AMPEROMETRIC MICROBIAL AND ENZYMATIC BIOSENSORS BASED ON CONDUCTING POLYMERS

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ABSTRACT

AMPEROMETRIC MICROBIAL AND ENZYMATIC BIOSENSORS BASED ON CONDUCTING POLYMERS

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In this thesis, six different biosensors based on conducting polymers of poly 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine [*poly(SNSNH$_2$)*] and poly(1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H*-pyrrole [*poly(SNSNO$_2$)*] were prepared. Electrochemical technique was used for polymerization of conducting polymers and two different immobilization techniques; crosslinking and adsorption were used for immobilizing enzyme or microbial in the conducting polymer matrices. The proposed biosensors were characterized and optimized. Optimum pH, thickness of conducting polymer and biological material amount were determined. Linearity, repeatability and operational stability experiments were performed. Carbon nanotubes and gold nanoparticles were also added to the biosensing system to see the effects of nanoparticles. The biosensors also used for ethanol and/or glucose biosensing in commercial samples. In the first part of thesis, a biosensor was designed by immobilizing *Gluconobacter oxydans* in *poly(SNSNH$_2$)* matrix on graphite electrode. The biosensor preparation method was a two-step procedure where the cells were immobilized by adsorption on the surface after the electropolymerization step.

Use of dialysis membrane to cover the surface after immobilization conserves the bioactive surface during the operation. The preparation is simple and not time consuming. Systems proposed showed good linearity and repeatability as well as high operational stability. Glucose amount in fruit juice, ethanol amount in vodka and whisky were determined. In the second part of thesis, a second biosensor was designed with electrochemical polymerization of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H*-pyrrole via cyclic voltammetry on graphite electrode. Afterwards, *Pseudomonas fluorescens* and *Gluconobacter oxydans* were immobilized successfully on the conducting polymer matrix separately. The proposed biosensors showed good linear range, and repeatability as well as high operational stability. In the third and fourth parts, gold nanoparticle and carbon nanotube effects were studied on *poly($SNSNH_2$)/glucose oxidase* biosensor, respectively. Covalent binding of glucose oxidase was achieved to *poly($SNSNH_2$)* by the help of glutaraldehyde on the top of graphite and carbon paste electrodes. Nanoparticle amount and optimum pH were determined for both biosensors. After analytical characterization, glucose amount in two fruit juices were determined with *poly($SNSNH_2$)/GOx/AuNP* and *poly($SNSNH_2$)/ GOx/CNT* biosensors. In the last part, biosensor was designed with immobilizing alcohol oxidase in *poly($SNSNH_2$)* matrix via crosslinking with glutaraldehyde on platinum electrode. The proposed biosensor was characterized and optimized in terms of thickness, enzyme loading, pH, AuNPs, CNTs, linear range, repeatability and operational stability.

Keywords: Amperometric Biosensors, Conducting Polymers, Enzymatic Biosensors, Microbial Biosensors

ÖZ

İLETKEN POLİMERLER BAZLI
AMPEROMETRİK MİKROBİYAL VE ENZİMATİK BİYOSENSÖRLER

TUNÇAĞIL, Sevinç
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Nisan 2010, 100 sayfa

Bu tezde, poli (4-(2,5-di(tiyofenil-2-yl)-1H-pirol-1-l)) benzenamin [*poli(SNSNH$_2$)*] ve poli(1-(4-nitrofenil)-2,5-di(2-tiyenil)-1*H*-pirol) [*poli(SNSNO$_2$)*] iletken polimerler bazlı altı farklı biyosensör hazırlanmıştır. İletken polimerlerin sentezi için elektrokimyasal polimerleştirme tekniği, biyolojik materyelleri tutuklamak için adsorbsiyon ve çarpraz bağlama olmak üzere iki farklı tutuklama tekniği kullanılmıştır. Tasarlanan biyosensörler optimize ve karakterize edilmiştir. Uygun çalışma pH'si, iletken polimer kalınlığı, biyolojik madde miktarı gibi parametreler optimize edilmiş, lineer çalışma aralığı, tekrarlanabilirlik ve operasyonel kararlılıkları belirlenmiştir. Altın nanopartikül ve karbon nanotüpler ile biyosensörler modifiye edilerek, nanopartiküllerin biyosensör cevabına etkisi incelenmiştir. Tasarlanan biyosensörler, doğal örneklerde glukoz ve/veya etanol tayininde kullanılmış, referans yöntem ile ya da etiket değerleri ile biyosensörden elde edilen veriler karşılaştırılmıştır. Tezin ilk kısmında, *Gluconobacter oxydans* mikropları, grafit elektrot yüzeyinde iletken polimer matrisinde adsorpsiyon yöntemi

ile tutuklanmıştır. Mikrobiyal biyosensörün hazırlanışı, $SNSNH_2$ monomerinin grafit elektrot yüzeyinde elektrokimyasal olarak polimerleşmesi ve mikropların yüzeyde adsorpsiyon yöntemi ile tutuklanması olmak üzere iki adımda gerçekleşmiştir. Diyaliz membranı elektrot yüzeyine geçirilerek biyosensörün kullanımı süresince mikropların korunması sağlanmıştır. Hazırlanışı kolay ve hızlı olan mikrobiyal biyosensör, iyi bir lineer çalışma aralığı, tekrarlanabilirlik, ve operasyonel kararlılık göstermiştir. Meyve suyunda glikoz tayini, vodka ve viskide etanol tayini, tasarlanan biyosensör ile yapılmıştır. Tezin ikinci kısmında, elektrokimyasal polimerleşme ile grafit yüzeyinde biriktirilen 1-(4-nitrofenil)-2,5-di(2-tienil)-1*H*-pirol iletken polimeri üzerine *Pseudomonas fluorescens* ve *Gluconobacter oxydans* mikropları adsorpsiyon yöntemi ile tutuklanarak iki ayrı biyosensör hazırlanmıştır. İki biyosensörün uygun çalışma pH'si, lineer çalışma aralığı, tekrarlanabilirliği, operasyonel kararlılığı belirlenmiştir. Tezin üçüncü ve dördüncü kısımlarında, *poli($SNSNH_2$)/glikoz oksidaz* biyosensörüne sırası ile altın nanopartikül ve karbon nanotüp etkisi incelenmiştir. Çapraz bağlama tutuklama yönetimi kullanılarak, glikoz oksidaz enzimi glutaraldehit yardımı ile iletken polimere çapraz bağlanmıştır. Altın nanopartikül etkisi grafit çalışma elektrodu ile, karbon nanotüp etkisi karbon pasta çalışma elektrodu ile incelenmiştir. Nanopartikül miktarı, pH, enzim miktarı gibi parametreler optimize edilip, biyosensör analitik olarak karakterize edilmiştir. *poli($SNSNH_2$)/GOx/AuNP* ve *poli($SNSNH_2$)/GOx/CNT* biosensörleri iki meyve suyunda glikoz miktarı tayininde kullanılmıştır. Tezin son kısmında, alkol oksidaz enzimi gluteraldehit yardımı ile çarpraz bağlanarak diğer elektrotlardan daha küçük elektrot yüzeyine sahip olan platin elektrot yüzeyinde, *poli($SNSNH_2$)* matrisinde immobilize edilmiştir. Tasarlanan biyosensör, iletken polimer kalınlığı, enzim miktarı, pH gibi parametreler ile optimize ve karakterize edilmiştir. Biyosensör cevabına, altın nanopartikül ve karbon nanotüp etkisi ayrı ayrı incelenmiş, nanopartikül miktarı optimize edilmiştir. Son olarak biyosensör ile vodka ve viskideki etanol tayini yapılmıştır.

