Advances in Chemical Engineering

Chapter 3

Label-free Electrochemical Detection of Oligonucleotide Hybridization Based on Composites of Intrinsically Conducting Polymers

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Abstract

We are focusing on the application of biosensor technology for the successful detection of selected DNA and mutated DNA sequences based on conduting polymer nanostructures. There are several tasks in the current research which need great concerns over the sensitivity, selectivity and throughput. Therefore, developing simple, efficient and cost effective methods for routine analysis of DNA hybridization is of great importance. Compared with other techniques electrochemical technique is an attractive and many advantages including high sensitivity, inherent simplicity and miniaturization and low-cost. It is well-known that electroactive conducting polymer (such as polyaniline, polypyrrole, poly (3, 4-ethylenedioxythiophene) is widely used in biosensors due to their unique physical and chemical properties and also low cost, easy preparation, and environment stability. In this book chapter, we have discussed a new sensing platform using nanostructure conduting polymers to detect target ssDNA and mutated ssDNA

sequences. A motivation behind this book chapter is an understand the basic concept of DNA hybridization and electrode fabrication, important parameter to improve the DNA hybridization efficiency including selectivity, sensitivity and low concentration detection and role of the nanostructured conducting polymer matrix in DNA sensing.

Keywords: DNA detection; electrochemical; biosensor; conducting polymers

1. Introduction

In the nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are polymers of nucleotides. Both DNA and RNA has two major purine bases, adenine (A), and guanine (G), and three major pyrimidines bases such as cytosine (C), thymine (T) and uracil (U). The chemical structures of the five major bases were given below in **Figure1**.

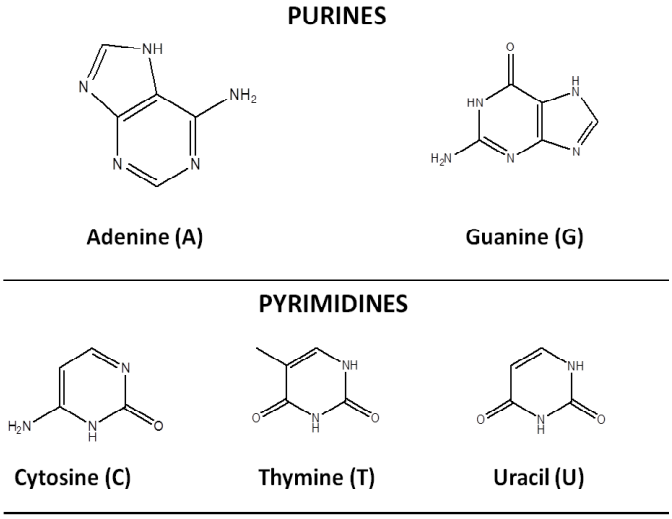

Figure 1: Chemical structures of major purine and pyrimidine bases.

Initially, James Watson and Francis Crick claimed double strand DNA helical structure in the year 1953 and create the new route in biological research for understanding the genome in living organisms [1].

DNA is the important basic biomolecules, which is used as chemical building block to store the gentic information in the cells and also it gives the blue print for entire characteristics of most living organisms. Researchers found that the DNA is the the most important molecular structure of the hereditary molecule in the cells. Based on the Watson - Crick Model, the DNA molecules present as helical structure with two polynucleotiede strands coiled around each other. Further, the sugar-phosphate backbone was present at the outside of the double helix structure and purine/pyrimidine bases were present in the inside of the helical structure.

The two single strand of the DNA double helix strcutre present in the opposite to each other. For example, one is the 5’ to 3’ direction, the other in the 3’ to 5’ direction. The 5’end having a phosphate group, which is linked with the 5’ carbon of its terminal DNA, whereas the 3’ end will usually having a hydroxyl on the 3’ carbon of its terminal deoxyribonucleotide.

The each signle strand DNA will bind to form as double helix structure through by the

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hydrogen bond between Adenine - Thymine, and Cytosine – Guanine. In addition, here three hydrogen bonds are involving between Cytosine – Guanine pairs. Similarly, two hydrogen bonds are involving between Adenine - Thymine pairs. The phosphate group of the DNA molecules have negative charge, which provides in electrostatic repulstion of the two strands. In order to join the two singe strands together, positive ions were much essential in solution for keep the negative charges neutralized.

The joining of two complementary single strands of DNA through hydrogen bonding to form a double-stranded DNA is called hybridization [2]. Further, the double stranded DNA was splite into two sing strands (dehybridize) when applying the particular temperature. This particular temperature of this transition is called the melting temperature (Tm), which is a more sensitive function of environmental conditions including ionic strength, pH and solvent conditions. Interestingly, when the temperature is reduced, the two strands will eventually come together by diffusion and rehybridize to form the double stranded structure [2].

2. Method of DNA Hybridization Detection

2.1. Conventional methods for the detection of DNA hybridization

It is well-known that the Southern blot method is conventionally used for the DNA sequences detection through gel-transfer hybridization process (**Figure 2**).

It is specifically fabricated to locate a particular sequence of DNA within a complex mixture by permanently attaching single-stranded DNA (ssDNA), which means denatured DNA to a solid support. Traditionally, a nitrocellulose membrane is widely used as the solid support, although a positively charged nylon membrane may also be used.

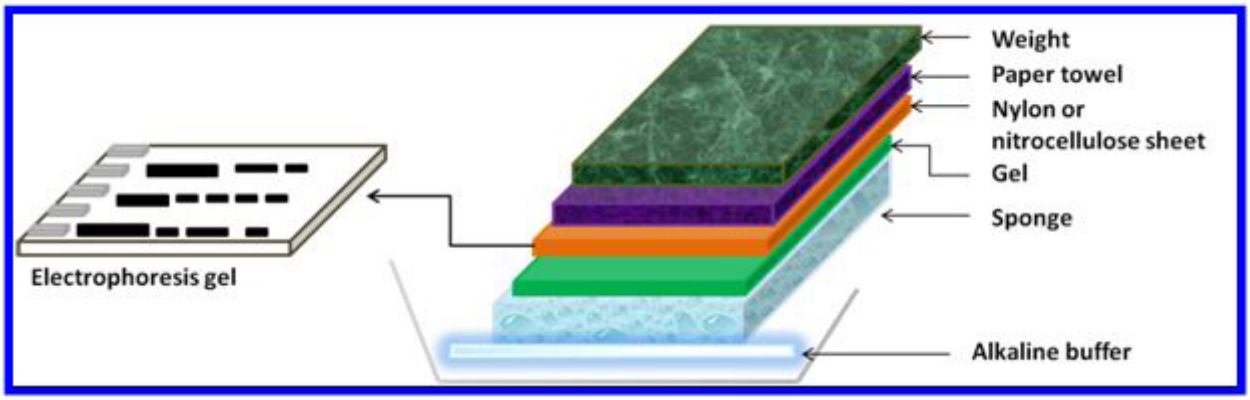

Figure 2: The schematic illustration of Southern blotting apparatus.

