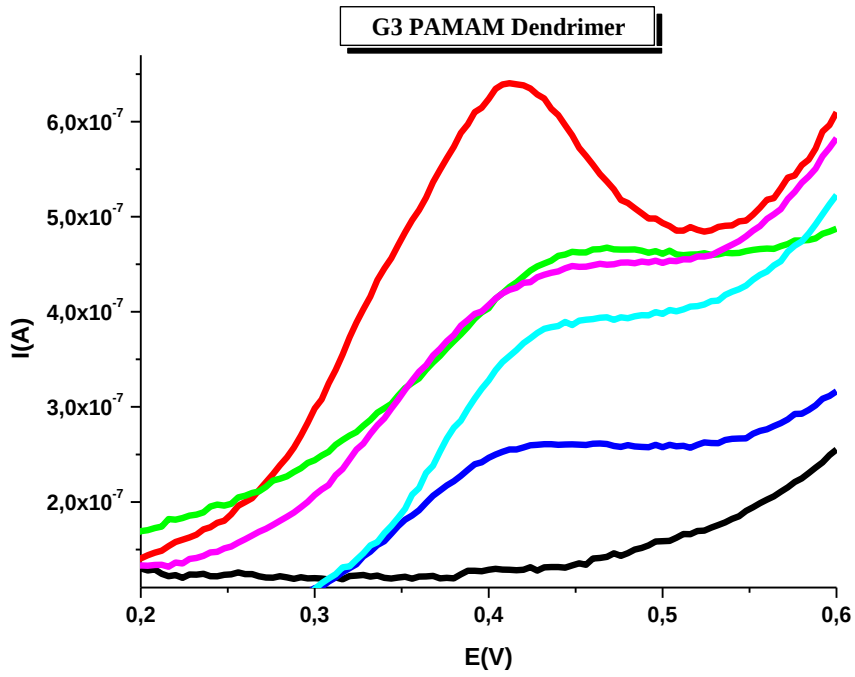
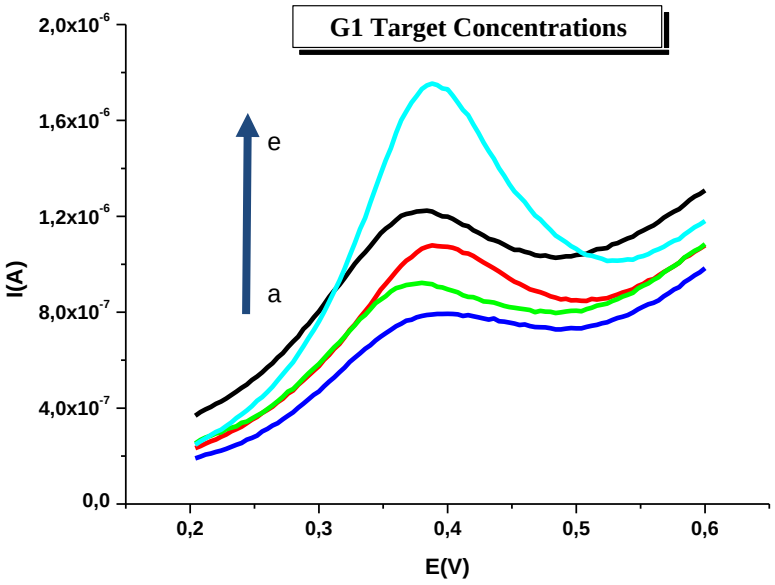


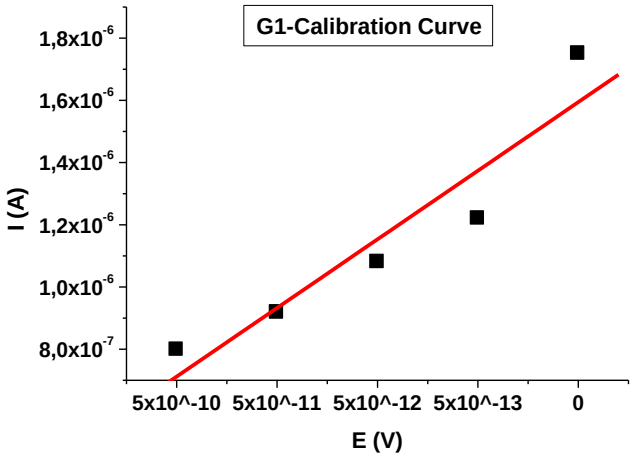
Figure 3.9 The DPV voltammograms for the oxidation signals of ferrocene during hybridization, point mutation and control measurements; (black line) bare electrode, (red line) modified with third generation (G3) of Fc-PAMAM dendrimer, (pink line) capture probe immobilized, (dark blue line) hybridization with complementary target sequence, (bright blue line) MM sequence which is one-point mutant sequence, (green line) NC sequence for control, respectively. The conditions are; hybridization time; 40 mins, hybridization temperature; 42˚C. DPV scan is from +0.20 to +0.60, non-stirred solution.