Anahtar Kelimeler: Amperometrik Biyosensörler, İletken polimerler, Enzimatik Biyosensörler, Mikrobiyal Biyosensörler

Dedicated to my parents, my grandfather and my brother.

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ABBREVIATIONS

CP	Conducting polymer
$SNSNH_2$	4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine
$SNSNO_2$	1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H*-pyrrole
poly($SNSNH_2$)	polymer of 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine
poly($SNSNO_2$)	polymer of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H*-pyrrole
P. fluorescens	*Pseudomonas fluorescens*
G. oxydans	*Gluconobacter oxydans*
GOx	Glucose Oxidase
AOx	Alcohol Oxidase
AuNP	Gold Nanoparticles
CNT	Carbon Nanotubes
EtOH	Ethanol
Glc	Glucose
Xyl	Xylose
Gal	Galactose
SEM	Scanning electron microscopy
AFM	Atomic Force microscopy
CVs	Cyclic Voltammograms
CV	Coefficient of variation
SD	Standard deviation

CHAPTER 1

INTRODUCTION

1. Biosensors

Analytical devices for analyzing, detection, quantification or monitoring of specific chemical species have gained a great deal of importance as a very hot topic "biosensors". A biosensor is a device that transforms chemical information, related with the concentration of a specific "analyte", into an analytically meaningful signal. Biosensors are used for direct measurement of the analyte in the sample matrix. Biosensors have two main components: a biological sensing element or biological recognition system called biocomponent/bioreceptor and the detection method called transducer.

The working principle of this device depends on producing an electronic signal, which has a direct relation with the concentration of the chemical which is under concern. The main aim of a biosensor is to transform a biological event into an electrical signal. "They are special chemical sensors in which the recognition system uses a biochemical mechanism. Generally a biosensor is considered as an integrated receptor-transducer device, which provides selective, sensitive, stable, repetitive, analytical information about the analyte using suitable biorecognition element" [1,2,3,4].

The first part of the biosensor is the "biorecognition part". This part mainly functions as a biochemical transducer. "Biorecognition system translates information from the biochemical reaction to a significant output with a defined sensitivity. The main function of the biorecognition part is to provide the sensor with selectivity and biocompatibility" [3].

In this point the biological material that used in biorecognition part is important. Enzymes, tissues, bacteria, yeast, antibodies, antigens, liposomes, and organelles are generated in biorecognition part of the biosensors [1,5,6].

The second part of the biosensor is the "transducer". Transducer is in direct contact with the biorecognition part. A transducer serves as a converter by converting the biochemical signal which occurs in biorecognition part to a meaningful, understandable signal as shown in Figure 1. Transducer is responsible for amplification storage and display of the signal [7]. There exist transducers related with the biological event occurred in biocomponent. Electrochemical, spectroscopic, thermal, piezoelectric are the most common transducers [8].

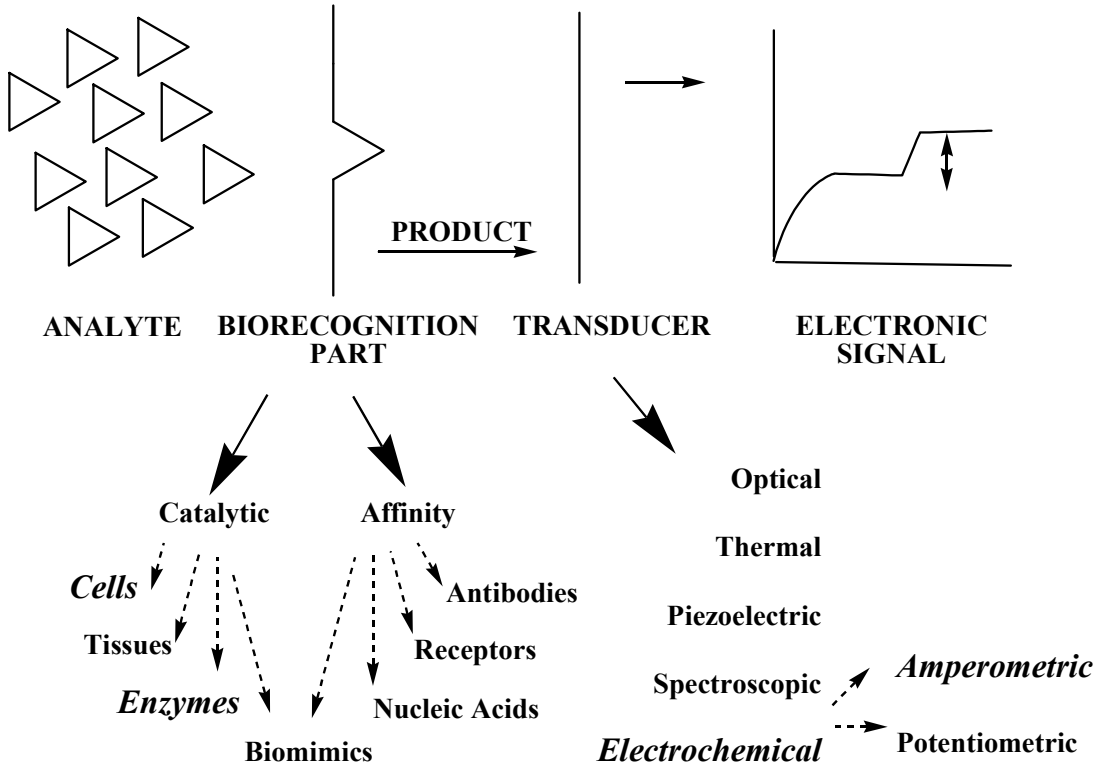


Figure 1. Simple representation of a biosensor

“Most of the biological molecules such as enzymes, cells etc. have very short life times in solution phase, therefore, they have to be fixed in a suitable matrix” [1]. Immobilization is a very important feature in designing the biorecognition part of the biosensors in constructing sensitive and stable biosensors. The immobilized biomaterial layer is chosen to catalyze a reaction, which generates or consumes detectable species [9]. “The success of the biosensor, the activity of immobilized molecules depends on surface area, porosity, hydrophilic character of immobilizing matrix, reaction conditions and especially on the immobilization method” [1]. The objective is to provide an intimate contact between the biological material and the sensing surface while maintaining and even improving its stability [10,11].

Immobilization means the localization of biological molecules during a continuous catalytic process. Immobilization is frequently used in industry. The cost of the biological material, the nature of the conversion process and the relative operational stabilities of the two forms determine the choice between the use of the free and immobilized ones [12]. Immobilized biological materials can be used multiple times. Their properties such as specificity, pH and temperature stability can be experimentally determined.

Biological materials should be well immobilized on the electrode surface to create biosensor [13,14,15]. Several physical and chemical ways (Table 1) can be used to do immobilization onto the electrode. Commonly immobilization can be achieved by several methods [1] like;

- ✓ Adsorption or entrapment behind a membrane, a solution of enzyme, cells or tissue, restrained with an analyte permeable membrane, covers the electrochemical detector.

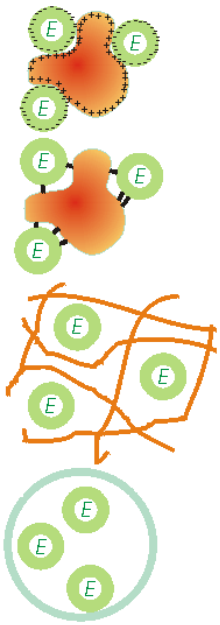
- ✓ Entrapment of biological material in a polymeric matrix, while electropolymerization takes place.

