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Development of Biosensor for Detection of Thrombin in Blood by Polyaniline Nanofibers

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by

Aditya Keshav Thakar

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Aditya Keshav Thakar

We, the thesis committee for the above candidate for the
Master of Science degree, hereby recommend
acceptance of this thesis.

Perena Gouma
Associate Professor Department of Materials Science and Engineering

Sanford Simon
Professor Departments of Biochemistry and Cell Biology and Pathology

Tadanori Koga
Assistant Professor Department of Materials Science and Engineering

This thesis is accepted by the Graduate School

Lawrence Martin

Dean of the Graduate School

Abstract of the Thesis

Development of Biosensor for Detection of Thrombin in Blood by Polyaniline Nanofibers

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Conducting polymers have always been a fascinating set of materials for researchers over the years. Their versatility in application makes them unique along with their easy processing techniques and low cost. Polyaniline is one of the most important members of this family. It has been widely used for various applications like corrosion resistance, EMI shielding, sensor development etc. Gas sensors and bio sensors have been developed by a lot of researchers having the basic working block as polyaniline. The ease of processing of polyaniline and

its capability to encapsulate bio molecules within has made it an even better option for bio sensor development.

This project aims at developing a new bio sensor making use of polyaniline as the base block for the detection of thrombin. Thrombin is a protease in the body that is responsible for thrombosis or excessive blood clotting. This is a study to try and crosslink polyaniline with glutaraldehyde and then covalently bond with a thrombin specific substrate. The cleavage of thrombin on the substrate will change the internal resistance of polyaniline and also be detectable in blood i.e. liquid media with the cleaved substrate molecules.

Polyaniline nano wires prepared by electrospinning have been used to enhance the sensor performance and reduce the sample required for testing. The polyaniline nano wires are hybrid nano wires prepared by using cellulose acetate as the binder for polyaniline. SEM and EDAX analysis were carried out for the Polyaniline/cellulose acetate films to thoroughly understand the structure of these films. The electrical resistance measurements were noted to get the basic concept of the bio sensor working. This biosensor when fully developed will be a disposable biosensor for detecting thrombin in blood.

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It is a matter of great satisfaction and pleasure to present this thesis on “Development of Biosensor for the Detection of Thrombin in Blood by Polyaniline nanofibers.” as my Masters’ Thesis.

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1. INTRODUCTION

I always wondered how the light automatically turns on feeling my presence in the room. How does the hand dryer start automatically when I stretch out my hand? The devices that that are responsible for these brilliant mechanisms have always been of great interest to me. In everyday life we see so many such devices that have now become an inseparable part of our lives. They aid us in our routine right from our home to our place of work or even during travel. A simple example may be the coffee vending machine which fills the glass only till a certain level or it does not start till a glass is placed under it. These devices sense needs and presence and deliver the required output much to our amazement in many cases. These “sensible” sensing devices are called Sensors.

A sensor is a device that takes physical, chemical or biological signal as input and gives an output signal in response these inputs. A sensor basically comprises of two components viz. the Detector and the transducer. The detector accepts the input signal which can be physical, chemical or biological. The detector can be a micron size material, nano particles or fibers, composite, metal oxide, polymer or any other material that can be useful in detection of the particular input signal. The transducer performs the task of converting the input signal into measurable signal such as electrical, optical, acoustic or any other measurable type of signal.

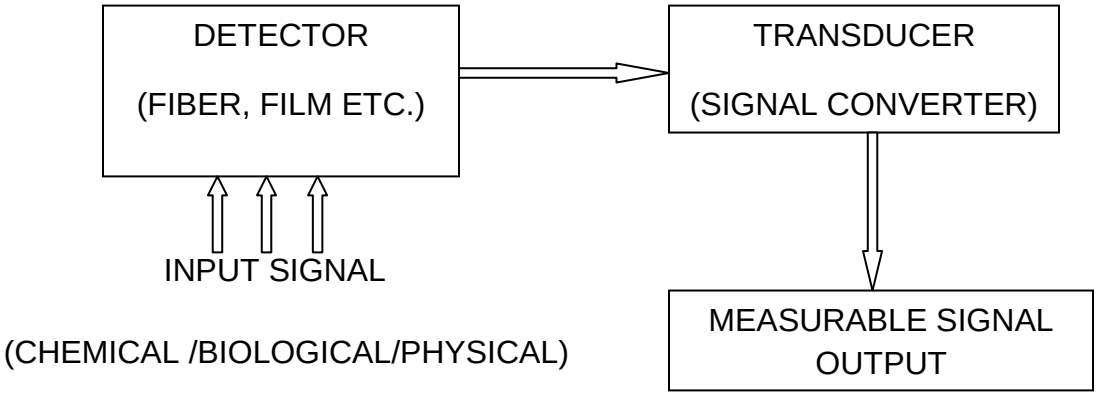


Figure 1: Sensor Working Principle

Sensors find most common applications in gas sensing, motion sensing, and heat sensing and even biological sensing. The application of sensors in the biological field has been a fascinating area of research for many years now. A very commonly used bio sensor is the diabetes sugar level sensor. A single drop of blood is enough for the detection and determination of sugar levels in blood. Hence the use of biosensors in the field of blood sensing has widened its horizons to a large extent. Another major blood related disease or problem is the formation of clots in the blood also called as blood coagulation. If not controlled at the initial phase of the clotting the development could prove fatal for the patient. Thus, it becomes important to know why and how these clots are formed and what exactly is responsible for their formation. Blood coagulation is facilitated by a protease in the body called thrombin. Over the years researchers have developed a range of sensors for thrombin detection. Polymers are one of the few methods used for sensor development in this field. Conducting polymers are a fascinating set of materials that have attained immense importance in the sensor development field. The fact that they are easy to process and light weight gives them added advantage over metals or other bulky materials. Polyaniline is one such conducting polymer that has been used by researchers for many years in various applications including sensor development. The applications of Polyaniline in the field of bio sensing are of great interest and are still being explored for even better possibilities[1, 2].

2. BIO SENSORS

Bio Sensors are sensors that use biological signal as input. This biological quantity can be protein, enzyme, antibodies, nucleic acids tissues and many more. This bio signal is also known as the analyte or the quantity to be measured or detected. The biological quantity reacts with the senor material or the receptor. The reaction is then converted into measurable signal by the transducer. The transducer converts the reaction of analyte with the receptor into optical, electrical or any other form of measurable signal [1].

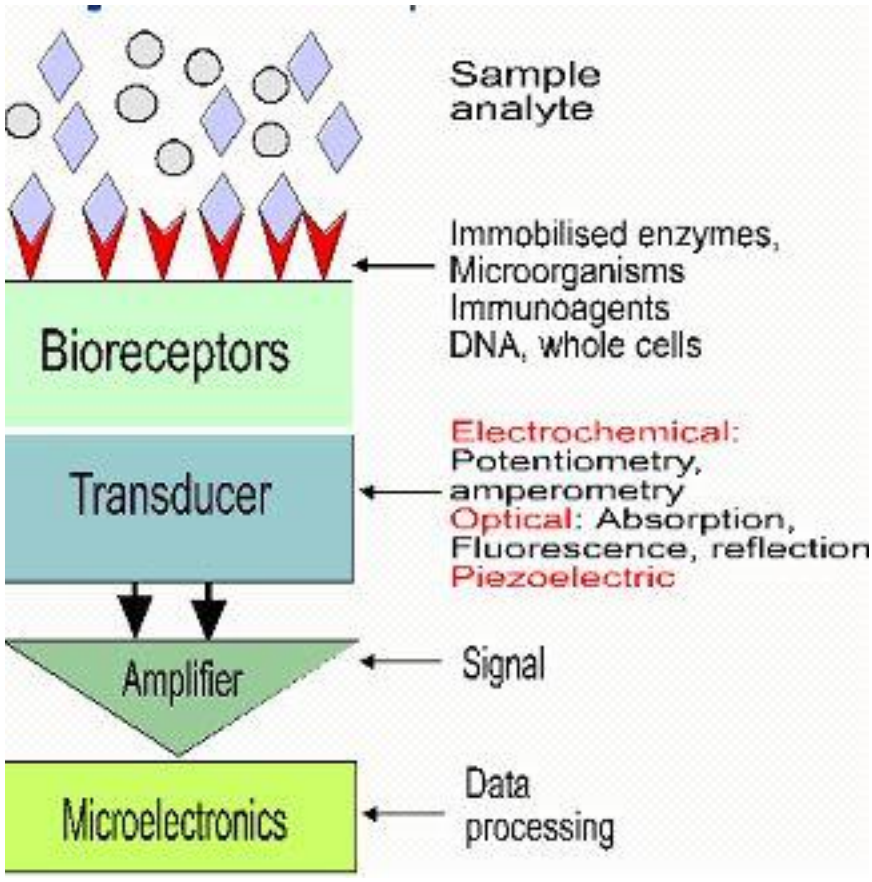


Figure 2: Bio-sensing Mechanism

3. THROMBIN

Thrombin is a serine protease protein present in the human body that plays a crucial role in blood clotting or blood coagulation. Firstly, it is important to understand the mechanism of blood clotting and the factors responsible for it and contributing to the formation of a clot [3, 4].