The denatured ssDNA fragments are kept on an agarose gel and split through electrophoresis. A thin sheet of nitrocellulose membrane is laid onto the gel and the separated DNA fragments are transferred to the sheet in a blotting set-up. The gel is sustained by a layer of sponge in a alkaline bath of buffer solution and this is further transferred via the gel and the nitrocellulose membrane through paper towels and weight stacked on top of the nitrocellulose thin sheet. The separated DNA fragments are transferred from the gel into the surface of the ni-

trocellulose sheet, where they adhere firmly and become permanently fixed after cross-linking with UV irradiation. The attached ssDNA over the nitrocellulose membrane has been further exposed into the labeled target DNA probes for a particular period time under good environment to enhance hybridization process. In general, different labled probe DNA was used including 32P, biotin/streptavidin or a bioluminescent molecule. For example,

If 32_p probe DNA is taken, an auto radiograph has been applied to evaluate hybridization where the DNA that has been hybridized to the labled probe will show up as bands on the autoradiograph. In the case of biotin/streptavidin detection is evaluated by colorimetric methods while bioluminescent visualization needs luminescence detection technique.

2.2. DNA hybridization biosensor

Biosensors are analytical instruments, ideally small and portable instruments that usually join the bio-recognition elements with the physical transducers (**Figure 3**), most generally electrochemical, optical, microgravimetry which utilize current, light or frequency to transduce the bio-recognition events, respectively.

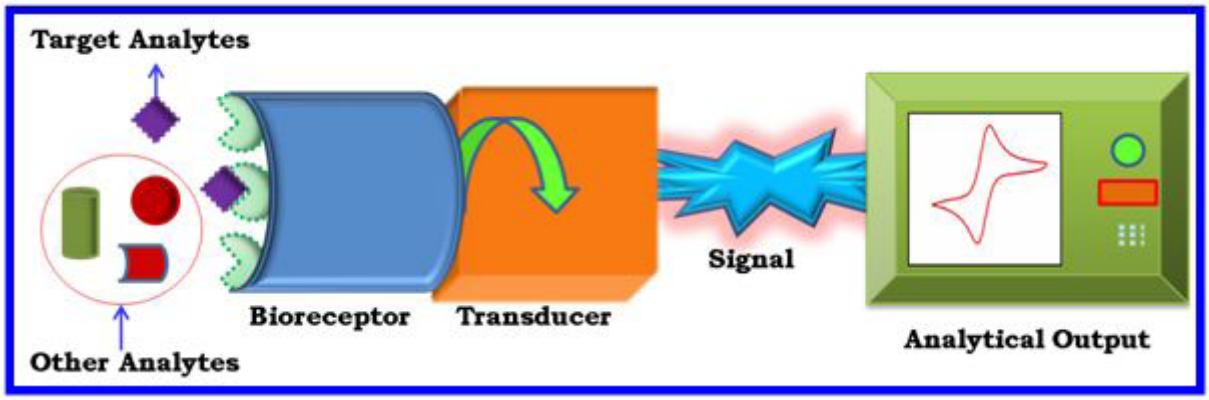

Figure 3: Schematic illustration of a biosensor which consists of a bioreceptor on a transducer attached to an analytical output

The sensing elements and/or receptors (antibodies, cell receptors, nucleic acids, imprinted polymers, porous nanostructure or catalytic reactions) were usually employed for enhance the specificity of the sensor [3].

As Southern blotting hybridization is labor intensive, time-consuming, requires expensive and hazardous probe labeling and normally needs to couple with expensive PCR instrument. Hence, this method is limited within hospital and research laboratories. This has been induced among research communites to develop an alternate detection technique with attractive features including simple, poratable, rapid, and high sensitivity and cost-effective. A DNA hybridization biosensor through electrical detection is able to fulfill these requirements. The electrical detection using most recent technological advances and nanostructure materials has provided great platform for the fabrication of portable DNA hybridization devices for rapid genetic screening and detection.

It is well known that the DNA hybridization biosensors represent a very important class

of affinity biosensor. A typical construction for a DNA biosensor consists of a probe ssDNA, which is fixed with the physical transducer. The probe ssDNA coupled transderuce will interact (hybridize with) corresponding complementary target DNA in the solution, i.e. the sample to be investigated.

2.3. Probe ssDNA immobilization

One of the most critical steps in the development of a DNA biosensor is the method used to attach the probe ssDNA on the physical transducer surface. A typical DNA biosensor is constructed by the immobilization of a probe ssDNA on a transducer surface to recognize its corresponding complementary (target) DNA sequence through hybridization event. DNA has to be attached on transducer in a way that the bases remain available for further bio-recognition event of the complementary target ssDNA strand. In this sense, the immobilized probe ssDNA should be vertical from the transducer (electrode) surface. Whereas, if it is attach the probe ssDNA horizontal onto the electrode surface, the bases of the DNA may restrict the interaction with corresponding complimentary target ssDNA. Hence, it is difficult to form a DNA double helix formation. So the probe ssDNA attachment on the transducer surface is an important role in the development of DNA biosensor. The following methods were commonly applied for the probe ssDNA attachment onto the transducer surface.