The selectivity of the present biosensor was investigated by using the signals of ferrocene oxidation and its electron movement within the structure of PAMAM dendrimer's generations as an modification electrode surface was used to immobilize capture probe DNA, to hybridize with the same concentration of complete complementary target DNA sequences, the one-base MM DNA sequences and the NC DNA, respectively, as shown in all the Figure 3.7, 3.8 and 3.9. A well-defined signal of electron movement was obtained for the complementary sequences. The signal for one-base MM DNA sequences was significantly weaker than that of the complementary sequences, and the NC sequences showed little response when compared with capture probe immobilization signal. For the application of DNA immobilized Fc-cored-PAMAM dendrimer's generations modified electrodes, the effects of generation of the ferrocene dendrimers were investigated by label-free trandusing mechanism and electrochemical sensing of DNA hybridization was studied (see Figure 3.7, 3.8, 3.9 respectively). The changes at the oxidation signal of the dendrimer were monitored in the presence of amino-linked probe alone and the hybridization between probe and target/NC/MM sequences. The dendrimer signal was decreased as a result of DNA hybridization between probe and its complementary sequence, target (Figure 3.7 green curve, 3.8 green curve and 3.9 dark blue curve). Due to the strong and specific binding of amino-linked probe with its complementary at the surface of the electrodes, a significant decrease at peak signal was observed in the presence of DNA hybridization. The selectivity of hybridization between amin-linked capture probe and MM sequences was detection for point mutation (Figure 3.7 black curve, 3.8 blue curve and 3.9 bright blue curve) and NC was also checked (Figure 3.7 red curve, 3.8 red curve and 3.9 green curve). In the results based on hybridization, better response was observed than similar ones [129-131] observed in the case of full-match hybridization.

3.1.5 Calibration Curves for G1, G2 and G3 of PAMAM Dendrimers by Differential Pulse Voltammetry

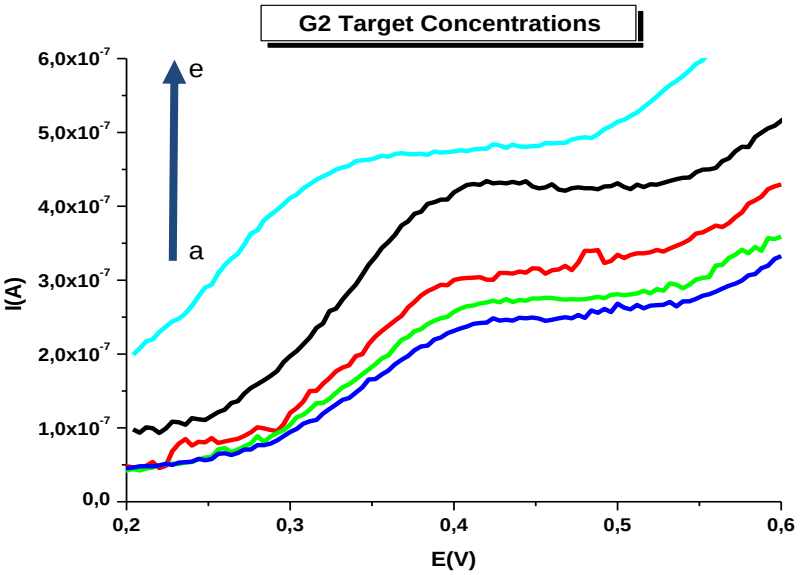


(A)

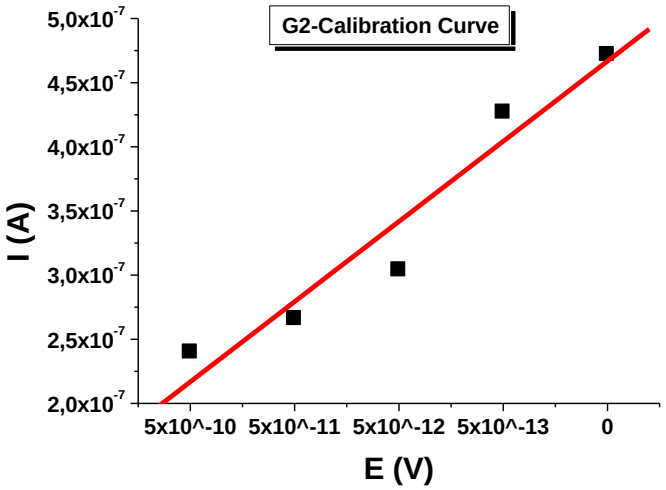


(B)

Figure 3.10 A) Effect of the target concentrations on the G1-Fc-PAMAM modified electrodes. The different concentrations of target sequences were applied on the capture probe. The immobilized capture probe concentration was $5x10^{-8}$ M. B) Calibration curve for G1-Fc-PAMAM coated electrode. The concentrations of complementary sequence solutions were (a) $5x10^{-10}$, (b) $5x10^{-11}$, (c) $5x10^{-12}$, (d) $5x10^{-13}$ and (e) 0 M, were injected on the sensors, respectively.

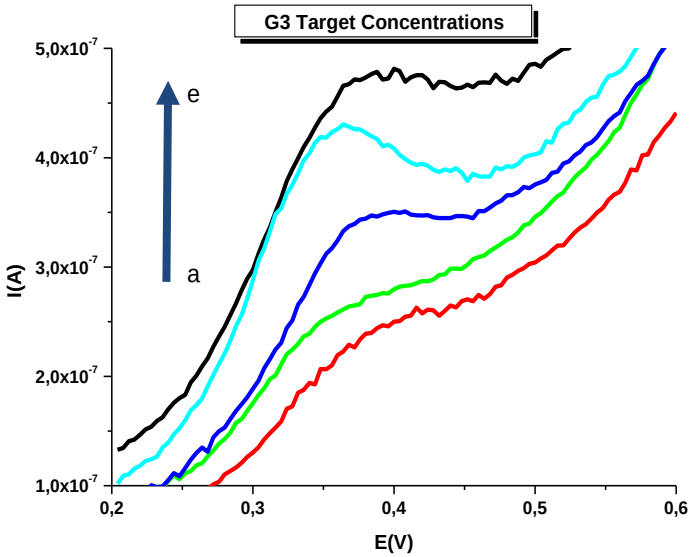


(A)