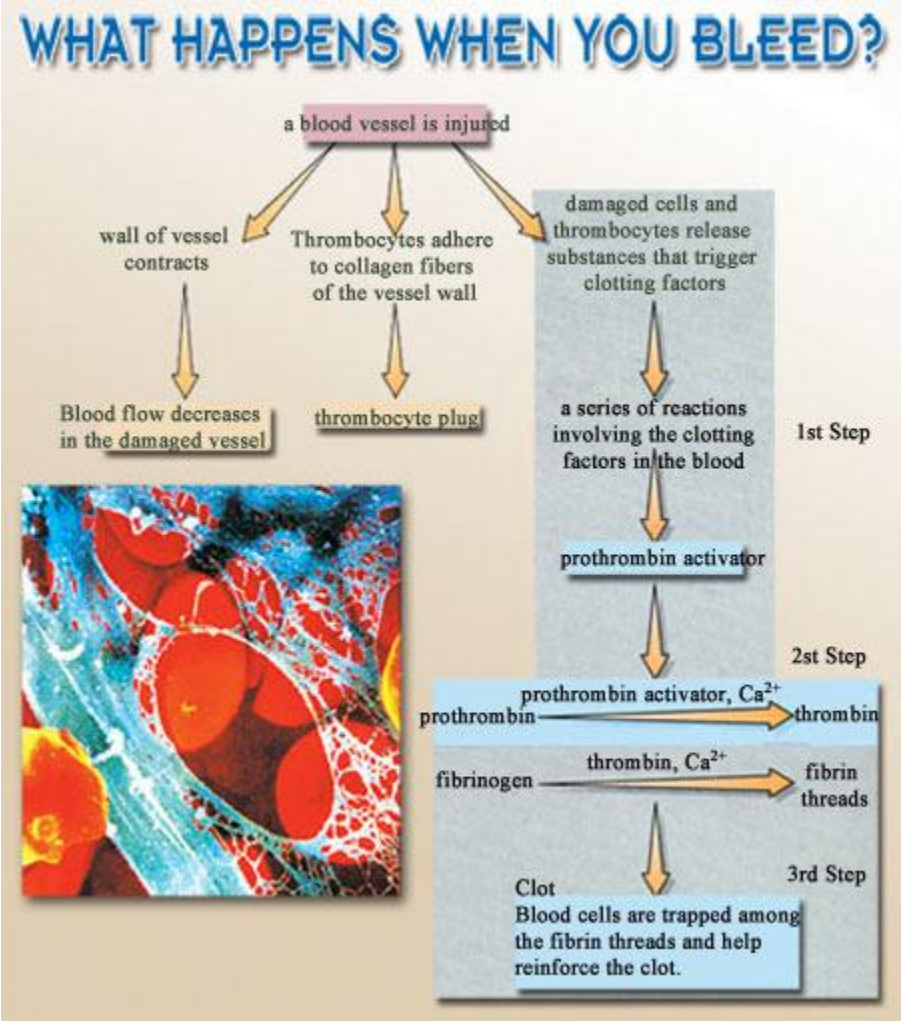


Figure 3: Mechanism of blood clot formation

Clotting is an important mechanism of the body that needs to operate well for our survival. Blood flows continuously in our body through the blood vessels. If by some means a blood vessel is damaged, the blood will start to flow away and it is important for this to be prevented. This is where the clot plays a crucial role. The clot arrests the blood flow locally and prevents the excessive loss of blood. This clot though should act locally only and it is important that it stops at certain level so that abnormal clotting can be prevented [3, 4].

Blood clotting needs to be understood with respect to two very important aspects viz. the platelets and the thrombin mechanism. The platelets are present in the blood stream. They are ready to act as soon as problem develops. The moment a blood vessel is damaged and bleeding starts, these platelets become sticky. The stickiness of the platelets indicates their activation. They line up along the damaged portion of the vessel preventing further flow of blood. The pile up of these platelets is called the “white clot.”

Thrombin system comprises of many proteins in blood which get activated when bleeding starts. These proteins undergo a series of reactions to produce a substance called fibrin. Figure 2 explains the formation of a blood clot with thrombin playing an important role in it. The mechanism of clotting due to thrombin system can be explained by a 3 step process as explained below.

- Formation of Prothrombinase: The prothrombinase formation depends upon the system that applies for blot clotting that time. The intrinsic system is the one where liquid blood comes in contact with a foreign body i.e. something that is not a part of the body. Extrinsic is the system is activated when the liquid blood comes in contact with the damaged tissue.

- Formation of Thrombin enzyme: Prothrombin is a plasma protein formed in the liver. The Prothrombinase converts this prothrombin to enzyme thrombin.

- Formation of Fibrin from Thrombin: Thrombin formed in the second step converts fibrogen which is another soluble plasma protein in liver into insoluble Fibrin. Fibrin being insoluble forms threads which in turn bind the clot. The threads of fibrin act as a web which has red cells entangled in it resulting in the formation of a “red clot.”

When the clot becomes mature, it consists of both platelets and the fibrin. The fibrin threads bind the platelets together which makes the clot stable [3, 4].

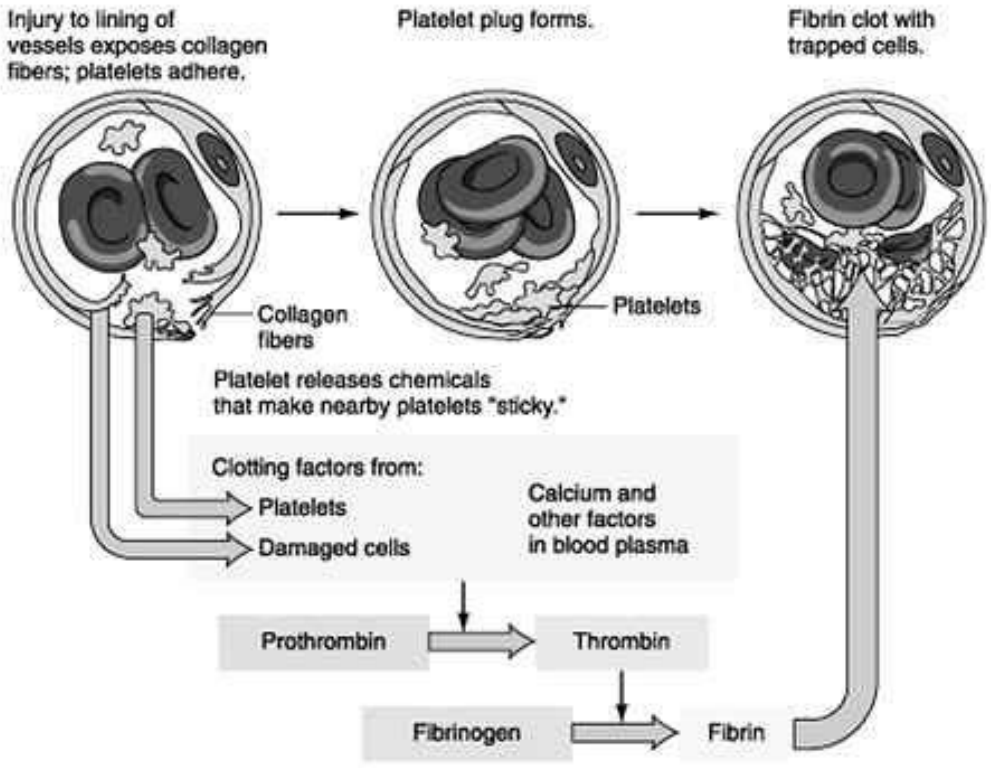


Figure 4: Three step Blood clotting mechanism

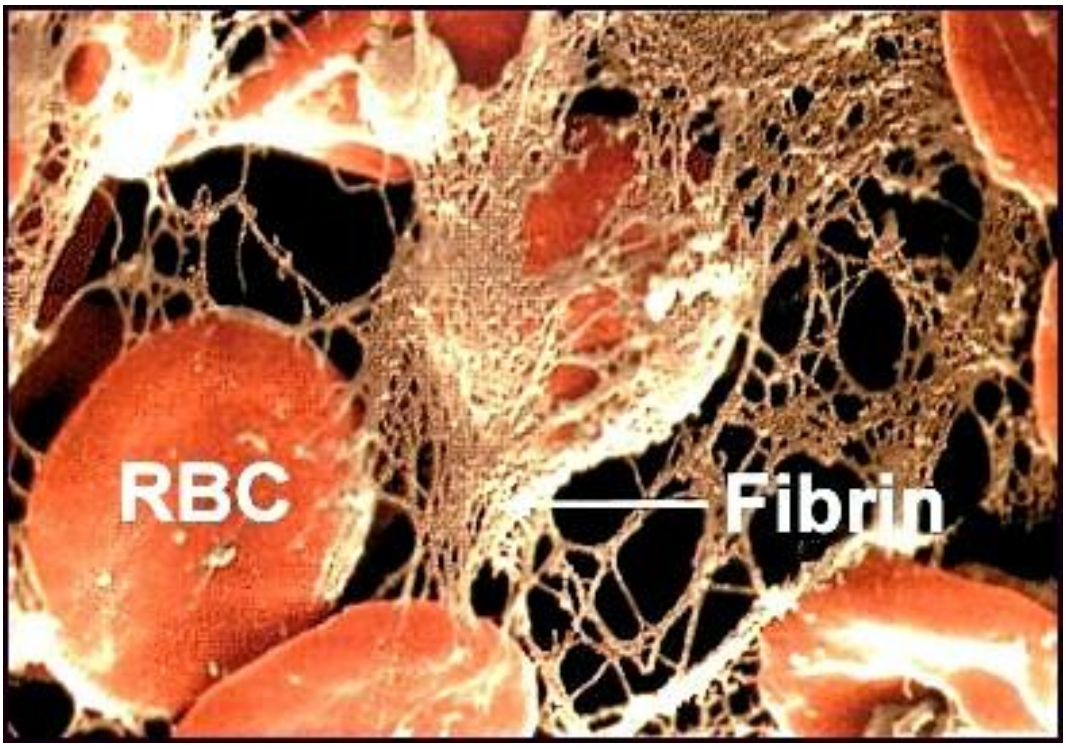


Figure 5: Blood clot showing Red Blood Cells entangled in Fibrin threads

Figure 3 shows the three step formation of the blood clot. The first part shows the damage to the blood vessel. The second part shows how the platelets block the flow of blood through the formation of platelet plug. The third part shows the formation of fibrin which then entangles the red blood cells to then form a larger clot. Figure 4 shows the image of actual blood clot where the threads of fibrin are clearly seen with the red blood cells entangled in them.

It is thus becomes very important to detect thrombin that it is in excess of required level. This is where the role of blood biosensors plays a pivotal role. Researchers over the years have developed various sensor materials to carry out the job some of which are given in the following section.