2.3.1. Entrapment in a polymeric matrix

In this method, the probe ssDNA can be retained in a matrix including agar gel, polyacrylamide, or conducting polypyrrole, which have been immobilized in advance on a solid support. The matrix has a mesh and porous size effective investigated by their large area of adsorption, which increases the amount of probe DNA strand attached, improving the sensitivity of the resulting system. However, the main problem in this method is the lack of probe DNA orientation, which decreases the accessibility to the target ssDNA. For example, Pividori et al. studied the nylon membrane has been used for the immobilize the probe ssDNA through adsorption [4]. Further, Li et al. used a polyacetic acid nanofiber membrane as a transducer substrate for probe DNA immobilization [5]. Similarly, Vivek et al. explored sol-gel matrix for the immobilization of the biomolecules [6].

2.3.2. Covalent binding

Another one of the most important method for DNA immobilization on transducer surface is covalent attachment. In this method, the probe ssDNA is attached via covalent chemical bonding between the transducer surface and a specifc functional group of the DNA, onto derivatized surfaces (e.x. glassy carbon or carbon paste modified electrode), functional groups (-COOH, -NH_2 etc.) substituted electro-active conduting polymers. In most commonly, coupling or cross-link reagents such as gluteraldehyde (GA), 1-ethyl-3-(3-dimethylaminopropyl)

carbodiimide (EDC) or a self-assembled monolayer were applied for creation of covalent bond between the probe ssDNA and modified transducer surface.

For example, Malhotra et al. gluteraldehyde (GA) used as a cross-linker for cross-link between the NH_2 modified probe ssDNA and electro-dposited thin film of polyaniline [7]. Similarly, Jadranka et al. investigated the electropolymerization of poly(pyrrole-co-4-(3-pyrrolyl) butanoic acid) onto which NH_2 modified probe DNA was anchored by the use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide coupling reagent [8].

2.3.3. Adsorption

This adsorption method is very simplest method. This technique involve based on the direct adsorption of probe ssDNA on the particular substrate including nitrocellulose, nylon membranes, polystyrene, metal surface and carbon. Adsorption mechanisms are most commonly classified as either physical adsorption and electrochemical. The physical adsorption is carried out by soaking the surface with the desired solution that needs to be immobilized and leaving the surface to dry at room temperature. In the case of electrochemical adsorption, DNA backbone has negatively charged groups, so that a positive potential applied to a substracte (electrode) attracts these ssDNA probe. It has the advantages of its ease of operation and it does not need any other chemicals or any other special nucleic acid modifications but its major limitation is the variability of the nucleic acid layer due to distortion of the molecule by adsorption and consequently the poor hybridization efficiency.

For example, Arora et al. investigated the application of physically adsorbed double stranded calf thymus DNA onto polypyrrole-polyvinyl sulfonate (PPy-PVS) film coated onto ITO glass substrate for sensing o-chlorophenol and 2-aminoanthracene [9].

2.3.4. Self-assembled monolayer

Self-assembled monolayer (SAM) of thiolated probe ssDNA or regular DNA is formed by spontaneous adsorption or chemical binding of molecules from a homogeneous solution onto a substrate. Most literature studies have used this method for the immobilization of DNA in the form of a SAM onto a gold surface through well-known thiol chemistry.

SAM of terminally thiol labled probe ssDNA onto gold surface offers a direct simple method of chemisorption of DNA probes onto transducer surface based on the formation of gold-thiol (Au-S) bonds [10]. 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, palladium, copper and platinum.

A mixed SAM has recent trend to fabricate the DNA biosensor, it shows the better hybridization discrimination efficiency when compared to conventional SAM. In this mixed

SAM method, the probe DNA with different thiol compounds used as diluent molecules such as 6-mercapto-1-hexanol (MCH), 3-mercaptopropionic acid (MPA) and so on [11,12]. The main advantage of this method, we can control the immobilized probe ssDNA orientation as well as probe ssDNA density onto the transducer surface.

2.3.5. Affinity interactions

It is well-known that streptavidin and avidin are the most stable proteins in nature. It has peculiar properties along with the ability of biotin to be incorporated easily into various biological molecules, allow streptavidin to serve as a versatile, powerful affinity tag in a variety of biological applications. Due to the reason behind that the strong binding between streptavidin/avidin and biotin, the both have been the most widely used affinity interaction in ssDNA immobilization [13,14]. Tetramer binding is created between streptavidin and biotin, yield in a very high affinity bond, with stability as high as a covalent bond. This strong binding does not affect by other external factors including pH or temperature, organic solvents and denaturing agent. But, the presence of the large protein molecules may possible to create a non-specific binding sites and compromise the sensitivity and selectivity of particular types of sensors [15]. For example, Singh et al. prepared chitosan-iron oxide film and used for immobilization of biotinylated probe ssDNA over chitosan-iron oxide film [16]. Similarly, nanostructured electroactive conduting polyaniline film on ITO glass plate has been fabricated using avidin-biotin as cross link for the sexually transmitted disease (STD) DNA detection [17].

2.4. Hybridization detection and amplification in DNA sensors

The conventional methods for identification of specific DNA sequences are based on hybridization with corresponding complimentary targe DNA, polymerase chain reaction (PCR), Southern blotting and various chemical methods. These are expensive, time-consuming techniques require highly trained person and lengthy sample preparations.

To overcome these difficulties several research groups have reported DNA biosensors based on probe ssDNA has been immobilized onto a suitable matrix coupled to a physical transducer. The transducers are generalized into three main categories: optical, microgravimetric and electrochemical techniques.

2.4.1. Optical DNA hybridization biosensor

Different types of optical DNA hybridization biosensors have been explored till now. These techniques commonly involve the use of fluorescent or surface plasmon resonance (SPR) spectroscopy depending on weather a fluorescent label is used in the probe ssDNA. Typical fluorescent DNA biosensor works on the emission signal from a fluorescent-label which is generally attached into either the DNA duplex or target DNA to transduce the hybridization via

the use of fluorometry. In most commonly, fiber optics have been used as the medium to transduce this signal produced from DNA hybridization process as they allow light transmission by series of internal reflection. A wide range of different optical transducers for DNA sensors has been extensively studied [18-20].