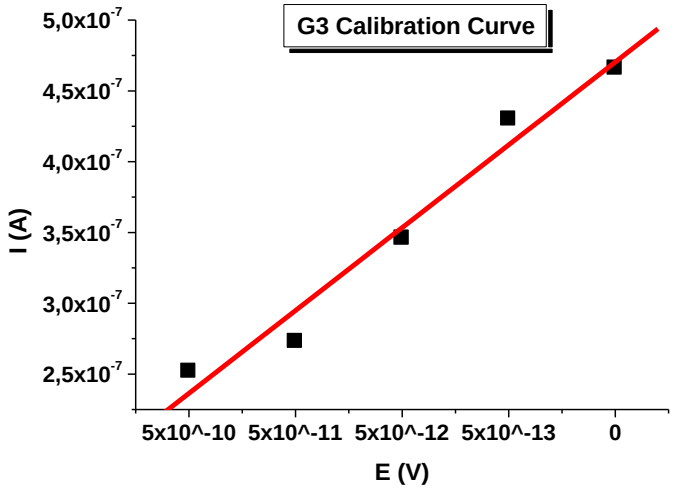


(B)

Figure 3.11 A) Effect of the target concentrations on the G2-Fc-PAMAM modified electrodes. The different concentrations of target sequences were applied on the capture probe. The immobilized capture probe concentration was $5x10^{-8}$ M. B) Calibration curve for G2-Fc-PAMAM coated electrode. The concentrations of complementary sequence solutions were (a) $5x10^{-10}$, (b) $5x10^{-11}$, (c) $5x10^{-12}$, (d) $5x10^{-13}$ and (e) 0 M, were injected on the sensors, respectively.



(A)



(B)

Figure 3.12 A) Effect of the target concentrations on the G3-Fc-PAMAM modified electrodes. The different concentrations of target sequences were applied on the capture probe. The immobilized capture probe concentration was 5x10-8 M. B) Calibration curve for G3-Fc-PAMAM coated electrode. The concentrations of complementary sequence solutions were (a) 5x10-10, (b) 5x10-11, (c) 5x10-12, (d) 5x10-13 and (e) 0 M, were injected on the sensors, respectively.

Figure 3.10, 3.11 and 3.12 shows that differential pulse voltammograms on single-stranded DNA probe-modified electrode after hybridization with different concentrations of complementary ssDNA sequences. DNA hybridization was carried out using this biosensing method. Firstly, the effect of amino- linked single stranded DNA concentration on the oxidation signals of dendrimer was studied in $5x10^{-8}$ M concentration. Then treated with various concentrations of complementary sequences from 0 to $5x10^{-10}$ M. The oxidation peak current decreased gradually and then levelled off as an just probe peak current in 0 M target sequences (actually its mean target-free hybridization buffer), when the concentration of single stranded capture probe was $5x10^{-8}$ M (see Figure 3.10 bright blue line, 3.11 bright blue line and 3.12 black line). The optimum hybridization concentration for 19-mer amino-linked probe was found as $5x10^{-10}$ M.

The sensitivity of the DNA biosensor was detected as shown in Figure 3.10, 3.11 and 3.12. The results showed that the signals of the reduction of ferrocene increased with the decrease of concentration of the target DNA (from a to e). With the PAMAM for the immobilization and hybridization of DNA and the label-free technique, the sensitivity was enhanced greatly and the detection limit was decreases greatly for DNA determination. By taking advantages of Fc-cored PAMAM dendrimer with abundant amine functional groups to tether single stranded DNA probe onto gold electrode and the good electrochemical properties, developed a new electrochemical DNA biosensor. The various generations of Fc-cored PAMAM dendrimer based DNA biosensor is sensitive and selective for target DNA with a low detection limit down to $5x10^{-13}$ M.

3.2 WORK PACK #2: AFFINITY IMMOBILIZATION BY NEUTRAVIDIN-BIOTIN INTERACTION

3.2.1 Electrode Characterizations

Experiments were carried out using an electrochemical analyzer GPESTAT. DPV measurements were performed in the SAB pH 4.8. The DPV parameters have been arranged to previous studies related to guanine oxidation detection.

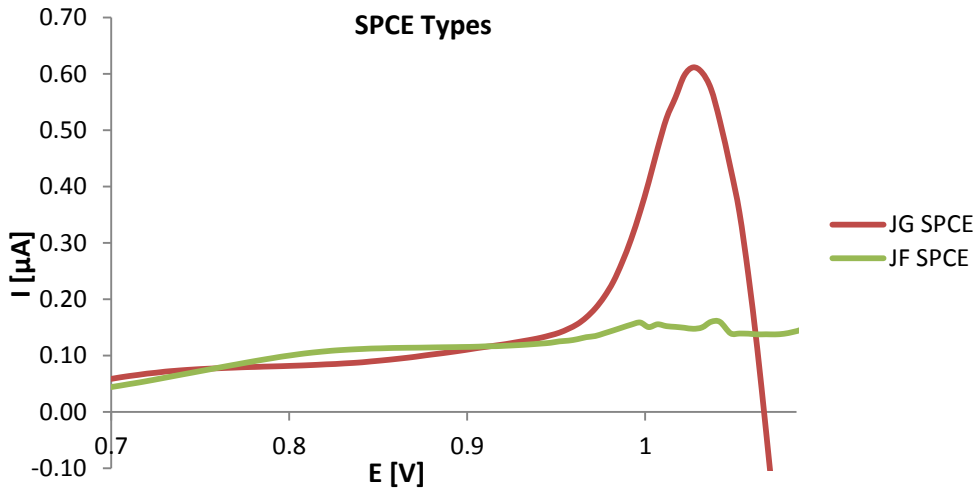


Figure 3.13 Comparison of the two different ink type electrodes.