✓ Covalent bonding or cross-linking of surfaces activated functional groups like NH_2, COOH or spacers, such as glutaraldehyde.

✓ Encapsulation of biological material, like droplets are surrounded by a coating of capsule, a small sphere with a uniform membrane around it.

Table 1. Immobilization Methods

Method	Advantages	Disadvantages
Adsorption	"No modification of biocatalyst. Matrix can be regenerated. Low cost"	"Binding forces are susceptible to change in pH, temperature and ionic strength"
Entrapment	"Only physical immobilization of biocatalyst near transducer. Low cost"	"High diffusion barrier, substrate accessibility to the enzyme is low"
Crosslinking	"Loss of biocatalyst is minimum. Low diffusional resistance. Stable under adverse conditions. Moderate cost"	"Produces very little of immobilized enzyme that has high intrinsic activity"
Encapsulation	"Provides a larger biocatalyst loading, protected against contamination and biodegradation"	"Only small substrate molecules are utilized with the intact membrane"



Chemically covalent linking biomolecules on transducer is an efficient method of immobilization using linkage reagent such as glutaraldehyde. By the help of covalent linking of biological receptor to electrode, sensor shows good stability under adverse conditions. The terminal aldehyde group in glutaraldehyde binds to the amine group of the biological material during the immobilization process [16,17,18,19,20].

1.1 Electrochemical Biosensors

When the transducer of a biosensor is electrochemical, the biosensor called as "electrochemical biosensor". The transducer is generally considered as "chemically modified electrode, since conducting, semiconducting, ionic conducting material is coated with a biological film" [3]. Electrochemical sensors correspond to a main subclass of chemical sensors where an electrode is used as the transduction element. The analytical power of electrochemical techniques and the specificity, selectivity of biological recognition part come together in electrochemical biosensors. Therefore, they provide fast, simple, and low-cost detection for biological events [21,22].

Depending on the type of transducer, electrochemical sensors can be classified as amperometric, potentiometric and impedimetric. Amperometric electrochemical biosensor is the most commonly studied and successfully commercialized class of electrochemical biosensors. Amperometric detection has proven to be very useful for quantification due to their good selectivity, sensitivity, rapid response, miniature size, and reproducible results [23].

Amperometry is based on the measurement of the current resulting from the electrochemical oxidation or reduction of electroactive species. The first amperometric biosensor was demonstrated by Clark who studied with an oxygen electrode [24]. Generally, the amperometric biosensing depends on measuring either the absolute value or the density of current in an electrochemical cell at a constant applied potential value on a Pt, Au or C based working electrode with respect to a reference electrode. The current difference before and after electrochemical reaction is directly related with the bulk concentration of the electroactive substrate or the product where oxidation or reduction takes place on the surface of a working electrode [3].

Amperometric biosensors are specific to their analyte, rapid in giving signal, simple to work, easy to fabricate. “They can be divided into three generations. In the first generation, the biocatalyst is bound or entrapped in a membrane; as a result biocatalyst is fixed on the surface of the transducer. Electroactivity of substrates and products is very important for the first generation of amperometric biosensors. First generation biosensors have the problem of interferences due to high applied potential which may oxidize or reduce electroactive nonspecific particles. In the second generation biosensors, biocatalyst is adsorbed on the transducer surface. Second generation biosensor generally uses mediators as electron carriers. Via mediators applied potential can be decreased. In the third generation biosensors, biocatalyst is directly bounded to the transducer. Direct electron transfer between the electrode and the biological materials is used. Therefore, generally interference problem does not occur” [3].

There exist two general categories of electrochemical biosensors depending on the biological recognition part: biocatalytic recognition element and bioaffinity (antibody-antigen interaction) recognition element. The biocatalytic-based biosensors are the most well known and researched biosensors. Three types of biocatalyst are commonly used;

- Enzymes, (the most common and developed),
- Whole cells (microorganisms, such as bacteria) or cell organelles (such as mitochondria)
- Tissues

1.1.1 Enzyme-based electrochemical biosensors

Enzymes are molecules of biological origin. They are homogeneous biological catalysts that increase the rate of specific catalytic reactions. Although their conformational structures may change during the catalysis, they are chemically unchanged and can be recovered from the solution at the end of the reaction. The catalyzed reaction takes place on a small part of the enzyme called the active site.

Hence, enzymes combine the recognition and amplification steps, as needed, for any sensing applications [25,26].

Enzyme electrodes are generally a layer of an enzyme (bioreceptor) which measures the concentration of a substrate with a suitable electrode (transducer). The enzymatic reaction transforms the substrate into a reaction product detectable by a transducer. The specificity of the enzyme for its substrate (enzyme substrate relation) and the analytical power of electrochemical devices come together on the surface of electrodes. Enzyme electrodes have been shown to be extremely useful for monitoring a wide variety of substrates of analytical importance in clinical, environmental and food samples [27].

The sensitive surface of electrode is in contact with an enzymatic layer; and it is assumed that there is no mass transfer across this interface. The surface of the enzymatic layer is in a solution containing the substrate under study. The substrate migrates to the layer of enzyme and through enzymatic reaction it is converted into reaction products [28].

Glucose oxidase

Diabetes has become the third most leading cause of death and disability in the world, affecting more than 150 million people. Glucose detection in human body is very important in clinical diagnosis of diabetes. For the treatment and control of diabetes, the amount of blood glucose has to be monitored and controlled properly [29,30]. For recent years, biosensors gained a lot of attraction due to their large area of applications. They can be used in diagnosis, food technology, biotechnology, genetic engineering, and environmental monitoring [31].

Glucose oxidase (GOx) is the mostly used in glucose biosensing for its practical usage and stability. GOx is extracted from *Aspergillus* and it contains two tightly bound flavine adenine dinucleotide redox centers that catalyze the electron transfer from glucose to gluconolactone and hydrogen peroxide [32]. (Figure 2).

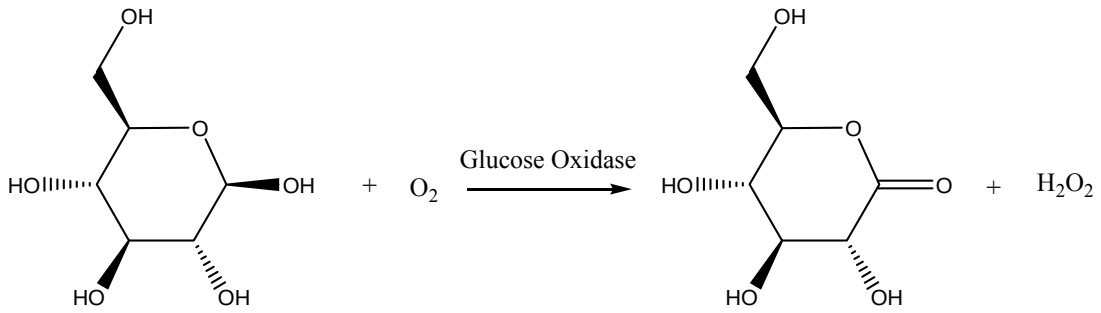


Figure 2. Reaction mechanism of glucose oxidase

The amperometric glucose biosensor is the most widely used among the different types of biosensors and they are suitable for biochemical analysis due to several advantages, such as simplicity in preparation and rapid response [33].