2.4.2. Microgravimetric DNA hybridization biosensor

As with using SPR for DNA sensing, the microgravimetric DNA biosensor is also able to offer label-free in situ detection of DNA hybridization through acoustic waves, surface acoustic waves or love waves. Acoustic wave identification using the quartz crystal microbalance (QCM) has been demonstrated by several research groups [21-25]. The QCM is well-known and popular as an extremely sensitive mass-measuring instrument as its resonance frequency decreases with an increase in mass on the QCM [26]. This QCM method can also applied to detect the single mismatch targe DNA sequences. For example, it is observed that 26-31 % decrease in resonant frequency when using single mismatch target DNA in the DNA hybridization study by QCM [23].

2.4.3. Electrochemical DNA hybridization biosensor

Electrochemical sensors have distinctive and very attractive advantages over the other detection methods (such as optical and microgravimetric sensing systems), including simple, rapid, low cost, point-of-care detection for selected target DNA and suitable for microfabrication technology [27-31].

Electrochemical biosensors combine the analytical power of electrochemical techniques (cyclic voltammetry, amperometry, electrochemical impedance spectroscopy, coulometry and so on) with the specificity of biological recognition processes (DNA hybridization). In general, the bioreaction produces an electrical signal that relates to the concentration of an analyte. For this purpose, a biospecific reagent is either immobilized or retained at a suitable electrode surface, which converts the bio-recognition event into a quantitative amperometric or potentiometric response. The combination of the electrode surface with a biomolecule provides new and attractive platform that are useful to solve the many challenging problem [32]

An impressive number of new designs for electrochemical DNA hybridization sensing have been emerged. Owing to their unique advantages, currently several publication and review articles can see in the literature. For example, good review articles were published by Kerman et al. [33], Drummond et al. [34], Wang [35] and Gooding [36] summarized the state-of-the-art and recent trend in electrochemical DNA hybridization biosensor technology. The most general strategy for electrochemical DNA hybridization detection is through the use of a redox-active labeled probe. The siginificant changes were observed from the affinity of the redox molecule after the interaction probe ssDNA when interaction with sample target DNA.

The labels range from redox-active DNA specific molecules, e.g. DNA groove binders [37] and intercalators [38-40], biological molecules such as enzymes [41-43] or metal nanoparticles [44-46]. In addition to that the label-free DNA detection is also possible through monitoring by the either the intrinsic redox-active properties (e.g. direct oxidation) of DNA bases (guanine or adenine) [47-50] or a changes in electrical properties on the transducer surface [51].

2.4.4. Amplification of DNA Biosensor

The novel elecroactive materials with special structure and modified substrates such as nano gold [52-55] quantum dots [56-58], carbon nanotubes [59-63], graphene [64-66], and CPs [67,68] have been used in a DNA hybridization biosensors as a signal amplifiers. Among them, conducing polymers (CP) are well-known as functional materials for biosensing applications due to their unique electrical, electronic, magnetic and optical properties, which are found only in inorganic system.

3. Brief Overview of Conducting Polymers (CPs)

Conducting Polymers (CPs) are polymers that inherently transmit the electricity and have attracted a great deal of attention over the past few decades. The first study about CPs was demonstrated by Letheby in the year of 1863 [69]. He has reported the chemical oxidation products of aniline in acidic media such as the human stomach. In the early 1900s, German chemists found and named different compounds as “aniline black” or “pyrrole black” and applied them on an industrial scale. However, detailed research about synthesis of polyaniline through chemical method was investiaged in 1962 by Moliner et al. [70]. Followed by, Bolto and co-workers were reported the iodine-doped polypyrroles in 1963 [71]. However, the electrical conducing properties of polyaniline and polypyrrole, as well as the relationship of their chemical structure remained unknown [72]. Interestingly, these issues were solved by Alan MacDiarmid, Hideki Shirakawa in 1977 by the discovery of highly conductive polyacetylene doped with iodine, which is the first study to demonstrate the conductivity of the polymers [73,74]. Following this great discovery, other different types of conducing polymers including polyaniline (PANi) [75], polypyrrole (PPy) [76], polythiophene (PT) [77], polyphenylene (PP) [78] and poly(phenylene vinylene) (PPV) [79] were found and studied in details.

3.1. Synthesis of conducting polymers (CPs)

Conducting Polymers can be widely synthesized through two methods including chemicallly and/or electrochemically oxidative polymerization of the appropriate monomers [80, 81]. Typically, chemical polymerization usually carried out by using some chemical oxidizing reagent ($FeCl_3$ or $(NH_4)_2S_2O_8$)), which is play a two role one is oxidize the monomer and secone role is provide a dopant anions. The chemical polymerization has the following advantages and disadvantages.

Advantages

Large-scale production possible

Post-covalent modification of bulk CP possible

More options to modify CP backbone covalently

Disadvantages

Can not make thin films

Complicated synthesis and purification process

Electrochemical polymerization commonly involves the formation of low molecular weight oligomers that are further oxidized by applying through lower potential than the initial monomer to form a polymer film on the conduting electrode surface (platinum, gold, glassy carbon and so on). It has the following merits and demerits.

Advantages

Thin film synthesis possible

Easy of synthesis

Entrapment of molecules in CP

Doping is simultaneously possible

Disadvantages

Difficult to remove film from electrode surface

Post-covalent modification of bulk CP is difficult

The different electrochemical techniques can be used including potentiostatic (constant potential) [82], galvanostatic (constant current) [83] and potentiodynamic (cyclic voltammetry) [84].

A counter ion is added during electro-polymerization to balance the positive charge created within the polymer chain. This process is usually called as doping and the counter ion is called dopant. The dopant can be provided by the oxidant employed during chemical polymerization or can involve electrolyte ions used during the electrochemical polymerization. The dopant incorporated into the CP during synthesis has a main responself for the effect of the conductivity, chemical and physical properties of asprepared CPs.

In addition to chemical and electrochemical oxidation polymerization, CPs have also been synthesized by methods including photochemical polymerization [85], plasma polymerization [86], enzyme-catalyzed polymerization [87], organometallic cross-coupling reaction [88] etc. However, most of these techniques for the preparation CPs involve the use of expensive chemicals and time-consuming.

3.1.1. Polyaniline

Polyaniline (PANi) known for approximately more than 150 years, PANi is the oldest and potentially one of the most useful electro-active CPs because of its much simple synthesis, environmental stability, and simple acid/base doping/de-doping chemistry. The polymeric structure of PANi is shown in **Figure 4**. It has three oxidation states, the fully reduced leucoemeraldine form (y = 1), the fully oxidized pernigraniline form (y = 0), and the half oxidized emeraldine form (y = 0.5) [75].