4. THROMBIN DETECTION SENSORS

Detection of thrombin is most commonly done by using aptamer based sensors. It is hence important to understand the concept of Aptamers. Aptamers are oligonucleic acid or peptide molecules that bind with specific target molecules. This binding is used in detection of various bio molecules like thrombin. Researchers over the years have made use of many aptamers for thrombin detection.

The immobilization of the aptamers on various materials has been successfully attempted by researchers and scientists. Hansang Cho et al used Surface Enhanced Resonance Raman Spectroscopy (SERRS) technique to detect thrombin. The sensitivity of the sensor was 10pM and sensor was highly selective to thrombin. They utilized gold nano particles to immobilize the thrombin aptamer and then recorded the change in surface resonance using Raman spectroscopy which occurred due to the displacement of the thrombin aptamer on the gold nano particle [5]. Mehmet Veysel Yigit et al used MRI signal contrast for thrombin detection. They used supermagnetic iron nano particles for the aptamer immobilization and observed the contrast in MRI signal after thrombin reaction. The sensitivity of the sensor was observed to be 225nM [6]. Xiao-Li Wang et al designed an aptamer- based extranuclease protein essay for the detection of various proteins. They used a thrombin aptamer for their experiments which consisted of 22-base-long single-strand oligonucleotide with the thrombin aptamer sequence at 3' terminal and 7 additional ones. The sensor had very high selectivity and sensitivity owing to this structure of the aptamer. They generalized the method for a range of proteins which can be detected with sensitivity and selectivity without any washes or separations [7]. Jung A Lee et al made use of pyrolyzed carbon films to immobilize the thrombin aptamer. The sensor is an electrical impedance biosensor with a sensitive to thrombin concentrations between 0.5nM to 500nM. The carbon pyrolyzed thin films were uses as working electrodes. The binding of thrombin with carbon results in the electron transfer and hence in impedance changes which are measured to detect

thrombin[8]. Xiaoying Wang et al used the electrogenerated chemiluminescence for thrombin detection. They used tri ruthenium doped silica nano particles as DNA tags for thrombin detection. The assembly of the aptamer was carried out on gold electrodes. The displacement of the DNA probe due to thrombin was recorded as the electrogenerated chemiluminescence. Thrombin concentrations ranging from 10fM to 10pM were detected using this sensor [9]. Ewa and Thomasz Heyduk developed nucleic acid based fluorescence sensors for protein detection. They successfully detected thrombin utilizing this sensor. The sensor is an aptamer-based fluorescence assay comprising of two aptamers co-associated for protein detection. Thrombin concentrations up to Pico molar were detected using this sensor [10]. Wenjuan Wang et al used gold nano particles for the immobilization of the thrombin aptamer. They used the gold nano particles as fluorescence probes and the aptamer as the probe. The gold nano particles were DNA modified with which they tested the ability of the biosensor under various parameters such as temperature and microenvironment [11]. Alessandra Bini et al immobilized the thrombin on piezoelectric crystals having gold on their surface whereas Hiaxin Chang et al tried detecting thrombin by fluorescence resonance energy transfer when thrombin reacts with a dye labeled aptamer assembled with grapheme [13, 14]. Platinum nanoparticles were used to functionalize nucleic acids by Ronel Polsky's group. These platinum nanoparticles were used to get amplified electrochemical signals for thrombin detection. The sensitivity limit was 1nM wherein the platinum nanoparticles acted as catalytic labels for the protein detection [15]. Douglas Toffelsen and his group came up with a new detection technique by detecting a Heparin dependent thrombin inhibitor in the human blood plasma itself while Nobuko Hamaguchi's group designed a new set of molecules by the name of aptamer beacons to detect thrombin. The aptamer beacons are fluorescent pair of molecules that show change in fluorescence upon reaction with thrombin [16, 17]. In 1989, M. Arai and his research group had come up with a sensor for detecting factor VIII gene at a thrombin cleavage site. The loss of factor VIII at a thrombin cleavage site was detected. This molecular deficiency was taken as a basis of direct detection of mild Hemophilia in a patient was developed [18]. Valeri Pavlov's group used thiolic nucleic acids for thrombin fictionalization and the detection was carried out by observing fluorescence changes. He also used gold nanoparticles as

electronic markers for the thrombin aptamers. Neil Rote designed a sensor to sense Phosphatidylserine which is present during thrombin-platelet activation and is directly related with spontaneous thrombosis [19, 20, 21]. Hui Yang developed a sensor for thrombin detection by making use of quantum dots. The quantum dots were labeled to the aptamer and this use of quantum dots reduced the sample requirement to 5µL and improved the sensitivity to below 1pM.

Bin Liu and Hoang- Anh Ho have developed sensors for thrombin detection using polymers. Bin Liu used the molecular orbital energies of conjugated polymers to detect thrombin. Hoang Ho used aptamer/conjugated polymer hybrids for the detection. Liu's group used water-soluble poly (fluorene-co-phenylene)s on the conjugated polymer backbone. Hoang used water soluble poly thiophene for his experiment in which he used single stranded DNA as the aptamer. Single stranded aptamer binds selectively with human thrombin and changes structure. This change in structure is enhanced by the polymer by means of optical signal [22, 23].

5. POLYANILINE

Polymers have revolutionalized the materials research field over the years. Their ease of fabrication, low cost and low weight has made them attractive options to replace metals and other bulk materials. Polymers can be used in the form of films, coatings, nano/ micro fibers and particles. They can be used in combination with other materials to fabricate ceramics, composites which are of immense use in many industrial and research applications. Conducting polymers have given the field of polymers a whole new dimension. They now offer even more attractive opportunities to researchers and scientists to make optimum use of their properties to make specific materials according to the requirement. Polyaniline (PANI) is one of the most important members of the conducting polymer family. Its semiconducting properties have made it a very convenient material to be used for varied applications. The applications for which Polyaniline had been used successfully include corrosion resistant coatings [29], electromagnetic interference shielding [30], electrochemical and biological sensing applications and many more [25].

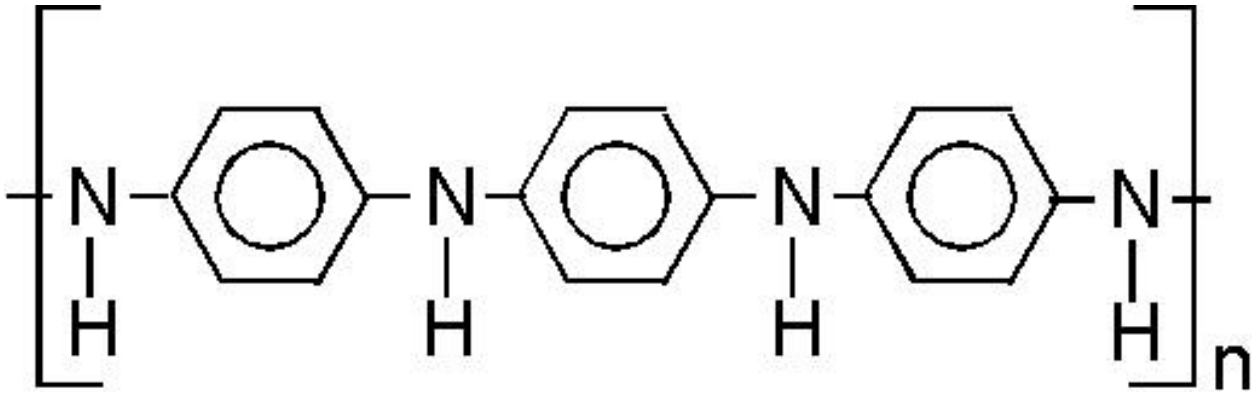


Figure 6: Structure of Polyaniline

The major advantage of polyaniline is its ease of processing. The fact that it can be synthesized to get thin or thick films, nano particles, composites and even can be drawn into nano fibers of diameters of a few nano meters gives it a distinct edge over a lot of other materials. The melt processing techniques of polyaniline include injection molding, blow molding, compression molding, fiber spinning etc. Solution processing techniques such as spin coating, casting, gels and electrospinning for nano fibers are also important techniques helpful in processing polyaniline. These different processes are used to get polyaniline to be in the required form for the particular application which includes textile industry, automobile industry, electronics and food packaging.

The applications of polyaniline can be even more efficient at nano level. Hence the use of polyaniline in the form of nano fibers has been of great interest to researchers especially for sensing applications. Jiaxing Huang investigated the various methods of synthesis of PANI nano fibers and their various applications. He has presented the methods to prepare pure PANI nano fibers by suppression of secondary growth of irregularly shaped particles. It was observed that the nano fiber structure was a natural part of the oxidation polymerization process of aniline. It formed the initial phase of polymerization which was a very important observation that could be used for making pure PANI nano fibers. The second phase of polymerization was the growth of irregular particles which on agglomeration gave polyaniline agglomerated structure. Huang observed that if this unwanted growth was controlled then pure PANI fibers can be obtained in their natural form [26].

Huang achieved the suppression of secondary growth with two techniques. In the first technique, the polymerization was only allowed to take place at the interface of the solution. The PANI used for the experiment is in its hydrophilic emeraldine salt form which diffuses away into the solution not allowing any scope for the secondary growth to occur. The second technique was aimed at stopping the polymerization after the nano fibers were obtained. This was achieved by fast mixing of monomer and initiator

solutions wherein the monomer molecules are swiftly consumed by polymerization thus not allowing the secondary molecules sufficient initiator molecules for their growth. Huang illustrated the applications of these PANI nano fibers for chemical vapor reaction sensing by using them to make composites with metals. He has also demonstrated the use of these nano fibers for flash welding [26].