Alcohol oxidase

The analysis of alcohol with high sensitivity, selectivity and accuracy is important in food technology, agricultural and environmental analysis, fermentation, wine industries, and clinical chemistry, blood, serum and urine analysis [34, 35].

Alcohol oxidase (AOX; Alcohol:O_2 oxidoreductase, EC 1.1.3.13) is an homooctameric flavoprotein and oligomeric enzyme consisting of eight identical sub-units, each containing a strongly bound cofactor, flavin adenine dinucleotide molecule. Alcohol oxidase is an important enzyme for methanol metabolism in methylotrophic yeasts species like *Hansenula, Pichia, Candida* [36,37,38].

Alcohol oxidase catalyzes the oxidation of low molecular weight alcohols to their corresponding aldehydes, using molecular oxygen as the electron acceptor. (eq 1.) Alcohol oxidase has strong oxidizing character against alcohols, and therefore the reaction is irreversible.

$$RCH_2OH + O_2 + AOX \rightarrow RCHO + H_2O_2 \qquad \text{eq 1.}$$

Biosensors based on alcohol oxidase are easy prepared since alcohol oxidase uses only molecular oxygen (O_2) as the cofactor. O_2 is involved in the reaction of oxidation of ethanol to acetaldehyde and hydrogen peroxide. Therefore, the catalytic reaction can be easily followed amperometrically [39,40,41].

1.1.2 Microbial electrochemical biosensors

In addition to enzyme-based electrochemical biosensors, microbial biosensors have gained great importance. Microbial cells show great potential for construction of biosensors because of their numerous advantages:

- ✓ The enzymes in microbial cells do not require being isolated; they are generally more stable in the microbial cell, where coenzymes and activators are already present naturally.
- ✓ Microbial cells exist universally and they can metabolize a lot of chemical compounds. Therefore, microbial sensors can be used for multiple sensing.
- ✓ Microorganisms have ability to settle in difficult conditions and ability to degrade. Thus, microbial biosensors have tolerance to change in pH or temperature much more than enzymatic biosensors [42,43].

Microbe-based biosensors are regularly used for analyzing Biological Oxygen Demand, [44] toxic agents, sugars and they have applications such as bioreactors [45], agriculture [46], biocontrol [47], biodegradation [48,49] and biosensors [50].

Gluconobacter oxydans:

Gluconobacter oxydans is one of the most used organisms in biotechnology. Its distinctive capacity to oxidize polyol substrates has led *Gluconobacter oxydans* to find applications in biosensor technology [51].

Gluconobacter oxydans are gram-negative bacterium in the family of Acetobacteraceae, and *G. Oxydans* gets its latin name from *oxys*, means "sharp acidic" and *dans* means "giving" [52]. Gram-negative prokaryotic cells have respiratory redox proteins located in the cell membrane and accessible from the periplasm. The outer membrane contains permeable porins. Membrane bound enzymes provide fast and highly efficient oxidation. *G. Oxydans* is an aerobe, having a respiratory type of metabolism using oxygen as the terminal electron acceptor [53]. (Figure 3). One of the most important features of *G. Oxydans* is that they do not have any pathogenic effect towards humans or animals [54].

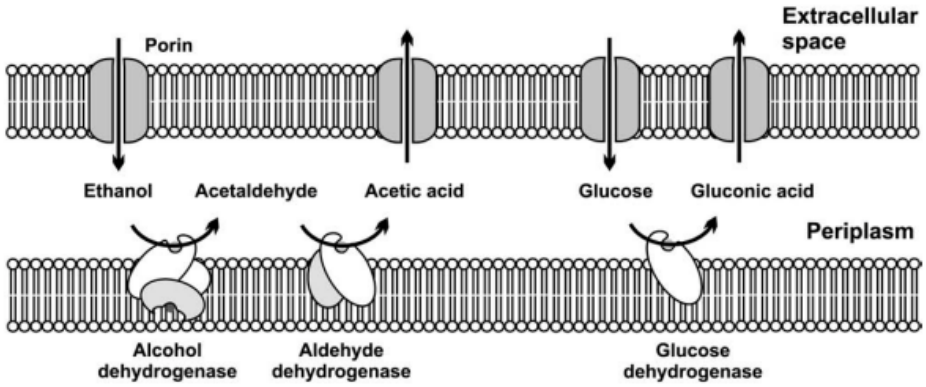


Figure 3. Membrane bound enzymes in *G. Oxydans* and reactions

Gluconobacter Oxydans holds a distinctive organization of metabolic pathways that makes them suitable for amperometric biosensor construction. In their cytoplasmic membrane, oxidative enzymes and the respiratory chain are surrounded. Substrates are oxidized in one or two steps in the membrane. *Gluconobacter Oxydans* contains multiple enzyme activities that are not available commercially like quinoprotein membrane-bound polyol dehydrogenases oxidizing D-glucose, ethanol, D-fructose, D-sorbitol, D-mannitol, glycerol and many other polyol-containing molecules [55].

Gluconobacter dehydrogenases are known to be ideal for use in biosensors due to their ability to stoichiometrically oxidize a wide range of substrates including mono- and poly-alcohols, multiple aldoses and ketoses, several disaccharides, triacylglycerols, and biological O_2 demand [56]. *Gluconobacter Oxydans* grow very fast in simple cultivation media, contain high enzymatic activities, stable during immobilization and do not need external cofactor [57].

Gluconobacter Oxydans can oxidize glucose via two pathways: Intracellular oxidation and dissimilation by oxidation by the pentose phosphate pathway and direct oxidation in periplasmic space by membrane-bound dehydrogenases. Glucose can be directly oxidized by membrane-bound glucose dehydrogenases (EC 1.1.99.17) to gluconate and 2-keto-D-gluconate. Similar to glucose ethanol can be also oxidized by two pathways. Especially direct oxidation in periplasmic space by membrane bound alcohol dehydrogenases acts on linear and branched monoalcohols up to C_4 to give the corresponding acids and ketones [58]. Various *Gluconobacter* based biosensors have previously been reported [59, 60, 61, 62, 63, 64].

Pseudomonas fluorescens

Pseudomonas fluorescens is a common gram-negative bacterium with a rod-shape. It is an aerobic and an interesting model to study since it uses organic compounds as their only source of carbon and energy [65,66]. *P. fluorescens* gets its name from Greek words pseudo, meaning "false" and *monas* meaning "single unit". It also contains a soluble fluorescent pigment called *"fluorescens"* [67]. In *Pseudomonas fluorescens*, glucose consumption follows two routes:

(i) "the direct oxidative pathway; which converts glucose to gluconate, 2-ketogluconate and then subsequently to 6-phosphogluconate by extracellular, high affinity, glucose dehydrogenase and gluconate dehydrogenase enzymes"

(ii) "the phosphorylative pathway; where glucose is converted to 6-phosphogluoconate by intracellular, low affinity, nucleotide-dependent glucokinase and glucose 6-phosphate dehydrogenase" [68,69].

The genus *Pseudomonas fluorescens* belongs to the γ subclass of the Proteobacteria. *Pseudomonas fluorescens* is a ubiquitous bacterium. They are substantially present in water and soil [70]. In amperometric electrochemical biosensors oxygen consumption in the presence of glucose due to the metabolic pathway of the *Pseudomonas fluorescens* is generally followed.