About Electrodes characterization; the differences between the different types of electrode were mainly due to the ink used during screen printed process. In the all experiments, JG electrodes were used to develop the DNA based-electrochemical sensor for breast cancer related DNA sequences. Actually, guanine oxidation could be detected by DPV [132] and its peak occurred around ~1.0 V.

Regarding the results of the DPV (shown in Figure 3.13), JG electrodes were the most suitable for the development of the electrochemical DNA sensor for breast cancer related DNA sequences. Indeed, JG electrodes proved to have the highest real guanine oxidation peak on the active surface.

3.2.2 DNA Analysis Based on Guanine Oxidation on The Screen Printed Carbon Electrode

Guanine oxidation could be detected by DPV and its peak occurred at around +1.0 V. In this part, firstly we applied screen printed gold electrode but an increase of the bare electrode signal could be seen. It matched with the gold oxidation peak that usually was between+ 0.65 V and + 1.35 V. Because the guanine oxidation and the gold oxidation were overlapping and making it difficult to distinguish the guanine oxidation from gold oxidation. Actually a way to avoid this problem the use of an electrochemical intercalator, redox indicator or organic dye allowing to move the interesting peak at another detection potential to overcome this problem but we were prefered to change the electrode surface. Because the detection mechanism is label-free based in our assay. So, the screen printed carbons electrodes were used for seen guanine oxidation peak.

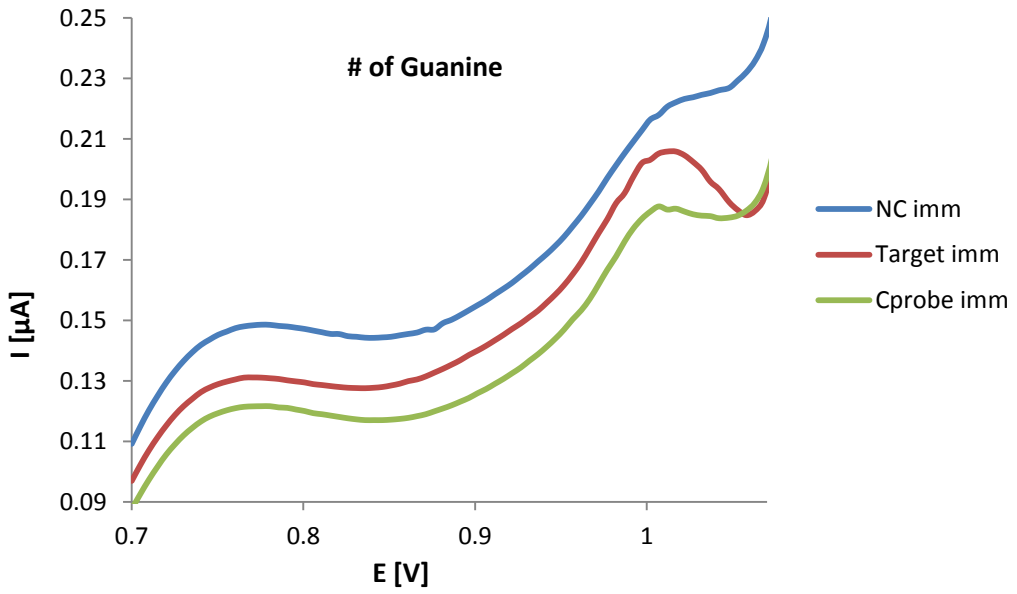


Figure 3.14 Single stranded DNA immobilization detection with depends on guanine number in the DNA sequences; the data is a result from an average of the 3 electrodes.

DPV was applied to detection of guanine oxidation depend on the guanine number in Figure 3.14, the capture probe DNA sequences immobilized on the screen printed electrode surface; they are single stranded so; we detected the peaks of guanine

oxidation because the guanine bases were unblocked. The capture probe sequence has just two guanine bases. The target sequence has four guanine bases and the NC sequence has thirteen guanine bases as well. The graph shows the high of guanine oxidation peak related to number of guanine. Depend on the graph the highest peak is coming from NC sequences and then target and the capture probe, it has very low peak [133].

In order to improve the efficiency of the proposed method for hybridization assay and mutation detections, BSA as a blocking agent in the corresponding step at the hybridization is used. In parallel, and also to avoid the non-specific adsorption the washing steps were improved.

The Figure 3.15 shows the typical differential pulse voltammogram for the guanine oxidation signal of during a hybridization assay, the guanine oxidation signal was reduces, because when occurred double stranded between the sequences; the guanine bases were blocked.

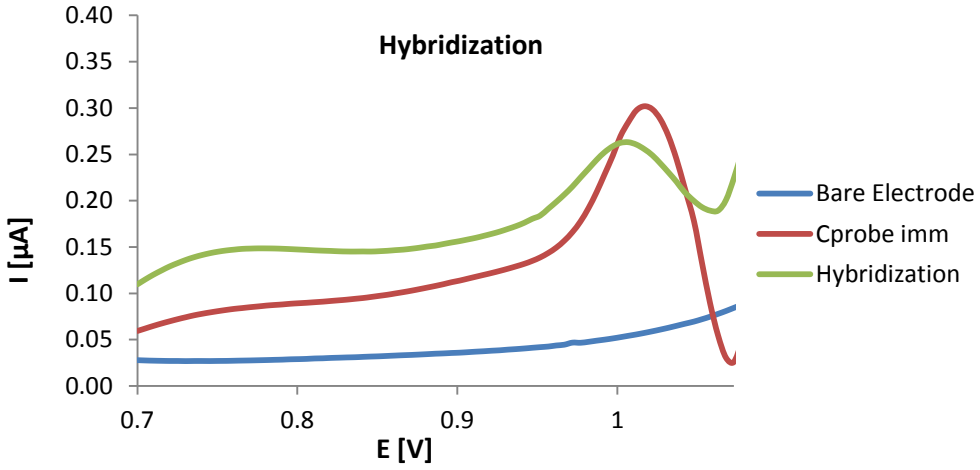


Figure 3.15 The peak of guanine oxidation differences; before and after hybridization with capture probe and its complementary (target sequences). Guanine oxidation detection by DPV was tested on the screen printed carbon electrode surfaces. Bare electrode, electrodes with probes immobilized through Neutravidin and electrodes on which the target DNA hybridized with the complementary sequences were tested with DPV.