Manuel J. Díaz-de León used the technique of electrospinning to prepare doped PANI nano fibers. He used Polystyrene and Polyethylene oxide as the binders for the fibers. 100mg Polyaniline was mixed with 129mg of 10-camphosulfonic acid (HCSA) and dissolved in 10ml of chloroform. The green color solutions were filtered after 6 hours of stirring to remove the non-dissolved parts. Two parts of this solution were prepared and polystyrene and polyethylene oxide were added to solution part 1 and 2 respectively. Thus, 2 homogenous solutions of PANI/PS and PANI/PEO were obtained after 4 hours of stirring. These solutions were deposited as nano fibers using the Electrospinning technique. Manuel used a pipette instead of syringe and hence used a copper wire to create charge within the polymer solution. The pipette was tilted downwards from the horizontal and a voltage of 15 -25 kV was applied to get electrospun nano fibers of PANI [27].

The electrospinning technique is a very easy and efficient way to prepare hybrid PANI nano fiber mats with different binding materials such as cellulose acetate (CA). These nano fibers have very good conducting ability and are hence of immense importance in the sensor development field. The use of these conducting polymer like PANI have for bio sensor development has been studied successfully and extensively by researchers over the years.

Polyaniline has been one of the most favored materials for bio sensor development as it offers some distinct advantages over many other materials. The first and foremost important properties for which it is such a popular choice are high conductivity and low cost. Other advantages offered by PANI are high surface areas, redox conductivity, chemical specificities, thickness control and the ease of deposition on any sensor electrode [25].

The conductivity of PANI can be explained on the basis of its semiconducting properties. PANI is a p-type semiconductor where holes are the majority carriers. Acid doping of PANI results in to polarized structures viz. bipolaron structure and the polaron structure. The polaron structure is more stable of the 2 structures. The nitrogen radical in the polaron structure acts as a hole. When the electron form the adjacent nitrogen jumps over to this radical, it becomes stable thus creating a hole in the adjacent nitrogen. This makes the hole move along the chain thus making the polymer conductive by the hopping mechanism. The same condition however is not applicable to the bipolaron structure. The holes in that structure are located very close to or adjacent to each other thus making the movement of holes impossible and makes the structure non-conductive [25].

PANI enables the integration of the biological receptor element so as to give detectable transducible signals. PANI is best suited to bio sensing applications owing to its chemical and structural stability around its amine groups which suits the immobilization of bio molecules. As the structure of PANI is stable, the bio molecules immobilized on it form a stable structure as well which is vital for the sensor to be effective. Nano structure of PANI has opened up even better possibilities for better bio sensor development. The nano size assures more surface area for the reaction and less amount of sample for testing. A significant improvement in the sensitivity of the sensor can be achieved with the use of nano size PANI [25].

PANI can be used very effectively for making immunosensors. The combination of antibody and antigen in connection with the transducer can help in detection of a wide range of analytes. PANI has been cross-linked using glutaraldehyde to get covalent immobilization of bio molecules on it. Glutaraldehyde and its cross-linking characteristics with PANI present a great avenue for bio sensor development.

6. GLUTARLADEHYDE

Glutaraldehyde is a 5' carbon linear dialdehyde. It is a pungent, sticky oily liquid soluble in water and alcohol. Applications of glutaraldehyde range from microscopy, leather tanning, sterilization, cytochemistry, pharmaceuticals etc. The most common use of glutaraldehyde is for cross-linking polymers for bio molecule immobilization.

Glutaraldehyde exists in 13 different forms in aqueous solution. All these forms are stable and capable of acting as cross-linking reagent to proteins. Glutaraldehyde has very high reactivity to proteins in the neutral pH range. This can be attributed to the reactive residues in the proteins and the different forms of glutaraldehyde in aqueous solution which give rise to different reaction mechanisms. Glutarladehyde reacts with most functional groups in proteins from amine, thiol, and phenol due to the nucleophilic nature of the most reactive amino acid side-chains. Glutaraldehyde reacts reversibly with amines over a large range of pH except for pH range 7 to 9 where the reversibility is low. Proteins contain lysyl residues containing ε-amino group which are responsible for the cross-linking of proteins with glutaraldehyde. The glutaraldehyde reacts with this ε-amino group of lysyl residue to form cross-links with proteins. The acid dissociation constant of the lysyl groups is greater than 9.5 but the presence of amino groups in unprotonated form at low pH are enough to react with glutaraldehyde to form cross-links [28]. Figure below illustrates the cross-linking mechanism of glutaraldehyde with proteins.

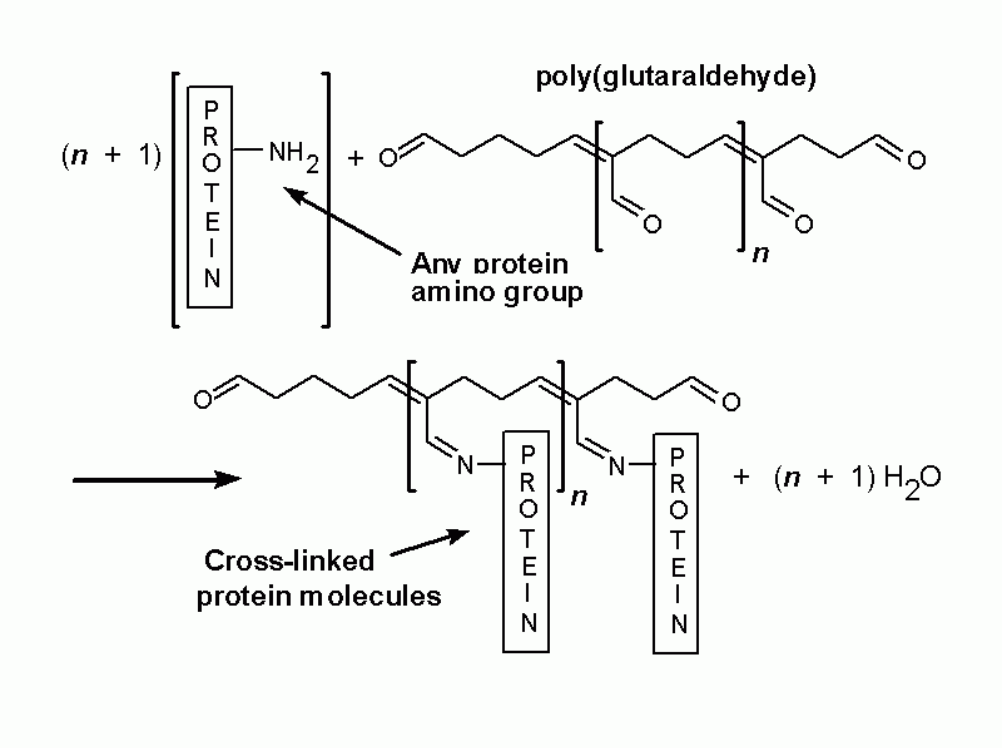


Figure 7: Cross-linking mechanism of Glutaraldehyde with proteins.

Glutaraldehyde is a commonly used cross-linker for immobilizing bio molecules on PANI surface by using the –NH2 group. Researchers have employed this cross-linker for different types of bio sensor development. Singh et al used this cross-linkage to develop cholesterol sensor while Dhand et al immobilized lipase to develop triglyseride sensor. Sai et al developed a protocol using quartz crystal microbalance for the immobilization of human IgG on PANI using glutaraldehyde cross –linkage [25].

In this project, we propose to develop sensing mechanism for thrombin using PANI nano fibers obtained from electrospinning. The immobilization of thrombin specific chromogenic substrate will be achieved by using glutaraldehyde as the cross-linking reagent.

7. MATERIAL SYNTHESIS AND CHARACTERIZATION

7.1 ELECTROSPINNING

Synthesis of polymers to prepare fibers has been used for a long time. These techniques include fiber extrusion and fiber spinning. Some fiber spinning techniques are gel spinning; melt spinning, wet spinning and so on. These techniques are capable of producing fibers of diameter in microns. The one technique that can prepare fibers with nano scale diameter is the process of Electrospinning1.

Electrospinning is the process used for producing polymer fibers with nano scale diameters. It is also referred to as Electrostatic Spinning. The fibers obtained from electrospinning are extremely fine fibers which have large surface area to volume ration along with small diameters and small pore size. Electrospinning finds applications in the medical industry for purposes like drug delivery, automobile industry, tissue engineering, composites, electronics, hygiene wipes, filtration and many more industrial and research fields1.

Electrospinning setup consists of a syringe, a programmable syringe pump, a needle, a high voltage power supply and a grounded collector screen. The syringe carries the polymer solution. The polymer solution can be of pure polymer in solvent or a polymer mixed with a binder or a composite solution consisting of polymer and other material to be encapsulated in the polymer. The programmable syringe pump performs the task of pushing the polymer solution out at a fixed rate depending on the polymer mixture to be electrospun. The needle is specifically made needle available in different gauge sizes. The gauge selection for the needle depends upon the viscosity of the polymer solution.

The high voltage power supply is capable of producing up to 30kV. The collector screen is grounded where the polymer fibers are collected[1].