2. Conducting Polymers in Biosensors

Conducting polymers are novel materials with a short history of research however, attracted much interest due to their combined properties of organic polymers and electronic properties of semiconductors [71]. Since 1977, increasing concern has been dedicated to the electrical properties of a new-class material, organic conducting polymers because of their facile synthesis, good environmental and long term stability of electrical conductivity and electrochemical properties [72]. The redox and unusual combination of the properties of metals and plastics make the conducting polymers a novel group of materials [73]. The advantage of using conducting polymers is that the polymer which is soluble enables a nondestructive analysis of the sample [74].

The physical and chemical properties of the materials used in the construction of biosensors have got significant influence on their performance [75]. As stated in article, “there is a great need to design the electrodes compatible with the biomaterial that can direct the rapid electron transfer at the electrode surface” [1]. Recently, conducting polymers appeared as a new field of research and development in designing biosensors [76,77,78]. “They have an organized molecular structure which permits them to function as a three dimensional matrix for the immobilization of catalysts retaining their biological activity for a long time” [79,80]. CPs have the ability to transfer electric charge produced by the biochemical reaction and they serve as the immobilizing matrices for biomolecules and provide a suitable environment for immobilization [81,82,83]. Moreover, conducting polymers allow rapid electron transfer. Conjugated π electron backbones offer free movement of electrons throughout the lattice [84].

Coating electrode with CPs improves the electrocatalytic properties of biological material, enhances rapid electron transfer and facilitates direct communication between electrode and the biological material [85,86]. (Figure 4).

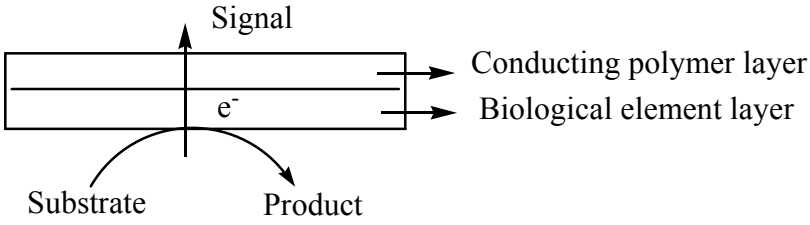


Figure 4. Conducting polymer based biosensors

Conducting polymers expanded the possibility of modification of the electrode surface, providing new properties to the biosensing system. Several papers were published to reveal that the electrodeposition of conducting polymers generates good matrices for the immobilization of enzymes [87].

Since conducting polymers offer different chemical structures and functional groups, they can be modified according to the need for immobilizing biological material. For that matter cross-linking method can be utilized using some functional groups like $-NH_2$, -COOH, to bind protein molecules directly [88,89].

During the process of electrochemical polymerization negatively charged molecules such as anions are present in the electrolytic solution. Biocomponent can also be embedded into the positively charged backbone as the dopants [90]. Hence during the electrochemical synthesis of conducting polymers, entrapment can be achieved with the direct deposition of the polymer on the electrode surface [91]. It is also possible to control the film thickness and therefore one can control the immobilized biomaterial amount [92].

Moreover, conducting polymers can improve rapidness, sensitivity, and adaptability of biosensors in analytical sense. They are also known to be biocompatible with aqueous solutions [93]. “It is known that electronics operate in a fundamentally different manner from biological systems; electronics is the domain of electrons and bio-systems are the domain of ions and molecules. Organic bio-electronics aim to combine and interface these worlds by utilizing the simultaneous electronic and ionic conduction present in conducting polymers [94]. The application of conducting polymers at the interface between biology and electronics is becoming an area of great importance. Specifically, the interaction of conducting polymers with both biomolecules (e.g. amino acids, proteins, enzymes, DNA) and living organisms (e.g. cells) are interesting for biomedical applications since the intrinsic properties of these materials can influence the molecular and cellular bioprocesses. From a practical point of view, the integration of conducting polymers with biological systems has been useful for the development of various applications such as implants for tissue engineering for nerve regeneration, actuators and biosensors etc.” [1,95].

“Electrochemical synthesis is the most preferred method for preparing electrically conducting polymers due to its simplicity and reproducibility. The main advantage of electrochemical polymerization in biosensing area is that the reactions can take place at room temperature which is very important for the biological material. T By varying either the potential or current with time, the thickness of the film can be controlled” [1]. Standard electrochemical technique utilizes a cell, containing a working electrode which is coated with a biocatalyst, a counter electrode and a reference electrode. The analyte is oxidized on the working electrode and electrons resulting from the oxidation are transferred to electrode. Various electrodes (platinum, glassy carbon, graphite, carbon paste) can be used in this three electrode system. (Figure 5).

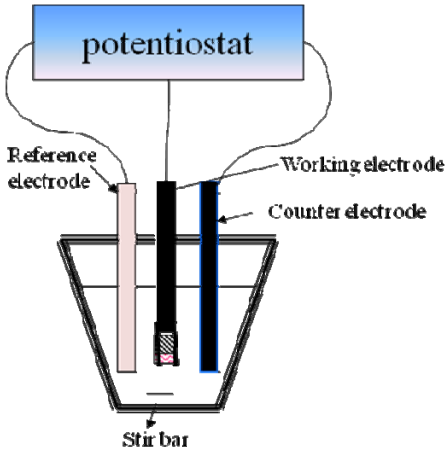


Figure 5. Electrochemical cell with three electrode system

3. Nanoparticles in biosensing area

The construction of materials in nanoscale is very important in nanotechnology and related applications like biosensors. Nanoparticles have several roles like immobilization of biomolecules; catalysis of electrochemical reactions; enhancement of electron transfer; labeling biomolecules and acting as reactant [96]. Nanoparticles are generally used to promote the electron transfer in redox proteins due to their electronic and structure properties. Hence they provide good communication between the electrodes and the redox proteins [97,98,99]. Nowadays nanoparticles are extensively used in the construction of electrochemical sensors. Among various kinds of nanoparticles, gold nanoparticles (AuNPs) and carbon nanotubes (CNTs) are the most favorable materials.

Gold nanoparticles (AuNPs) exhibit attractive properties in the application of biosensors due to their small size, high surface area, high surface energy, ability to do electron transfer between proteins and metal particles and their function as electron conducting pathways and good biocompatibility [100]. Due to their large specific surface area and high surface free energy, nanoparticles can adsorb biomolecules strongly.

Importantly, the adsorption of such biomolecules onto the surfaces of nanoparticles can retain their bioactivity because of the biocompatibility of nanoparticles. AuNPs have the advantage in preparing biosensors since they provide a stable immobilization platform where biomolecules retain their bioactivity. AuNPs allow direct electron transfer between electrode and redox proteins thus, there is no need for electron transfer mediators in designing biosensors [101]. They have been used as the matrix for the immobilization of biological materials, to help retain the enzyme bioactivity and enhance sensitivity of the biosensors. Moreover, it was reported that gold nanoparticles can immobilize proteins through the covalent bonds formed between the gold atoms and the amine groups and cysteine residues of proteins [102]. Their unique chemical properties offer extensive use in catalytic, biological, and sensing applications. Gold nanoparticles with different shapes can be obtained with 10-100nm dimensions [103]. It was reported that due to their excellent catalytic activity and good biocompatibility, AuNPs have been used as electrode modifiers for the construction of electrochemical biosensors via all immobilization methods [104,105].