Firstly, the assay developed was a model system as an initial strategy proposed. At this assay the binding of capture probe DNA to the surface is achieved via Neutravidin-Biotin interaction and then hybridized with the target sequences. After the hybridization the guanine bases were blocked and the guanine oxidation signal was decreased (green line in Figure 3.15). That means; interaction of capture probe and its target (perfect complementary) DNA sequences at the screen printed carbon electrode surface, there was a decreased at guanine signal. This decrease marked a possible damage in the oxizible groups of guanine that could be caused mutations basically on guanine bases [134].

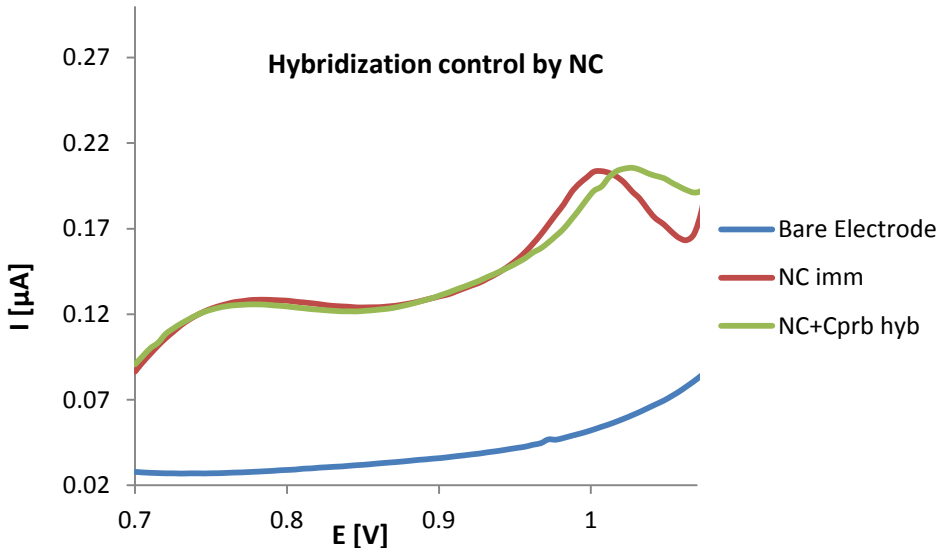


Figure 3.16 Peak of guanine oxidation differences; before and after hybridization with Capture Probe and on the Non-Complementary sequences. This peaks close to each other; that is no hybridization and no guanine blocking.

Figure 3.16 shows the control, because firstly the Capture probe was immobilized on the electrode surface and then NC sequence treated with sequences; the result is no hybridization , because when compare the highes of the peaks, they were very close to each other. Because guanine bases unblocking.

3.2.3 Analysis of Different Concentration of DNA Sequences

The experiments of target concentrations were revealed good linear behavior in the examined concentration range from 5 to 40µM after the 40 minutes hybridization time at the 37˚C. The dependence of current guanine peak responses on the electrode surface, as well as calibration curves are shown in Figure 3.17

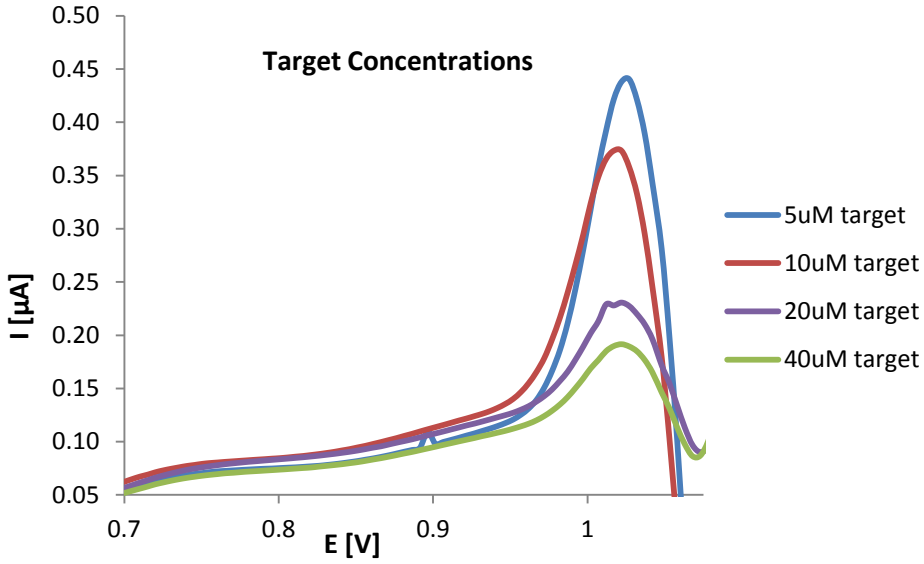


Figure 3.17 Effect of the target concentrations. The different concentrations of target sequences were applied on the capture probe. The immobilized capture probe concentration was 40µM.