The set-up for the electrospinning experiment is shown in figure 8. The polymer solution is pumped out at fixed rate by the programmable syringe pump. The rate is such that the solution exits the needle as a drop. This drop has its surface tension which tries to pull it inside due to the solution viscosity. When high voltage is applied, the droplet is

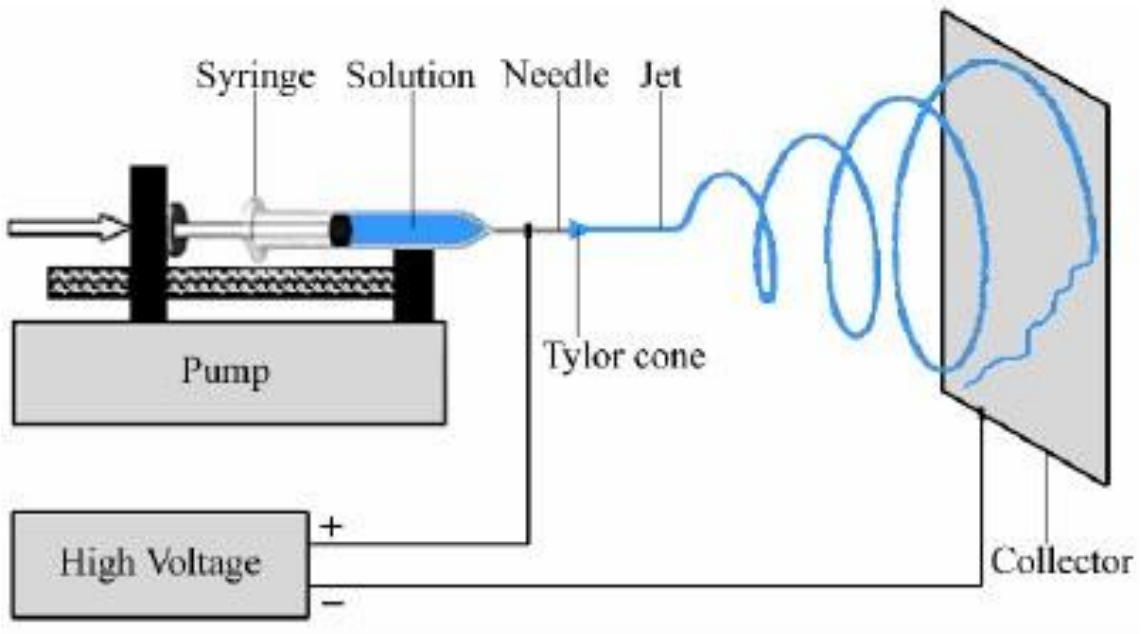


Figure 8: Electrospinning set-up and fiber formation

pulled towards the grounded collector screen and fibers are pulled out of it. The fibers are pulled towards the collector screen. When the fibers are about to reach the screen, the solvent evaporates and a non-woven mat of fibers is collected on the collector screen. The droplet at the point of fiber release from it resembles a cone which is called the Taylor cone. Solution viscosity plays a vital role in the formation of the Taylor cone which in turn is crucial for fiber formation[1].

The electrospinning process can be carried out with a horizontal set-up (Figure 8) or with a vertical set-up. The added advantage of the vertical set-up is that the polymer solution can be drawn towards the screen under the influence of gravity. The tilting of the syringe from the horizontal axis at a suitable angle is also another modification possible for electrospinning fibers1. Manuel J. Díaz-de León had used a tilted syringe/pipette from the horizontal when he prepared PANI nano fibers[27].

The composite nano fibers can be obtained by two techniques. The first technique requires the polymer fiber mats to be immersed into the composite solution to get the desired structure. In this method, the composite particles are adsorbed on the polymer fibers. The second technique mixes the polymer and the composite solutions to get a new solution. This solution is then electrospun to get the desired composite fibers. The drawbacks of the first technique are the time required for soaking which can be even more than 36 hours and the adsorption of the composite which cannot be controlled. The product obtained after soaking requires heat and chemical treatment to get the final result which cannot be controlled to get the desired composite structure of fibers. The second technique manages to eliminate these disadvantages. The mixing of the polymer and composite solution can take place thoroughly and the fibers drawn then do not require any soaking and further treatment.

Electrospinning process is dependent on 3 process parameters viz. needle to collector distance, the solution flow rate and the applied voltage. The more the distance between needle and collector, lesser is the fiber diameter and lesser are the chances to form beads in the structure. The electric field strength is however inversely proportional to this distance. The flow rate has to be less to get fibers of smaller diameter. Surface charge density and volume charge density decrease with increase in flow rate while electric current increases with increase in flow rate. The applied voltage directly affects bead formation and is inversely proportional to the fiber diameter. AC potential improves the fiber uniformity.

The most important element of the electrospinning process is the polymer solution. Hence, the characteristics of the solutions will directly affect the process and the fiber formation. Viscosity, solvent, ionic strength, temperature and concentration of the polymer are the main parameters that need to be looked at during the process. Viscosity has a parabolic relationship with the fiber diameter and spinning ability. Viscosity is important for the formation of Taylor cone for fiber formation. They type of solvent decides the evaporation time and solidification rate of the polymer fibers. Increase in ionic strength increases charge density and reduces the bead density. Higher temperature promotes uniform fiber formation without beads however it reduces the viscosity of the solution. Concentration of the polymer has direct effect on the fiber diameter. The cube of polymer solution is directly proportional to the fiber diameter. Thus, the optimum control of these parameters is helpful in getting fibers with diameter of a few nano meters and also reduces the formation of beads.

We have utilized Electrospinning technique to prepare nano fiber hybrid mats of Polyaniline (PANI) and Cellulose acetate (CA) as the base for the bio-sensor.

7.2 POLYANILINE-CELLULOSE ACETATE (PANI-CA) HYBRID MATS PREPARATION

Leucoemeraldine base Polyaniline was purchased from Sigma-Aldrich. Cellulose Acetate was purchased from Fluka. The mixing of these two chemicals was carried out to obtain the PANI-CA solution for electrospinning. The proportion of mixing was 80 wt% of CA and 20 wt% of PANI. The process of making the solution has been illustrated in figure 9.

PANI and CA were dissolved in separately in two different solvents. 0.30gm Ca was dissolved in 2ml of acetic acid. The mixture of CA and acetic acid was sonicated in the

ultrasonication bath for 3 hours till all the CA had dissolved in the acetic acid to give a viscous and colorless solution. 0.08gm PANI was dissolved in 2ml of acetone. The mixture was sonicated in the ultrasonication bath for 3 hours till almost all the PANI had dissolved in the acetone. The solution was then centrifuged to separate the undissolved PANI from the solution.

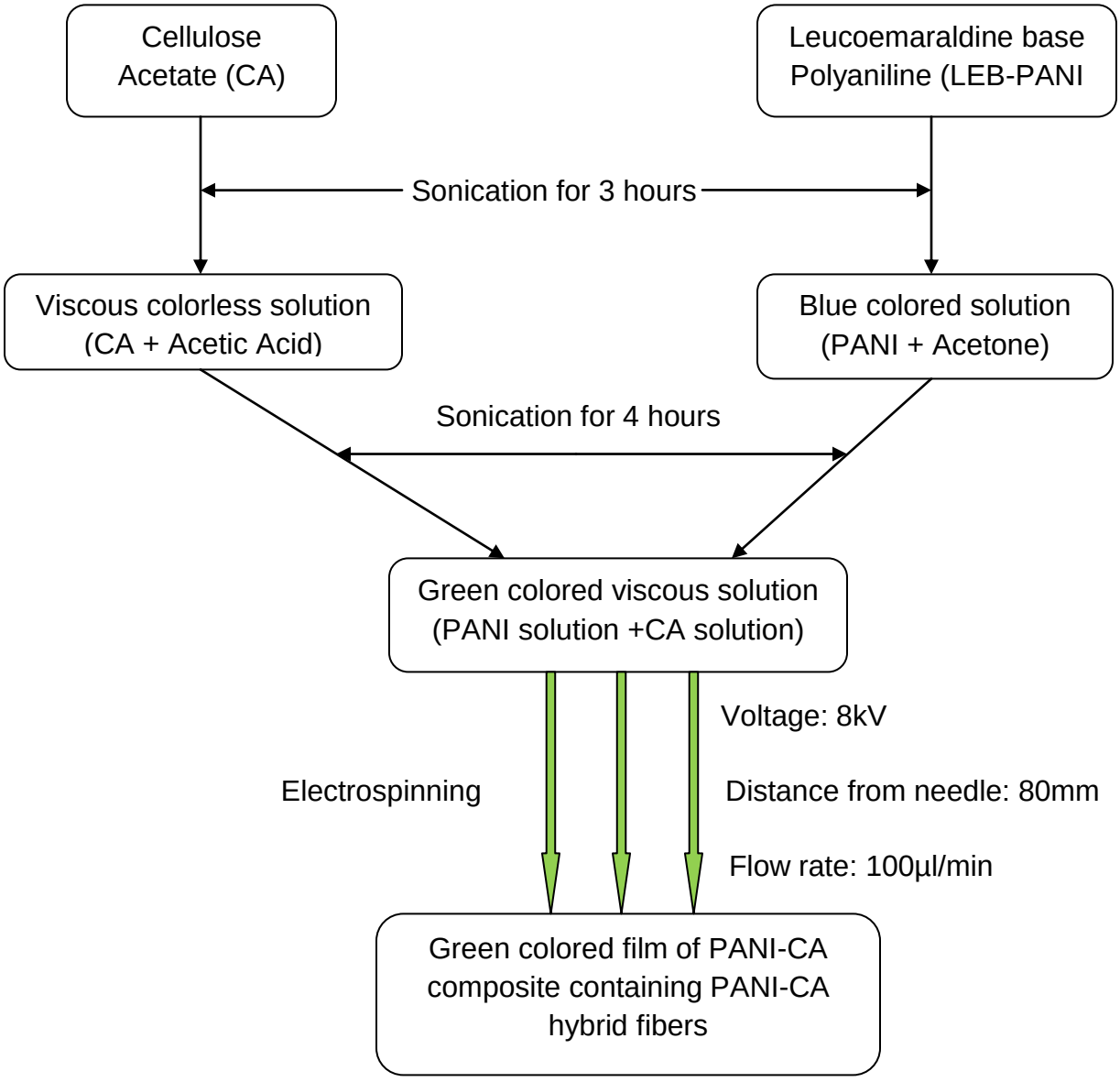


Figure 9: Preparation of PANI-CA hybrid composite films

The PANI solution was less viscous than the CA solution and was blue in color. Hence, 1 ml of the PANI solution in acetone was taken and added to the 2ml solution of CA in acetic acid. This mixture was again sonicated in the ultrasonication bath for more than 4 hours to completely mix both the solutions and get a green colored viscous solution of PANI-CA composite. This solution was then deposited as fiber mats with the help of electrospinning. The applied voltage was 8kV and the distance between the needle and collector was 80mm. The needle used for the deposition was a 22 gauge needle. The flow rate used for the process was 100µl/min. The film deposited on aluminum foil was also green color and peeled off from the aluminum foil after deposition during the first experiment of electrospinning. The second time onwards, the film had the same appearance but did not peel off the aluminum foil.