Carbon nanotubes (CNTs) have multiple functions for the development of various biosensors; they favor the electrocatalytic oxidation/reduction of H_2O_2, enhance amperometric signals due to the increased interfacial electron transfer and large surface areas [106,107,108]. Because of their unique properties, such as enhanced electron transfer, high electrical conductivity, high mechanical properties, ability to grow on different substrates, CNTs have been intensively researched in electrocatalytic and sensing applications. The electrodes based on CNT exhibit highly sensitive and selective responses to generated hydrogen peroxide or oxygen consumption by enzymatic reactions [109,110]. Moreover, CNTs attracted much attention due to their high chemical stability, unique electronic properties, and relatively high mechanical properties [111,112]. Conducting polymer and CNTs attracted interest in recent years since the incorporation of CNTs into conducting polymers can lead to produce new composite materials with combined properties of each component; hence their synergistic effect would be useful in some particular applications [113].

“The carbon nanotube based biosensors combine the bioselectivity of redox enzymes with the inherent sensitivity of amperometric transductions, and have proven to be very useful for the biosensing” [68,114]. CNTs have the simple chemical composition and atomic bonding pattern, and have diversity in structure and properties. These unique properties make CNT extremely attractive for electrochemical biosensors [115,116].

CHAPTER 2

EXPERIMENTAL

2.1 Reagents

The strain of *Gluconobacter oxydans* (*G. oxydans*) was obtained from German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. Yeast extract was purchased from Oxoid (Hampshire, England; www.oxoid.com) Glucose oxidase (GOx; β-D-Glucose: Oxygen 1-oxidoreductase; from *Aspergillus niger* Type II, E.C 1.1.3.4; 21200 Unit/g solid), Alcohol oxidase, (AOx, Pichia pastoris, 28 Unit/ mg protein, 37mg protein/ mL) was purchased from Sigma. Graphite rods type RW00, with 3.05 mm diameter and 13 % porosity were obtained from Ringsdorff Werke GmbH, Bonn, Germany. Wet emery papers were Tufback Durite as P1200. Platinum, auxiliary and reference electrodes were purchased from BASi Analytical Instruments. Spectrophotometric Trinder reaction kit (Cromatest, Glucose MR, Cat. No. 1129010), $LiClO_4$, $NaClO_4$, dialysis membrane (cut off 12000), $AlCl_3$, succinyl chloride, benzene-1,4-diamine propionic acid, nitromethane, iron (III) chloride, propylene carbonate, poly(methylmethacrylate), dichloromethane, toluene, D-glucose, ethanol and gold colloidal (~ 0.75 A_{520} units/mL, 10 nm) were purchased from Sigma (St. Louis, USA). Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Multiwalled CNT (diameter; 110 –170 nm, length; 5-9 μm, mineral oil and graphite powder were purchased from Sigma-Aldrich (St.Louis, MO, USA) and used without any pre-treatment. All other chemicals were of analytical grade and purchased either from Merck (Darmstadt, Germany) or from Sigma (St. Louis, USA).

2.2 Instrumentation

2.2.1 Chronoamperometry measurements

Chronoamperometry measurements were carried out with a Radiometer electrochemical measurement unit (Lyon, France) and Palm Instruments (PalmSens, Houten, The Netherlands). A platinum electrode was used as an auxiliary electrode and an Ag/AgCl (3 M KCl saturated with AgCl as an internal solution) as the reference electrode. Depending on the biosensing system, graphite, platinum and carbon paste electrodes were used as working electrodes.

2.2.2. Cyclic Voltammetry measurements

For cyclic voltammetry experiments Palm Instruments (PalmSens, Houten, The Netherlands) was used with three electrode including working electrode, counter electrode and reference electrode configurations.

2.2.3. Surface Characterization

Scanning electron microscope (SEM) (JEOL JSM-6400) and Atomic Force microscope (Vecoo multimode V) were used for surface imaging of the microbial and enzyme electrodes.

2.3 Experimental Procedures

2.3.1 Synthesis of 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine

For synthesis of the monomer, 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine ($SNSNH_2$), firstly 1,4-di(2-thienyl)-1,4-butanedione was synthesized with the double Friedel–Crafts reaction in the presence of $AlCl_3$ and CH_2Cl_2 at 15 ºC. In the presence of catalytic amount of propionic acid, toluene 1,4-di(2-thienyl)-1,4-butanedione and benzene-1,4-diamine was refluxed for 4 h (yield 78%) in a round-bottomed flask with an argon inlet and magnetic stirrer. Resultant mixture was stirred and refluxed for 24h under argon. After evaporation of the toluene, the desired compound as a pale yellow powder was obtained by flash column chromatography (SiO_2 column, elution with dichloromethane) (Figure 6) [117,118,].

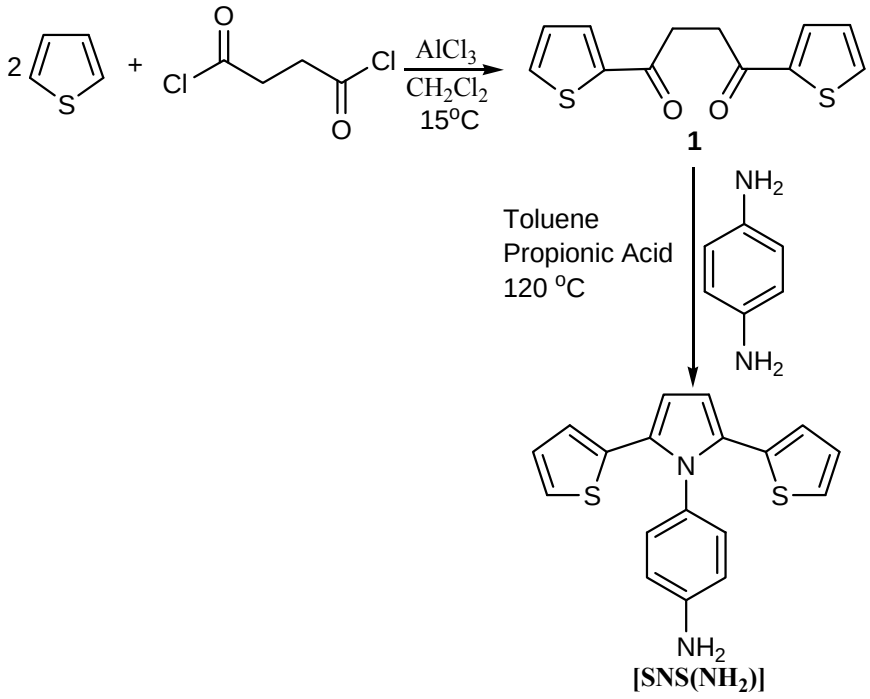


Figure 6. Synthesis of 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine ($SNSNH_2$)

2.3.2 Synthesis of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H*-pyrrole

For the synthesis of monomer 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H*-pyrrole ($SNSNO_2$), a round-bottomed flask equipped with an argon inlet and magnetic stirrer was equipped with the 1,4-di(2-thienyl)-1,4-butanedione, 4-nitroaniline, p-toluene-sulphonic acid and toluene. The reaction mixture was stirred and refluxed at 110°C for 24h under argon. After evaporation of toluene and flash column chromatography, $SNSNO_2$ monomer was obtained as pale brown powder as reported previously (Figure 7). [119,120].