The sensitivity of this DNA biosensor was explored by using the immobilized capture probe to hybridize with the BRCA1 gene target sequence of different concentration. The average oxidation peak current difference of guanine bases after hybridization was linear with logarithmic value of the target sequence concentration ranging from 5 µM to 40 µM, with the regression coefficient of 0.95. The sensitivity of this sensor is superior to that based on screen printed carbon electrode with the detection limit of 5 µM for three point mutation and also using one point mutant sequence, treated on the capture probe, the detection limit 40 µM.

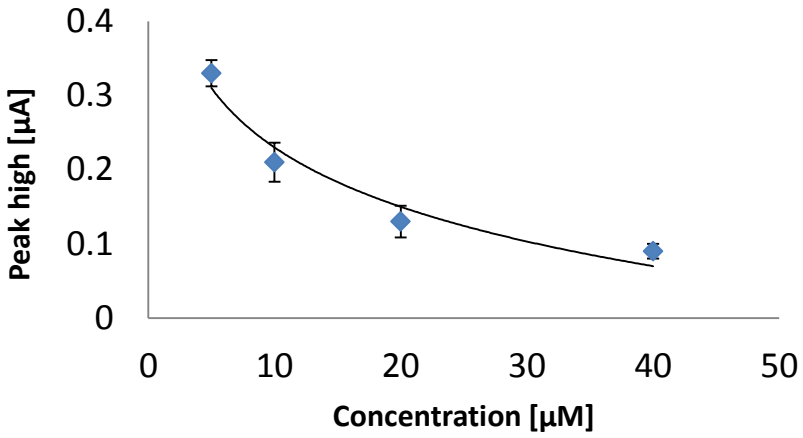


Figure 3.18 Calibration curve for the hybridization detection through neutravidin-biotin probe immobilization. Target concentrations of 5 µmol/mL, 10 µmol/mL, 20 µmol/mL and 40 µmol/mL were injected on the sensors. DPV potential of between 0.7 and 1.1 V was applied in the presence of 0.5 M sodium acetate buffer and 0.1 M KCI. The final current values are plotted and the bars indicate the standard deviation. The data is a result from an average of the 3 electrodes.

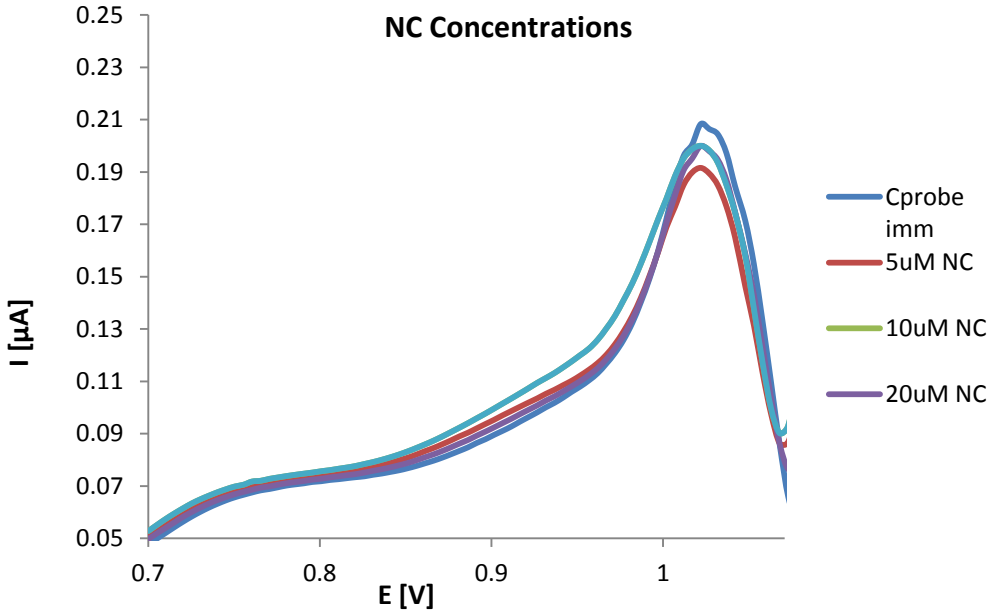


Figure 3.19 Confirmation with Non-Complementary sequences by DPV with probe immobilization though neutravidin-biotin interaction using b-BSA blocking solution on the whole sensor. Probe concentration was 40 µM and target concentrations of 5 µM, 10 µM, 20 µM and 40 µM were injected on the sensors.

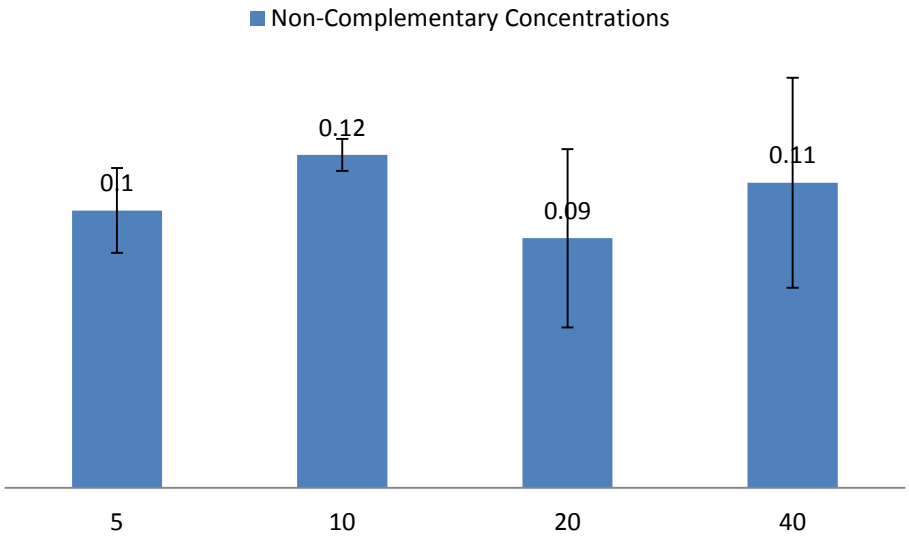


Figure 3.20 Histogram showing the results of the hybridization performed by Non-Complementary sequences with different concentration. The data is a result from an average of the 3 electrodes.