The resistance of the PANI-CA was measured and SEM analysis was carried out to observe the structure of the films and the formation of fibers.

7.3 RESISTANCE MEASUREMENTS

The PANI films had to be coated with silver paste for the purpose of resistance measurement. The silver paste was applied as to lines on the films to which 2 gold electrodes were attached. The silver paste was applied by using a hand-made mask which was used to draw the shapes required for the measurements. After the application of silver paste and the gold electrodes, the sample was kept in the oven at 1000C for 5 hours for the silver paste to dry out. The resistance measurements were taken using the Keithley 6517 A electrometer/high resistance meter. The measured resistance of the PANI-CA films was observed to be between 34-36MΩ.

7.4 SCANNING ELECTRON MICROSCOPY (SEM) ANALYSIS

The SEM analysis for the PANI-CA films was carried out to observe the structure of the films. The SEM used was a Hitachi Field Emission Scanning Electron Microscope (FESEM). The purpose of the analysis was to know the size and distribution of fibers in the structure.

SEM analysis was carried for 2 samples viz. the first peeled of film and the unpeeled off film. The samples were coated with gold before they were inserted into the SEM. The coating of the samples was done in Argon plasma. The samples were placed at an angle in a small vacuum chamber. Argon gas was then let in such that there was 99% vacuum and 1% Argon in the chamber. A high voltage was then applied to the chamber to activate the Argon plasma. There is a golf foil present at the top of the chamber near which the blue color Argon plasma can be clearly observed. At a high voltage, the atoms of argon in the plasma are vibrating at high velocity and as they are closer to the gold foil, they start knocking out the gold atoms from the foil. The result of this activity, gold is sprinkled down on everything in the chamber and covers the samples kept inside forming the required conductive coating. Figure 10 shows the gold coated samples stuck on a slide using conductive carbon tapes.

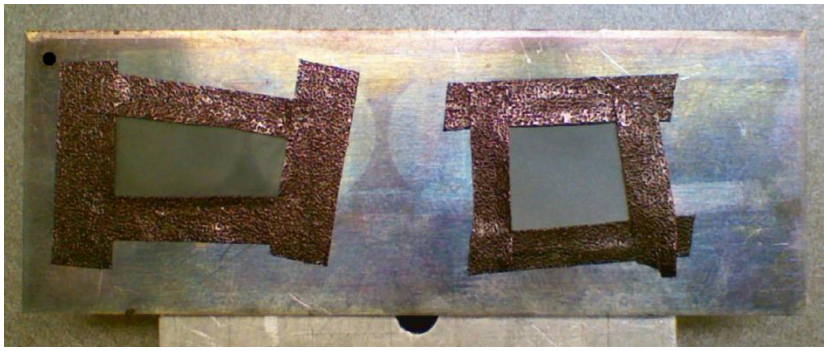


Figure 10: Gold coated PANI-CA electrospun films

7.5 CROSS-LINKING OF POLYANILINE WITH THROMBIN SPECIFIC SUBSTRATE

Thrombin from bovine was purchased from Sigma-Aldrich. The Thrombin specific chromogenic substrate, Biophen CS-01(81) Chromozyme Thrombin, was purchased from Aniara Diagnostica.

The cross-linking reagent used for PANI was Glutaraldehyde. The protocol to crosslink glutaraldehyde with PANI was followed in accordance with Sai et al[31]. Sai et al used a simple protocol to utilize glutaraldehyde as cross-linking reagent for PANI for immobilizing bio molecules on PANI for a piezoelectric biosensor. We modified the actual protocol used by Sai et al according to the need of our experiment keeping the basic principle same.

The PANI-CA film is cut in five small parts and put into small wells for reacting with glutaraldehyde and the thrombin specific substrate. In the first step, PANI-CA films were treated with 1% glutaraldehyde in Phosphate buffer solution (PBS) for 1 hour. The films were then washed by PBS twice to ensure the removal of excess glutaraldehyde. In the second step, four of the PANI-CA films were incubated with 0.5 mg/ml thrombin substrate in PBS for another hour, followed finally by PBS wash couple of times. One film was not treated with the substrate but only treated with glutaraldehyde. The films were then completely ready for testing the cleavage of thrombin to the covalently bonded thrombin substrate[31].

10mg thrombin was dissolved in 5.5ml PBS. Three of the PANI-CA films previously treated with glutaraldehyde and the thrombin substrate were then treated with this thrombin solution[31]. One of the samples treated with substrate was taken for resistance measurement to get a reference value of resistance. This value will then be compared

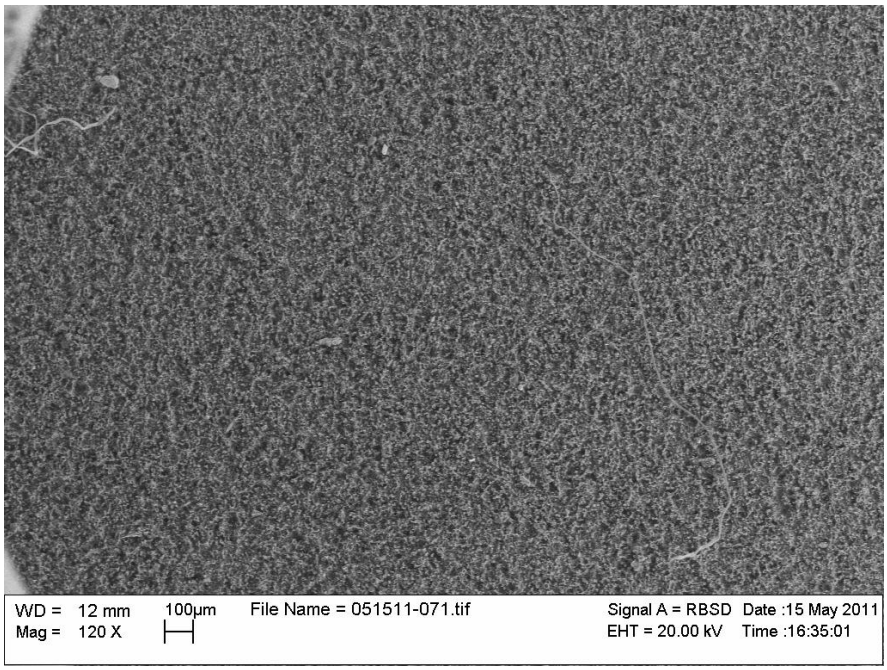
with the value of resistance obtained from the any of the other films after treatment with thrombin to get an idea of the change in resistance caused by the thrombin cleavage. 1ml thrombin solution was added to each of the three wells containing the films. The solution was kept in there for 2 hours. Allocates of 100µL were taken from each sample well at 15 minute intervals. These allocated can be useful for analysis with the spectrophotometer.

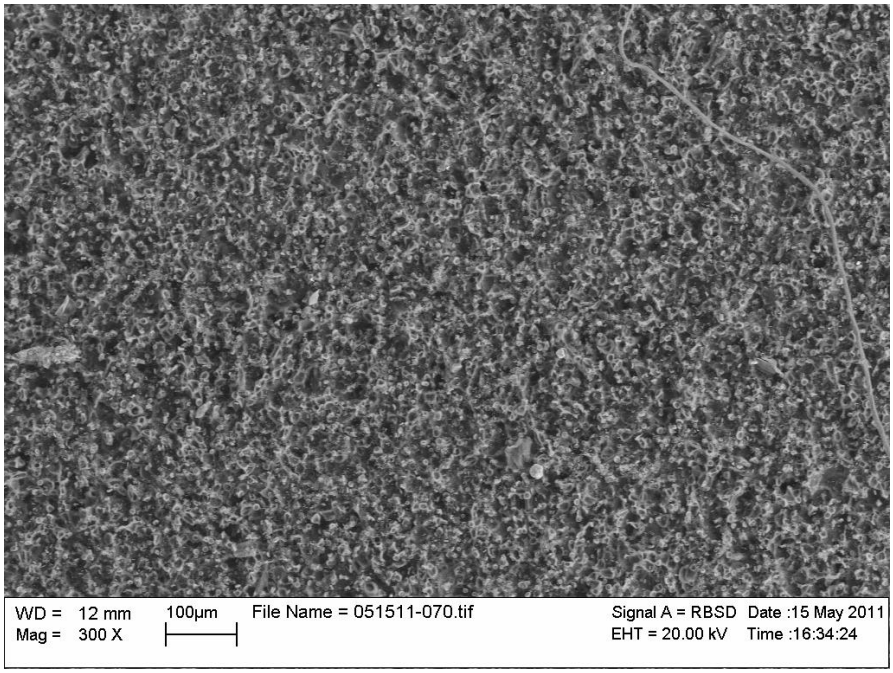
8. RESULTS AND DISCUSSION

- PANI-CA hybrid films were successfully prepared using the electrospinning technique. These films were the base block for bio sensor development. The first PANI-CA film prepared was green in color and it peeled off the aluminum foil immediately after deposition. The second film however did not peel off the foil. The non peeling of the latter films could be deciphered as the lack of solvent in the polymer solution than the previous case. Aisha Bishop and Dr. Perena Gouma have previously worked on the synthesis of PANI-CA hybrid films. In their research, CA and PANI were mixed together in acetone and then electrospun to get fiber mats of PANI-CA[32, 33, 34]. The color of the mats was white and the fibers were well defined with no beads. The SEM images of our sample and their sample are clear evidence of the difference in structure with respect to bead formation. The parameters used for electrospinning were the same in both the experiments except for the needle which was 22 gauge in our experiment and 20 gauge in their experiment[32, 33, 34]. Manuel J. Díaz-de León had used concentrated sulphuric acid to dissolve PANI and then utilized polystyrene (PS) and polyester oxide (PEO) as binders. He also had got a green colored solution which he electrospun to get a green colored film of fibers. The fibers were well defined when observed under the AFM[27]. Our results differ with Manuel J. Díaz-de León on the structure of the films with the beads in our structure being the notable difference. The parameters that were different in our synthesis were the applied voltage for electrospinning, the inclination of the syringe with the horizontal and the flow rate which affected the final results. Saima Khan from University of Ohio used camphorsulfonic acid to dope PANI and chloroform to dissolve PANI and then electrospun the solution to get green colored fiber webs. The fibers were extremely well defined and the absence of beads was once again highlighted to be the main difference in structure[37].