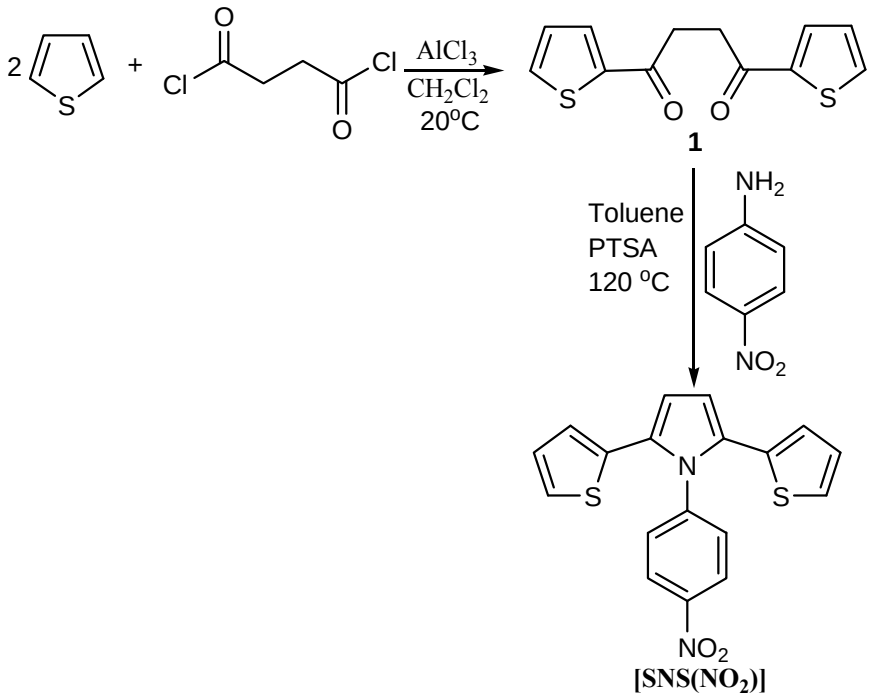


Figure 7. Synthesis of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H*-pyrrole ($SNSNO_2$)

2.3.3 Cell cultivation of *Gluconobacter oxydans*

The strain of *G. oxydans* was maintained on agar containing 100 g L^{-1} D-glucose, 10 g L^{-1} yeast extract, 20 g L^{-1}calcium carbonate, 20 g L^{-1} agar. By aerobic cultivation at 28°C on a rotary shaker in 250 mL flasks filled with 50 mL of media, the cell biomass was obtained. The growth medium was prepared with 5 g L^{-1} glucose and 5 g L^{-1} yeast extract and the culture was inoculated from the slant agar. Afterwards the culture was incubated for 12 h to reach the late exponential phase. At 600 nm, the cell growth was followed spectrophotometrically. The cells were collected by centrifugation for 10 min at 3500×*g*, resuspended in sterile 0.9 % NaCl solution and centrifuged again. For the biosensor preparation biomass was used [117,121,122,123].

2.3.4 Cell cultivation of *Pseudomonas fluorescens*

Pseudomonas fluorescens was prepared by the following procedure [124]: *"Pseudomonas fluorescens* was sub-cultured in nutrient agar, and transferred to mineral salt medium containing 1 g/L glucose. Mineral salt medium with 0.244% Na_2HPO_4, 0.152% KH_2PO_4, 0.050% $(NH_4)_2SO_4$, 0.02% $MgSO_4.7H_2O$, 0.005% $CaCl_2.2H_2O$ and trace element solution (10 mL/L), 0.1% NH_4NO_3, 0.05% $(NH_4)_2SO_4$, 0.05% NaCl, 0.05% $MgSO_4.7H_2O$, 0.15% K_2HPO_4, 0.05% KH_2PO_4, 0.0014% $CaCl_2.2H_2O$, 0.001% $FeSO_4.7H_2O$ and trace element solution (1 mL/L) were used as growth media for *P. Fluorescens.* pH 6.9 was used in the growth media. After 16 h, by centrifugation the biomass was harvested and suspended in mineral salt medium and then re-centrifuged. During the experiments bacterial cells in logarithmic phase were used and the cell growth was followed spectrophotometrically at 560 nm" [119,125].

2.3.5 Preparation of microbial biosensor based on *poly($SNSNH_2$)* and *Gluconobacter Oxydans*

"Graphite rods were polished on wet emery paper and washed thoroughly with distilled water, sonicated for 2-3 min, rinsed with bi-distilled water and dried at 105°C. Continuous cyclic voltammograms of $SNSNH_2$ with different charges were obtained at graphite electrode in acetonitrile solution containing 5 mg/mL $SNSNH_2$ monomer and 0.1 M $NaClO_4$ and 0.1 M $LiClO_4$ at scan rate of 0.5 Vs^{-1}" [117].

"G. oxydans suspensions were spread over the polymer coated electrode for the immobilization of microbial cells on conducting polymer matrix. The electrode allowed drying at ambient conditions for 1 hour. The electrode was washed to remove unbound cells. The layer was covered with a dialysis membrane, pre-soaked in water and the membrane was fixed tightly with a silicone rubber O-ring. Daily prepared electrodes with fresh cells were used in all experiments" [117].

2.3.5.1 Modification of microbial biosensors with Gold Nanoparticles

For the preparation of gold nanoparticle (AuNP) modified microbial sensors**,** *G. oxydans* suspension was mixed with 2.5 μL of gold colloidal solution with the average size of 10 nm. This gold nanoparticle *G. oxydans* mixture was used as the biological sensing material. After the polymerization of *poly($SNSNH_2$)* with the given procedure, different amount of gold nanoparticles absorbed onto the surface of conducting polymer. The rest of the procedure for preparing electrode was same as mentioned previously. To see the conducting polymer effect, control experiments were performed with a polished *G. Oxydans* adsorbed graphite electrode without conducting polymer (Figure 8).

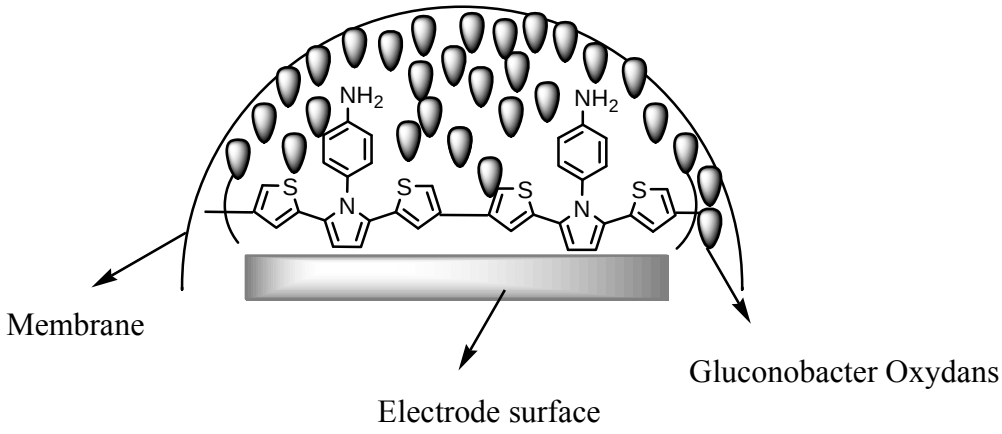


Figure 8. Preparation of *poly(SNSNH$_2$)/G.Oxydans* biosensor

Control experiments were done using entrapped cells behind dialysis membrane on the polished graphite electrode without the polymer.