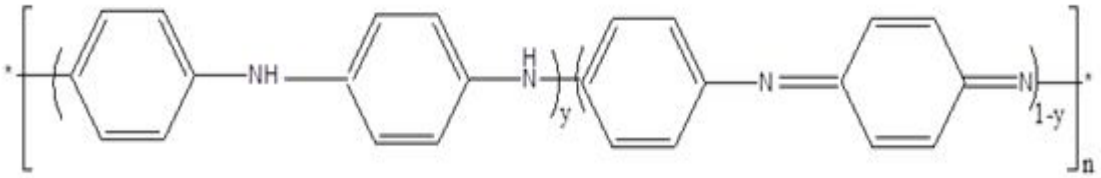

Figure 4: Polymeric structure of polyaniline

3.1.2. Polypyrrole

Polypyrrole (PPy) comprising of five-membered hetrocyclic rings is one of the most promising CPs (Figure 5). PPy was first chemically polymerized in 1916 by oxidation with H_2O_2 to give an amorphous black powder known as pyrrole black [89,90]. Later, Bolto et al. reported highly electrically conductive iodine doped polypyrrole in 1963 [71]. Since then numerous extensive reports with attractive properties have been developed on all aspects of this type of CP because of their easy synthesis, tunable conductivity, reversible redox property, high mechanical stability and good environmental stability.

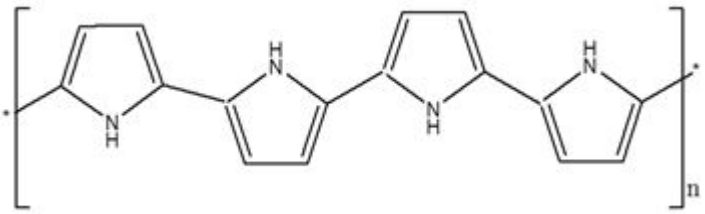

Figure 5: Polymeric structure of polypyrrole

3.1.3. Poly (3,4-ethylenedioxythiophene)

Poly (3,4-ethylenedioxythiophene) (PEDOT) is a conducting polymer based on the 3,4-ethylenedioxythiophene (EDOT) monomer, having the chemical structrue shown in **Figure 6.** This derivative of polythiophene was fabricated in the second half of the 1980s by the scientists at the Bayer AG research laboratories in Germany [91]. Since then the PEDOT polymer has been attracted considerable attention in many potential areas due to it is high electrical conductivity and stability in the oxidized state.

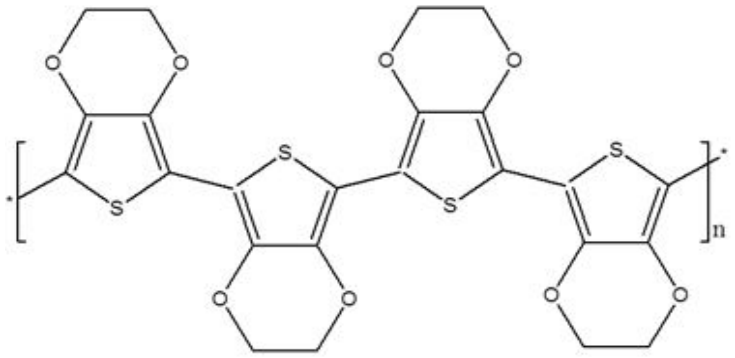

Figure 6: Structure of PEDOT

3.1.4 Preparation of PEDOT

PEDOT is most commonly prepared by chemical or electrochemical oxidative polymerization methods. Chemical oxidative polymerization of EDOT monomer has been carried out using several ways and oxidizing reagents. For example S. Armes and R. Corradi [92] have synthesized PEDOT by using $FeCl_3$, $Ce(SO_4)_2$ and $(NH_4)_2Ce(NO_3)_6$ as oxidizing reagents. They have stated that oxidizing reagent concentration should be higher than the monomer concentration to get good yields and conduting of the product. Followed by, ferric toyslate used as oxidant at an elevated temperature (110˚C) in combination with imidazole as a base and formed a black, insoluble and infusible PEDOT film that demonstrated conductivity up to 550 S/cm was reported [93]. Jonas and Krafft has been developed soluble PEDOT based on $Na_2S_2O_8$ as the oxidizing agent in an aqueous solution of poly (styrenesulfonic acid) (PSS) [94].

In electrochemical polymerization results in the formation of a highly transmissive sky-blue in colour, doped PEDOT film at the anode was obtained [95]. The electrochemical polymerization of EDOT is normally carried out in organic solution and/or aqueous micellar media. For example, PEDOT films have been synthesized from alkyl ammonium or lithium salts as supporting electrolyte in acetonitrile or propylene carbonate as elecrolyte [96]. The generally accepted mechanism of electro-polymerization of PEDOT is similar with that of polypyrrole formation mechanism. However unlike pyrrrole, only the α-α’ coupling of the 3,4-ethylene-dioxythiophene is expected due to the blocked structure of the monomer. Hence, PEDOT is expected to have few defects than PPy.

4. Fabrication of Nano-Structured Conducting Polymers

In recent developments in nanoscience and nanotechnology, micro/nano-strctured electro-active conduting polymers have played an important role because of their unique physical and chemical properties. The nanostructure has several advantages when compared to that of bulk polymeric materials including high surface area, low density, along with special chemical and physical properties. The nanostructure CPs fabricated through template based method either hard or soft-template method.

4.1. Hard-template methods

The first prpration of conducting polymer nanofiber and nanotube nanostructure has been reported by Martin [97]. Following this great method, a different nanostructure CPs including polyaniline [98,99], polypyrrole [99,100] and poly (3,4-ethylenedioxythiophene) [99,101] has been reported. The commonly used hard-templates are aluminium oxdie membrane and track-etched polycarbonate membrane to fabricate the nanostructure CPs.