The effect of NC sequence concentration on the oxidation signals coming from guanine base was also studied in various NC concentration from 5 to 40 µM. the guanine oxidation signal was generally leveled off in the all concentration, it demonstrated that no hybridization between NC sequences and capture probe.

3.2.4 Analysis of Point Mutation Detections

The capture probe immobilization response peak was saved for the control from hybridization response using the Autolab software. The result which is an analytical signal due to the reduction of guanine base at potential +1.0 V was saved. The DPV peak height at the potential of +1.0 V as the analytical signal was used in the all different concentrated target sequence hybridization process.

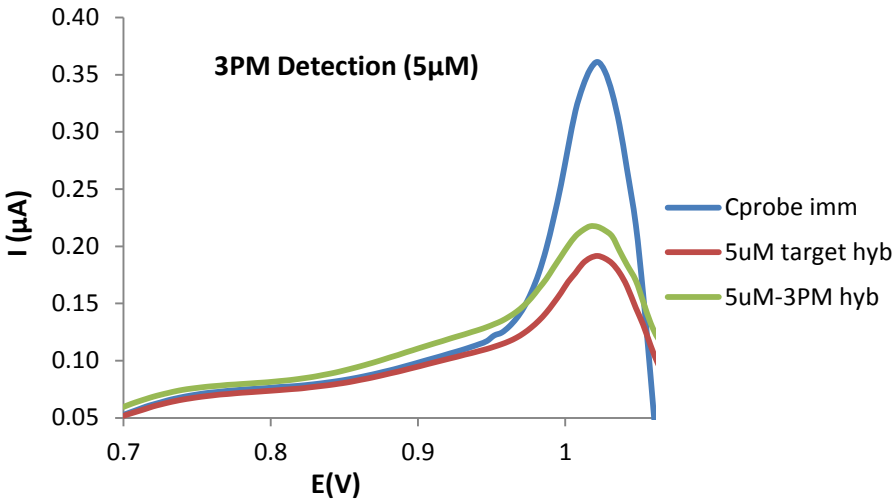


Figure 3.21 The use of three-point mutant sequences and complementary target sequences by DPV with probe immobilization though neutravidin biotin interaction using b-BSA blocking solution on the whole sensor. Probe concentration was 40 µM and target concentrations of 5 µM was injected on the sensors. The data is a result from an average of the 3 electrodes. The developed assay provide a reliable discrimination against NC DNA as well against target sequences and three-point mutation sequences.

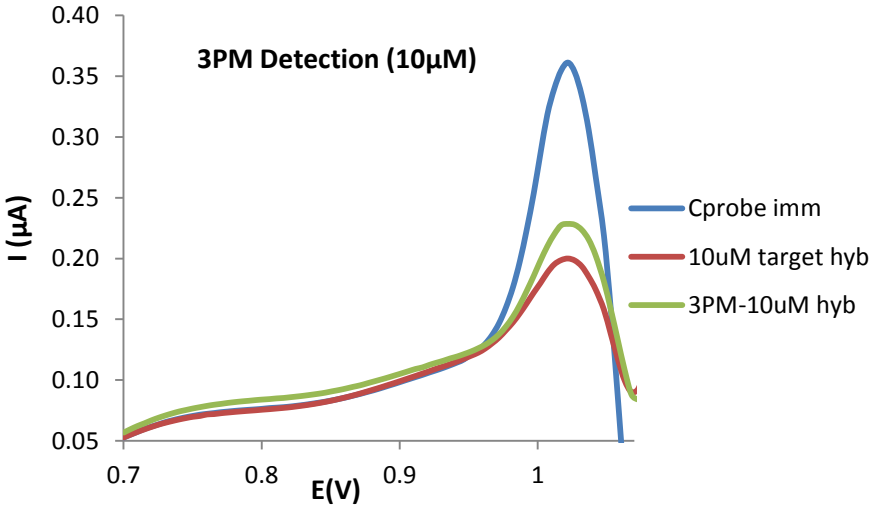


Figure 3.22 The use of three-point mutant sequences and complementary target sequences by DPV with probe immobilization though neutravidin-biotin interaction using b-BSA blocking solution on the whole sensor. Probe concentration was 40 µM and target concentrations of 10 µM was injected on the sensors. The data is a result from an average of the 3 electrodes.