- Resistance measurements were carried out on the PANI-CA films prepared by electrospinning. The resistance of the film was found to be in between 34-36 MΩ. The reason for this high resistance can be attributed to the presence of beads in the electrospun film structure. The resistance value however was not used for finding the resistivity and the conductivity of the fibers. This value of resistance is for the entire film and electrical conductivity has to be with respect to the fibers. The fiber and length and cross-sectional area are of immense importance. The presence of beads affects the continuity of the fibers. The fiber length cannot be calculated directly. The average diameter can be taken from the SEM images but the length of the fiber is not so easy to calculate. Saima Khan used the four probe method for conductivity measurements. She deposited a single fiber on the four probe system and observed that fiber under microscope. The highest conductivity measured by her was 3.2 S/cm for a single fiber[37]. Manuel J. Díaz-de León calculated the conductivity to be 0.76 S/cm which is close the value in bulk[27]. The presence of beads scatters the conductivity making it a non-uniform distribution. The formation of beads can be dependent on solution viscosity, flow rate, applied voltage and the distance between the needle and the collector. The viscosity of a solution if increased to an optimum level, the formation of beads can be eliminated[35]. The solution viscosity is crucial for Taylor cone formation. A better Taylor cone is obtained with a highly viscous solution. The lower viscosity solution will tend to have more beads due to the absence of Taylor cone as the fibers cannot be pulled out of a droplet. The droplet stays at the tip of the needle owing to its surface tension which is due to the viscosity of the polymer solution. The application of higher voltage and higher distance between collector screen and needle can be used to get longer fibers and could help in avoiding bead formation[35].

- The formation of beads was thought of as the main reason behind the high resistance of the PANI-CA film. This formation of beads was confirmed in the SEM analysis of the PANI-CA films. The figures 11 to show the structure of

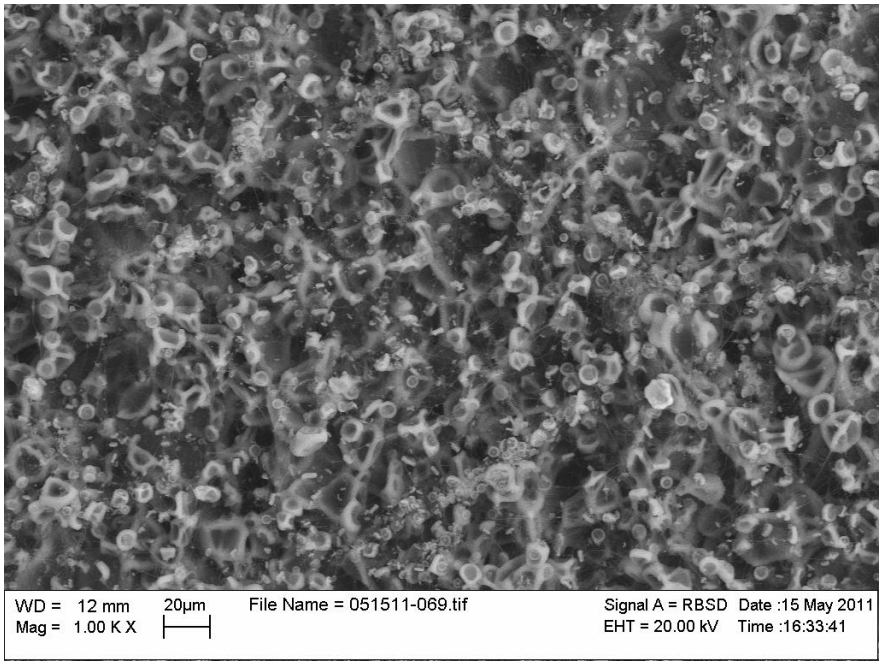




Figures 11 and 12: Lower magnification images of PANI-CA

- PANI-CA films prepared by electrospinning. SEM analysis was carried out for 2 films. The first sample was the film that had peeled off the aluminum foil and second was the film that did not peel off the aluminum foil.

  The lower magnification images (120X and 300X) are inconclusive about the presence of beads in the structure. These images show uniform distribution of polymer over the entire film without agglomeration in one part of the film.



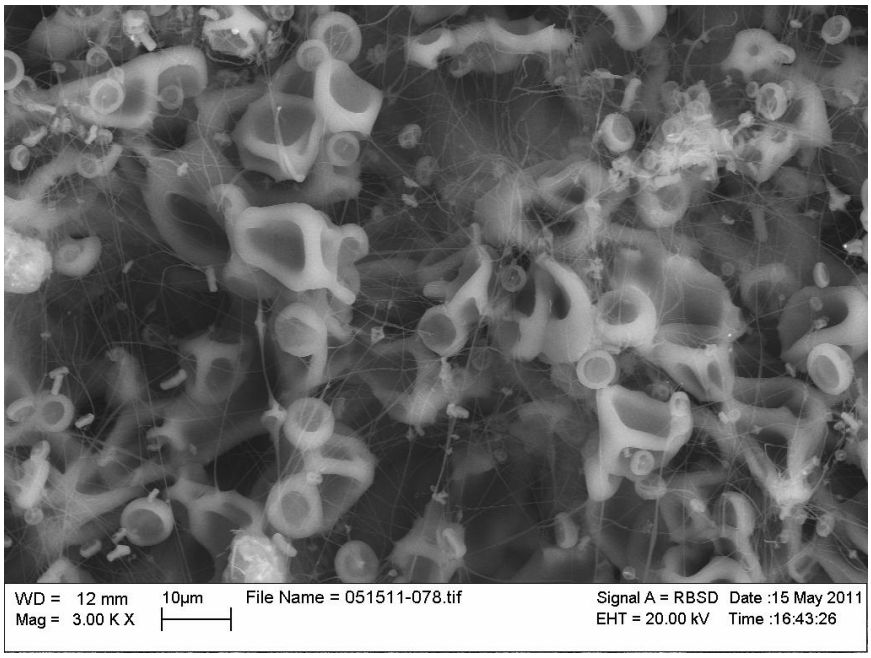


Figure 13 and 14: PANI-CA films at 1KX and 3KX

Figures 13 and 14 are the images of PANI-CA films at slightly higher magnification. These images shot the formation of beads in the structure.