The hard-template methods are the most commonly used and the most efficient approach for the fabrication of highly controlled and uniform nanostructures. However, the used hard-template commonly has to be eliminated with help of strong acids/bases or organic solvents or with high temperature after the preparation. These kinds of removal steps may increase the cost and restrict for large scale synthesis. Further, these processes have severely affected the nanostructure of the resulting products.

4.2. Soft-template methods

The soft-template method is also commonly known as self-assembly techniques. Surfactant self-assembly in a solution has been studied in details including both theoretically and experimentally because of their importance in synthesis of micro/nano-structures with controlled dimension [102]. The self-assembly ability of surfactants in a bulk solution therefore creates the possibility of surfactant micells serving as soft-templates to form CP nanostructures. The main advantages of this method are that the soft templates promote the CP to grow in a tubular form and need not be removed after the polymerization. However, the soft templates are often not quite stable, the versatility for different systems is poor and the multiformity of final products is obvious [103,104].

5. Electorchemical Detection of DNA Hybridization Based Nanostructured Conduting Polymers

5.1. Role of nano-structured CPs in biosensor applications

It is well-known that electro-active CPs acts as versatile platform for sensing applications because they not only posses unique properties but also can be applied as immobilization matrix, receptors and transducers in biosensors fabrication process. In recent years, studies of CP-based sensors have shown a trend towards the development of nano-structured CP based sensors, owing the ability to tailor the sizes and structures. Because of their very high aspect ratio, high electronic conductivity and small size, nanowires and nanotubes offer the potential of high sensitivity, low power operation, and massive redundancy in nanosensor array. CP nano-structures not only retain these unique properties, but also have the characteristics of nanomaterials (e.g. large surface area, size and quantum effect), which further increases the

merit of CP in designing and making novel sensors.

5.2. Nano-structued materials in DNA hybridization biosensor

Over the past few years, nano-structured materials and technologies have played an important role for the design of new types of DNA sensing methods and devices, which have led to excellent improvements in terms of high sensitivity, selectivity, multiplexing capacity, and simplicity. Moreover, multifunctional nanostructured materials can be composited together to desing the versatile sensing platform, to meet the demands of fast, simple, and inexpensive methods for DNA biosensing. For example, Fang et al. [105] has been reviewed the applications of carbon nanotubes (CNTs) in electrochemical DNA hybridization biosensors specifically. He stated that in this review CNT plays a two significant role: (i) using CNTs as sensing platform for immobilizing DNA molecules as well as powerful signal enhancement to amplify signal where produced from the DNA hybridization process; (ii) CNTs help as effective carrier and/or indicator to concentrate proteins and/or electroactive analytes for electrochemical sensing of DNA hybridization. Followed by, Wang's group designed a CNT-based dual amplification route by using a chronopotentiometry for ultrasensitive electrical bioassay of DNA. The application of CNT amplifiers was combined with the preconcentration feature of CNT transducers to provide a dramatic improvement of the sensitivity of the sensor [106]. Zhang et al. [107] fabricated a high sensitive DNA hybridization biosensor using MWCNT-AuNP nanohybrid synthesized through layer-by-layer covalent attachment. Further, they have achieved a limit of detection to be 6.2 pM. Similarly, Sun et al. described the dendritic nanogold-reduced graphene oxide nano-composite for the electrochemical DNA hybridization detection by differential pulse voltammetry technique [108].

5.3. Nano-structured CPs in DNA hybridization biosensor

Different nano-structured conducing PANi nanotube have been fabricated by Zhang et al. [109] and successfully applied for the electrochemical detection of DNA hybridization. Feng et al. demonstrated gold nanoparticles and polyaniline nanotubes membrane and used for the DNA hybridization with high sensitivity [110]. Similarly, Zhou et al. fabricated sulfonated polyaniline nanofiber and AuNP for label-free potentiometric DNA hybridization biosensor [111]. Zhou et al. [112] reported DNA hybridization detection by electrochemical impedance spectroscopy using AuNP/CNT/PANi nanofiber.

It is well-known that the fabrication of nanostructured CPs is another issue currently limiting their application in DNA hybridization biosensor. In most commonly, hard and soft-template approaches were broadly used in the synthesis of CP nanostructures. However, simple, efficient, controlled and large-scale method for the preparation of nanostructured CPs are still lacking.

Recently, PPy nanotubes with excellent electrical conductivity have been prepared by using the fibrillar complex of $FeCl_3$ and methyl orange (MO), acting as a reactive self-degraded template. This novel template is stable in acidic aqueous solution of MO and can be dissolved under mild neutral aqueous conditions after the polymerization of monomers on its surface. In other words, it formally acts as a "hard-template", but effectively as a "soft-template". This can be considered an alternative to conventional soft and hard-template methods. This method was introduced by Yang et al. to prepare the PPy nanotubes with diameter as small as 50 nm [113]. $FeCl_3$/MO is a key template material for the fabrication of nanotubes because it can provide large effective surface area, nanometer size structures with high aspect ratio and can be fabricated in large scale with inexpensively and reproducibly.

The PPy nanotubes have been used as a core for the fabrication of PPy-PANi & PPy-PEDOT core-shell nanotubes. These core-shell nanotubes were synthesized by in-situ chemical oxidative polymerization of monomers on the surface of PPy nanotubes to form core-shell nanotubes [114 -116]. The as-prepared core-shell PPy-PANi nanotube has been used for the DNA hybridization detection. The detailed electrode fabrication process for the DNA sensor was shown in **Figure 7**. In the first step, the asprepared core-shell PPy-PANi nanotube has been modified over the gold transducer surface (electrode B) and then the polymer nanocomposite modified surface was soaked into gluteraldehyde (GA) solution to activate the surface (electrode C). It is well-known that gluteraldehyde has been widely used as crosslinker in biosensors that can covalently attach the capture probe ssDNA onto the modified electrode surface. Followed by, NH_2 modified probe ssDNA was immobilized (electrode D) over the gluteraldehyde activated polymer nanocomposite surface through cross-link between aldehyde and NH_2 functional groups. Finally, the probe ssDNA modified surface further utilized for the detection of DNA hybridization with target DNA. The hybridization event has been monitored through differential pulse voltammetry in the presence of methylene blue as intercalator. Methylene blue (MB) is a phenothiazine dye that is broadly used in electrochemical DNA biosensors. In general there are three different binding modes between MB and DNA. MB can interact with the negatively charged anionic phosphate backbone of DNA by electrostatic binding, intercalation with the major or minor grooves of the dsDNA helix and preferential binding between MB and guanine bases.