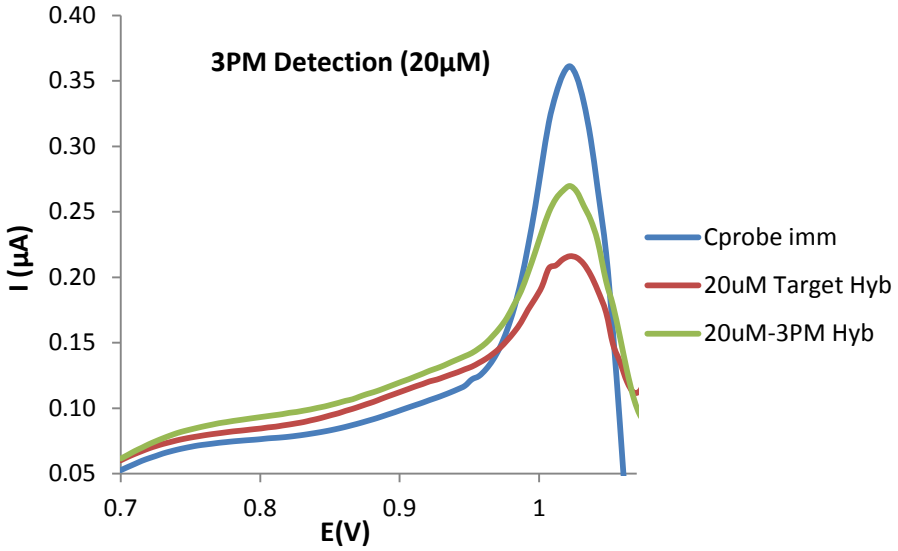


Figure 3.23 Use of three-point mutant sequences and complementary target sequences by DPV with probe immobilization though neutravidin biotin interaction using b-BSA blocking solution on the whole sensor. Probe concentration was 40 µM and target concentrations of 20 µM was injected on the sensors. The data is a result from an average of the 3 electrodes.

Detection of one and three point mutation by DPV with probe immobilisation though neutravidin-biotin interaction using b-BSA solution on the whole sensor. A voltage between 0.7 - 1.1 V was applied using the mixture of 0.5 M Sodium Acetate buffer and 0.1 M KCl in 1:1 volume ratio. Each sample concentration was repeated for three times using three individual electrodes. In the Figure 3.24 blue, red, green, purple and bright blue lines show the Capture Probe immobilization, hybridization with perfect complementary sequence, three-point mutation detection, one-point mutation detection, non-complementary sequence interaction, respectively.

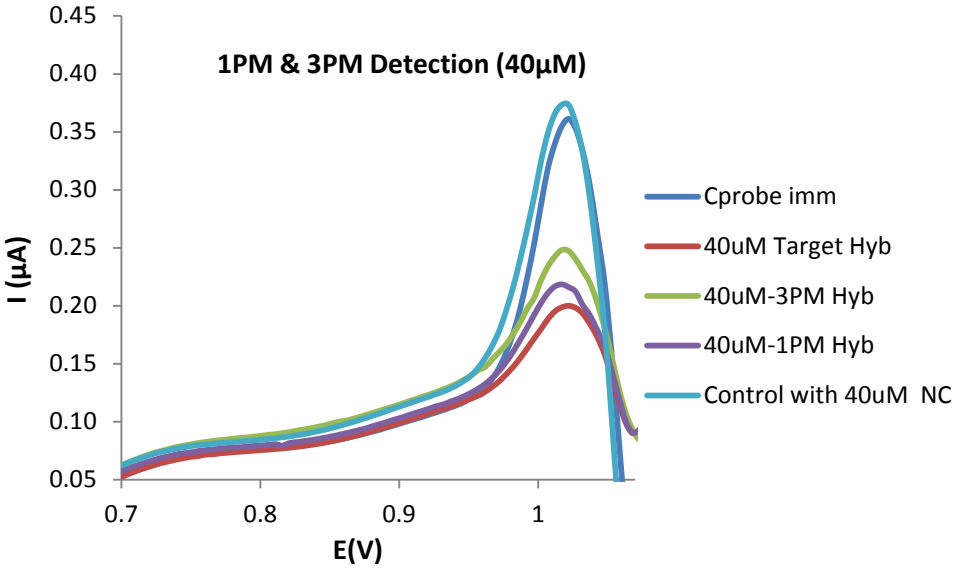


Figure 3.24 Use of one-point and three-point mutant sequences, complementary target sequences and NC sequences (for control) by DPV with probe immobilization though neutravidin-biotin interaction using b-BSA blocking solution on the whole sensor. Probe concentration was 40 µM and target concentrations of 40 µM was injected on the sensors. The data is a result from an average of the 3 electrodes.

The different DNA-based biosensor strategies developed show an excellent discrimination agains NC DNA as well as for one and/or three base mismatches. In this cases, when replaced the target DPV signals with very low intensity or even null response were observed. These indicate that we could selectively identify the target DNA sequence without intercalator.

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CHAPTER 4

CONCLUSION

In this study, DNA hybridization assay and genetic mutations detection through two different sensor surface based on label free transduction mechanism was described using commercially available oligonucleotides. The main aim of the study was to demonstrate the importance of biosensor technologies for diagnosis of breast cancer by detection of one-point and/or three-point mutations that occurs mostly in various region of BRCA1 gene. The specific and sensitive hybridization reactions were successfully achieved in the different concentration ranges. Furthermore one-point mutation could be significantly detected using interaction between capture probe and complementary target sequences by conventional three electrode system and screen printed electrode system instrumentations with different immobilization techniques. When compared the results, first of all between different generations of PAMAM dendrimer, the results shows that first generation is more useful from the other generations. Moreover when we compare it with affinity immobilized DNA hybridization on the screen printed electrode, the second way gave better results than the conventional system. And the obtained hybridization responses gave parallel results. The performed experimental procedure was neither time-consuming nor with labeling. Label-free electrochemical methods used for detection may be very promising taking into account their high sensitivity, selectivity, simplicity, low cost of BRCA1 gene point mutations related to breast cancer and available of portable instruments. As a final conclusion, the development of surface and then capture probe immobilization onto the developed place and then hybridization assay with target sequences and mutant sequences, all the electrochemical analysis strategies were successfully demonstrated. The future development of DNA biosensor (BRCA2, p53) and immunosensors (CEA, HER-2) based on the already developed strategies.

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