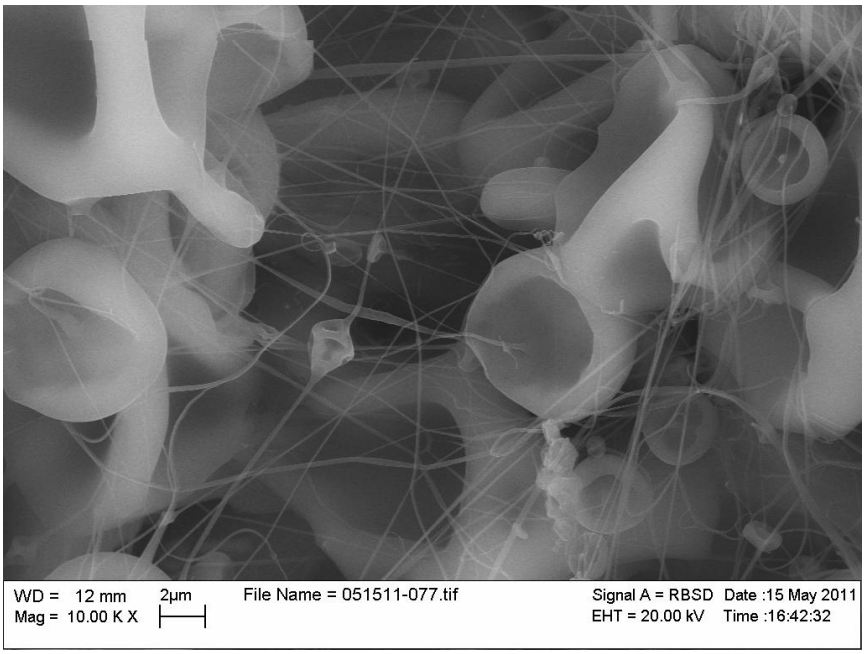


Figure 15: PANI-CA films at 10KX

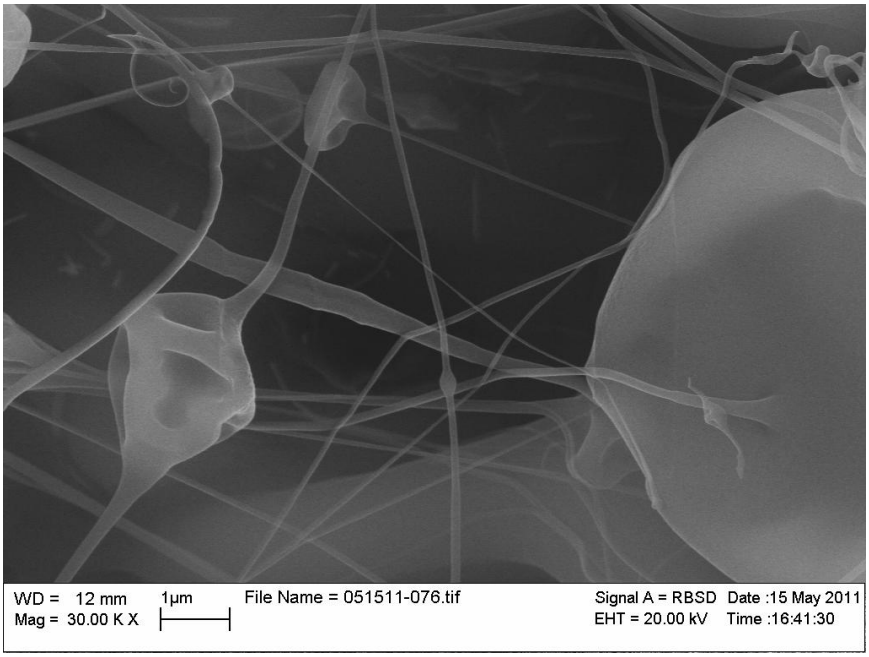


Figure 16: PANI-CA image and 30KX

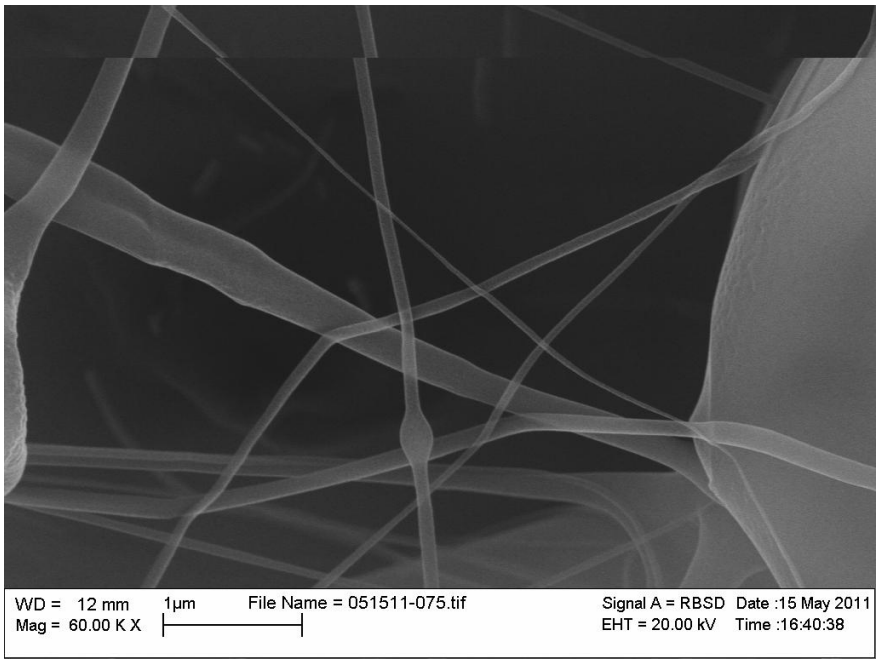


Figure 17: PANI-CA films at 60KX

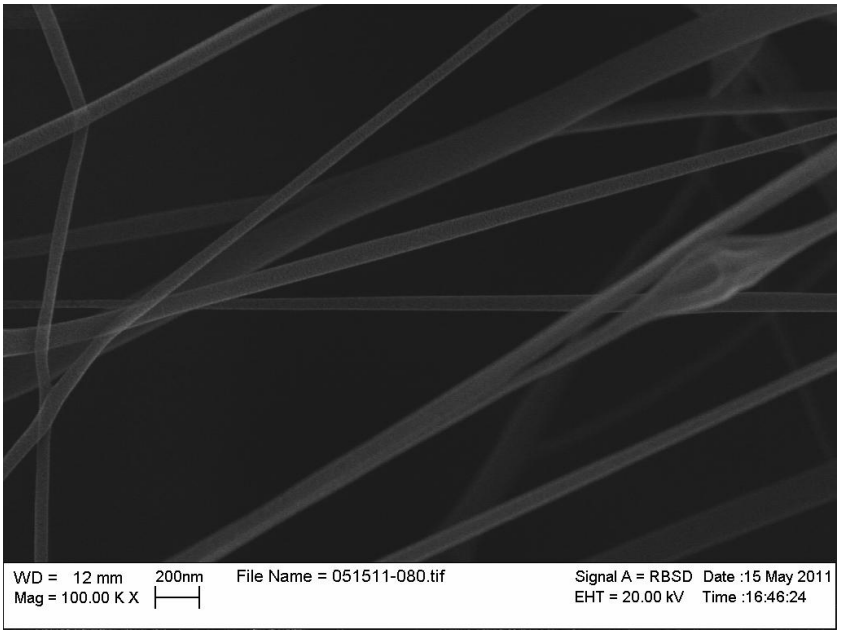


Figure 18: PANI-CA images at 100KX

The image at 3KX clearly shows the formation of beads along with fibers of nano diameter. SEM images of Aisha Bishop's sample clearly show the difference between the two films[32, 34]. Our sample has structure with beads connected by the means of fibers whereas Aisha's sample shows distinct network of fibers which are very well defined. Higher magnification images can help get a better view of the structure with closer look at the fibers and the beads.

Figure 18 shows the fibers with diameters ranging from 50nm to 150nm. The higher magnification images of the samples show the formation of beads in the structure. The continuity of the films is affected by the presence of beads.

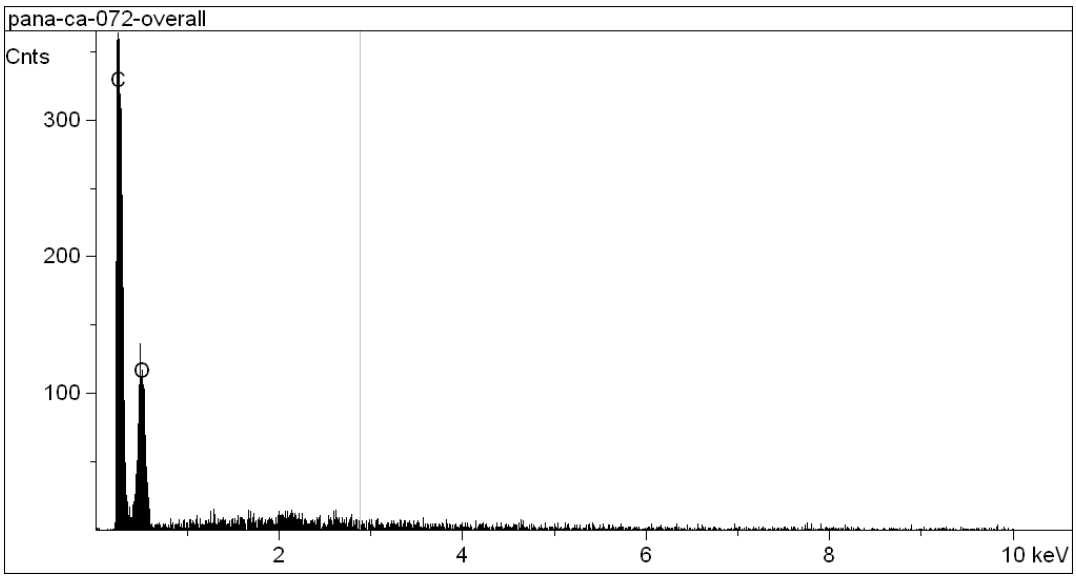


Figure 19: The EDAX analysis of PANI-CA film

The EDAX analysis was conducted on the PANI-CA films to check the presence of any unwanted element in the sample. The results show no other unwanted element in the analysis except for two peaks indication carbon and oxygen. An advanced analysis like FTIR would serve better indentifying the unwanted elements if any in the sample.

- The PANI-CA films were treated first with glutaraldehyde to achieve cross-linking. The process was followed with reference to Sai et al[31]. The glutaraldehyde now acts as a double connector as it now connects the substrate to the PANI-CA films. The PBS is used as the solvent for glutaraldehyde and for the thrombin specific substrate. The films that were treated with substrate were then treated with thrombin solution in PBS for 2 hours. Allocates were taken every 15 minutes as samples for the spectrophotometer. Conductivity of the PANI-CA films before the addition of thrombin was measured similar to the forst measurement of PANI-CA films. The resistance of the film with the peptide substrate came out to be in between 26-27 MΩ i.e. showed a decrease in its original value from 34-36 MΩ.

This value of resistance with the peptide film on will now act as reference for the further readings. Addition of thrombin was then carried out and again resistance measurements were taken. The resistance decreased yet again from 26-27 MΩ to 23-24 MΩ. This decrease in resistance could mean two things; the first would be that the structure has been altered with the cleavage of thrombin to the sensor or the thrombin has bound with the PANI-CA film. This change was observed in the form of drop in resistance of PANI-CA films. Three readings were taken for each type of film to confirm the range of values obtained. The PANI films were green when they were first made and then turned dark blue on further treatments with Glutaraldehyde and thrombin substrate. After the films were treated with thrombin and kept overnight, they turned green again.

9. FUTURE SCOPE OF WORK

- The fibers formed in the electrospinning process consist of beads along with fibers. These beads affect the continuity and hence the electrical conductivity of the fibers. One of the main objectives of future research would be to try and change the solution viscosity and electrospinning parameters to obtain fibers without bead formation. Greater solution viscosity should ideally eliminate most of the fibers that could then make it easy to get exact and absolute values of conductivity which would help in further designing of the sensor.

- The resistance of the PANI-CA films was found to be in 34-36 MΩ. With the reduction in beads and some doping with some acid, this resistivity should be brought even further down thereby increasing the conductivity.

- The films can be tested with FTIR for the presence of any unwanted material that was not found from the EDAX tests conducted on the samples. The beads are a complex structure. We cannot make out from SEM images or from the EDAX spectra about the exact composition or anything else about them. This is where FTIR could prove to be a useful characterization technique to get more precision and assurance about the results.

- The change in resistance of the PANI-CA film i.e. the sensor after thrombin cleavage or after the binding of thrombin is evidence that the sensor can give change in electrical signal for reaction with thrombin. an optical density measurements of the allocates taken in the experimental work could give us a clear indication on whether the thrombin has cleaved or has it bound with the surface. A better structure of the sensor with continuous and uniform fibers could allow exact conductivity measurements. The nano fibers will help in reducing the sample required for testing which is this case is blood which makes it a very promising sensor. The use of fine nano fibers will ensure quick response time and excellent sensitivity as well.

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