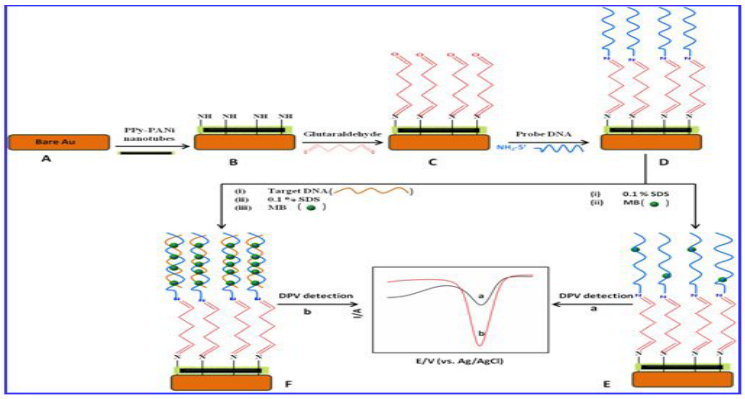

Figure 7: Fabrication procedure of this electrochemical DNA biosensor. (A) Bare Au, (B) A + PPy-PANi nanotubes, (C) B + GA, (D) C + ssDNA, (E) D + non-complementary and (F) D + complementary target [114]. Reproduced from Ref [114] with permission from the Royal Society of Chemistry.

Here, intercalation binding of MB with the major/minor grooves of the dsDNA helix structure have key role to get the greater DPV signal (electrode F) when compared to that of un-hybridized surface (probs ssDNA; electrode E). Further, the fabricated DNA sensor can detect mismatched target DNA with greater changes (**Figure 8**). In **Figure 8** shows the DPV response of the probe ssDNA (curve a) modified surface after interact with complemetarnty (curve b), non-complementary (curve c), single mismatched and double mismatched targe DNAs. Each target DNA has exhibited clear different peak current. It is suggested that PPy-PANi nanocomposite modified electrode has effectively distinguish the complementary, non-complementary and mismatched target DNAs. The fabricated sensor surface showed good linear range (1.0×10^{-9} to 1.0×10^{-13} M) and detection limit (50 fM)

Similarly, poly (3,4-ethylene dioxy thiophene) coated polypyrrole nanotubes (PPy-PEDOT) has prepared by chemical oxidative polymerization method and then silver nanoparticles self-assembled over the PPy-PEDOT nanocomposite to form PPy-PEDOT-AgNP nancomposite. The formed silver nanoparticles over the PPy-PEDOT was well dispersed, which is much benefits to attached the probe ssDNA with enough spacing between each HS-ssDNA for efficient coiling of target DNA.

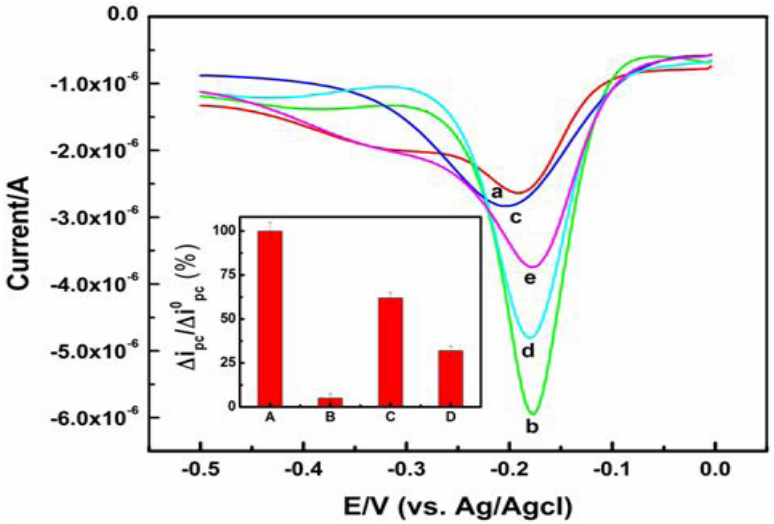

Figure 8: DPV of intercalated MB reduction peak current at (a) PPy-PANi-GA-ssDNA, (b) PPy-PANi-GA-dsDNA (com), (c) PPy-PANi-GA-dsDNA (non-com), (d) PPy-PANi-GA-dsDNA (SMM) and (e) PPy-PANi-GA-dsDNA (DMM). Inset: Corresponding bar diagram of normalized change in Ipc. Reproduced from Ref [114] with permission from the Royal Society of Chemistry.

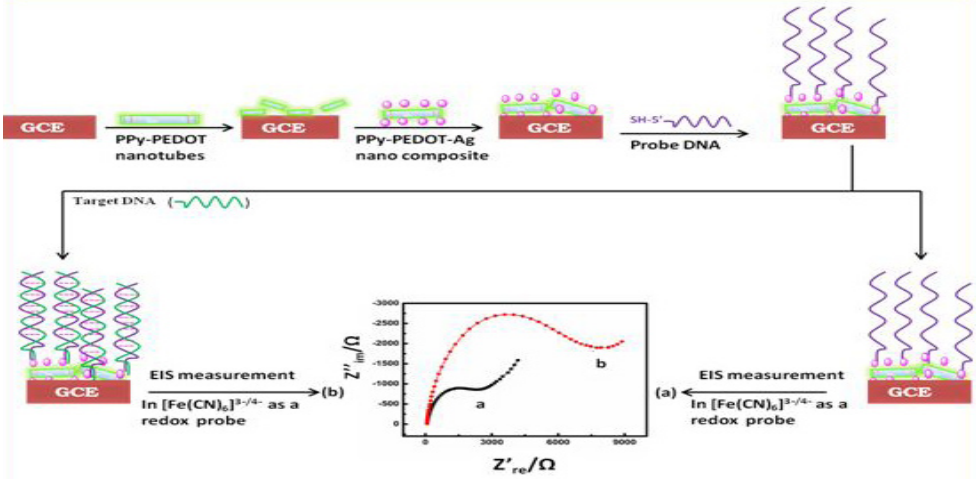

Figure 9: Schematic illustration of HS-ssDNA covalently immobilized onto the PPy PEDOT-AgNP nanocomposite by the Ag-thiol interaction at room temperature. Reproduced from Ref [116] with permission from the Elsevier.

The detailed electrode fabrication process has been explained in **Figure 9**. In this process, initially PPy-PEDOT-AgNP nanocomposite modified over the glassy carbon electrode by simple drop-casting method and then SH-ssDNA was immobilized through self-assembled monolayer. The ssDNA modified surface finally used to detect the different target DNA by electrochemical impedance spectroscopy in presence of $[Fe(CN)_6]^{3-/4-}$ as redox probe. **Figure 10** showed the electrochemical impedance spectroscoapy of different modified surfaces and inset figure 10 showed change in the charge transfer resistance for different modified electrode surface.

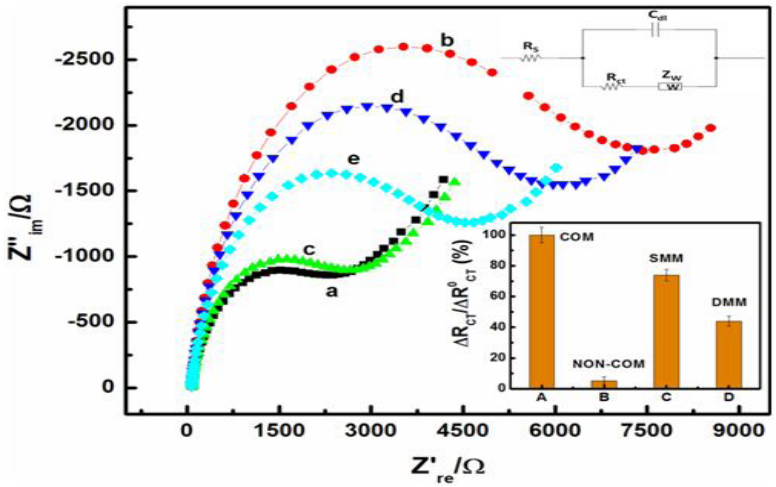

Figure 10: EIS behaviour of (a) PPy-PEDOT-AgNP-S-ssDNA, (b) PPy-PEDOT-AgNP-dsDNA (com), (c) PPy-PEDOT-AgNP-dsDNA (non-com), (d) PPy-PEDOT-AgNP-dsDNA (SMM) and (e) PPy-PEDOT-AgNP-dsDNA (DMM) in presence of 1mM $[Fe(CN)_6]^{3-/4-}$ in PB (p^H 7.0) solution. Inset: Corresponding bar diagram of normalized change in RCT. Reproduced from Ref [116] with permission from the Elsevier.

From the bar diagram clearly observed that the R_{CT} value much higher for complementary target DNA than other modified surfaces. It is due to the fact that complementary target DNA form perfect helical structure with probe ssDNA and the electrode surface has become more negatively charge created. Hence, the negatively charge electrode surface has strongly repel the negatively charged redox probe. Hence, the R_{CT} values were much higher than other modified electrode surface. The PPy-PEDOT-AgNP modified electrode surface exhibited good linear range from 1.0×10^{-11} M to 1.0×10^{-14} M with detection limit of 5.4×10^{-15} M. The

derived values is superior than that for MWCNT-Ag, PANi-Au, MWCNT-PPy-Au and PPy-PANi-Au nancomposite reported in literature [117-119].

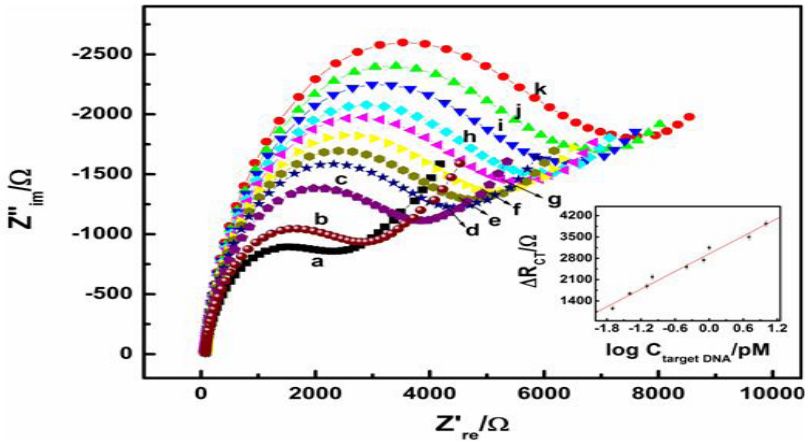

Figure 11: EIS detection of different target concentration using the PPy-PEDOT-AgNP-S-ssDNA. (a) 0, (b) 1.0 × 10-14, (c) 2.0 × 10-14, (d) 4.0 × 10-14, (e) 8.0 × 10-14, (f) 1.0 × 10-13, (g) 4.0 × 10-13, (h) 8.0 × 10-13, (i) 1.0 × 10-12, (j) 5.0 × 10-12 and (k) 1.0 × 10-11 M. Inset: Variation of ΔRCT with log (Ctarget DNA). Reproduced from Ref [116] with permission from the Elsevier.

6. Conclusion and Perspectives

Based on the unique properties of electroactive conducting polymers have been ultilized for DNA hybridization biosensor application. The conducting polmers were modified with nanoparticles (gold or silver) and/or using cross-link for probe ssDNA immobilization. The conducting polymers nanostructures can produce a synergic effect with enhance catalytic activity, conductivity and stability. Therefore, the preparation of conducting polymers with 1-D nanotubes has been utilized as platform for prbe ssDNA immobilization and hybridization with target ssDNA. In addition, the electrochemical method, which promising advantages of label-free detection of DNA hybridization event, should be a promising direction for the fabrication of portable DNA sensor tool. The role of conduting polymers in DNA hybridization biosensor not only provide an high sensitivity but also provide stable immobilization of probe ssDNA for ultra-low detection of target ssDNA. We hope these kinds of DNA hybridization biosensor based conducting polymer nanostructure have siginificant impact and hold a potential for future application in medical diagnosis.

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