2.3.6 Preparation of microbial biosensor based on *poly(SNSNO$_2$)* and *Gluconobacter Oxydans* & *Pseudomonas fluorescens*

"Graphite rods were polished on wet emery paper and washed thoroughly with distilled water, sonicated for 2-3 min, rinsed with bi-distilled water and dried at 105°C. Electrochemical polymerization of ($SNSNO_2$) was potentiodynamically carried out with a potential range between 0.0 V and 1.1 V in acetonitrile medium containing $NaClO_4$ (0.1 M) and $LiClO_4$ (0.1 M). For immobilization of microbes, 25 µL of intact cells (*G. oxydans* with 35×10^9cell titer and *P. fluorescens* with $15x10^{10}$ cell titer) was spread over the polymer coated electrode and allowed to dry for 1 h. Afterwards, the electrode surface was covered with a dialysis membrane, pre-soaked in water. With a silicone rubber O-ring, the membrane was fixed tightly" [119]. (Figure 9).

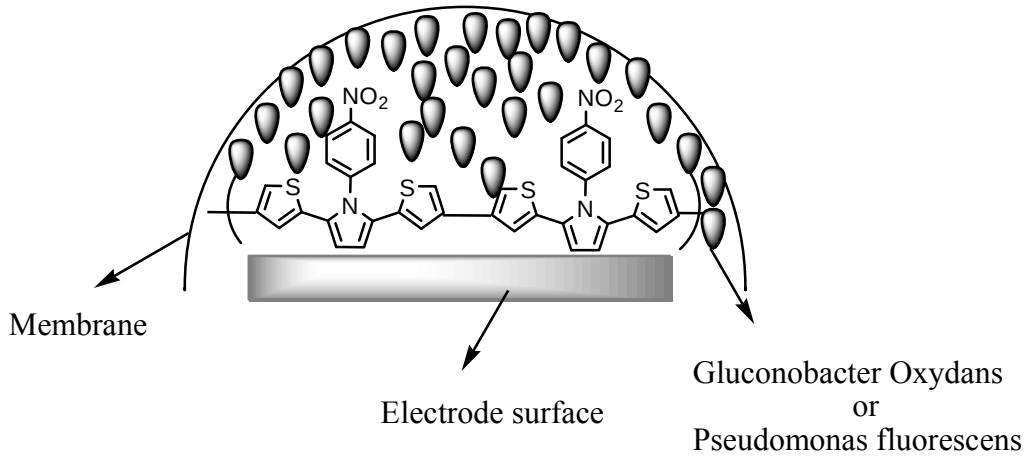


Figure 9. Preparation of *poly(SNSNO$_2$)*/*G.Oxydans* or *P.Fluorescens* biosensor

2.3.7 Preparation of enzymatic glucose biosensor based on *poly(SNSNH$_2$)*, glucose oxidase, Gold Nanoparticles

"Graphite rods were polished on wet emery paper and washed thoroughly with distilled water, sonicated for 2-3 min, rinsed with bi-distilled water and dried at 105°C. Continuous cyclic voltammograms (CVs) up to 100 cycles, with charges of 1.05×10^{-3} C obtained at graphite electrode in acetonitrile solution containing 5 mg/mL $SNSNH_2$ monomer and 0.1 M $NaClO_4$ and 0.1 M $LiClO_4$ at scan rate of 0.5 Vs^{-1}" [119].

For the immobilization of glucose oxidase, proper amounts of GOx and 1% glutaraldehyde in (10 μL) in potassium phosphate buffer solution (50 mM, pH 7.0) were spread over the surface of graphite electrode and allowed to dry at ambient conditions for nearly 1 hour.

After polymerization of $SNSNH_2$, different amount of gold nanoparticles (1%, 0.5%, 0.1%, 0.05%) with absorbed onto the surface of conducting polymer. For the immobilization of glucose oxidase, proper amounts of GOx and 1% glutaraldehyde (10 µl) in potassium phosphate buffer solution (50 mM, pH 7.0) were spread over the surface of AuNP modified polymer coated electrode and allowed to dry at for nearly 1 hour. (Figure 10).

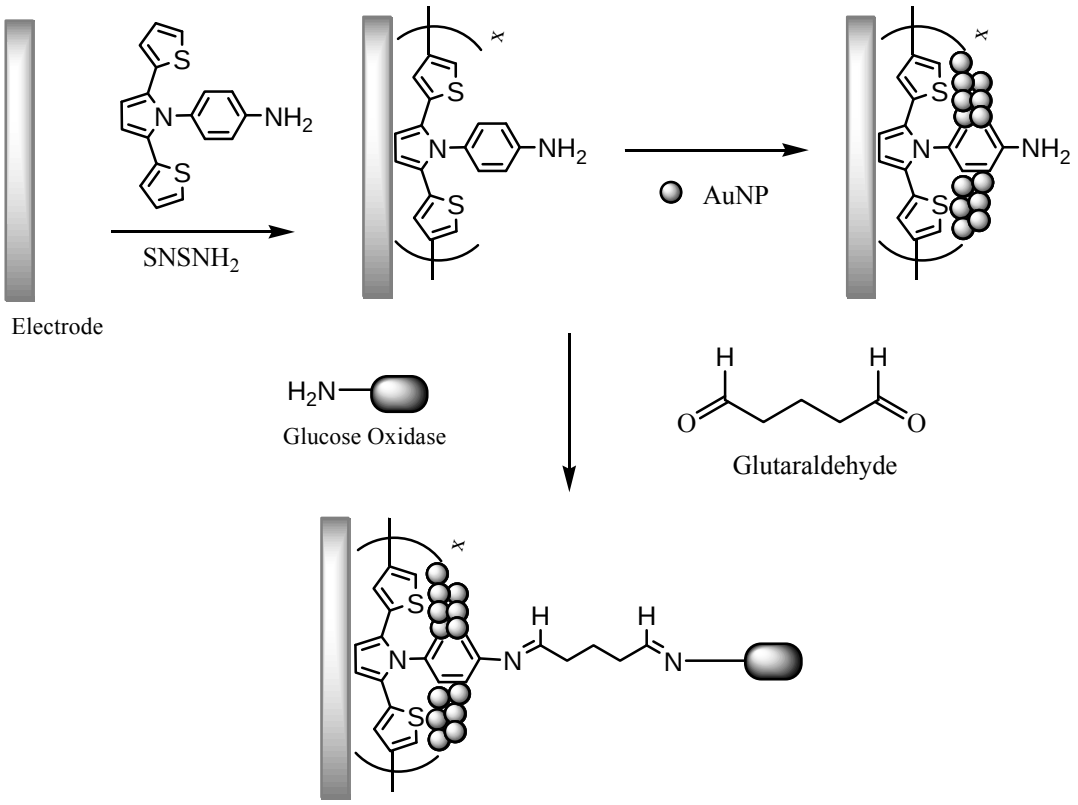


Figure 10. Simple representation for preparation of *poly(SNSNH$_2$)*/GOx/AuNP

2.3.8. Preparation of enzymatic glucose biosensor based on *poly($SNSNH_2$)*,GOx and CNTs

Initially, proper amounts of graphite powder and mineral oil (74%:26%) were mixed to obtain the carbon paste electrodes (CPE). A portion of the resulting paste was then packed in the cavity (3.0 mm diameter and 5 mm depth) of a Teflon tube. Electrical contact was established via a copper wire. The surface of the paste electrodes were smoothed on a weighing paper and rinsed carefully with distilled water.

"Conducting polymer was coated on the carbon paste electrode surface through running 100 cycles by cyclic voltammetry, with a total charge of 1.05×10^{-3} C. The polymerization was achieved in acetonitrile solution containing 5 mg/mL $SNSNH_2$ monomer, 0.1 M $NaClO_4$ and 0.1 M $LiClO_4$ at a scan rate of 0.5 Vs^{-1}" [117].

To obtain CNT modified *poly($SNSNH_2$)*/CNT/GOx electrodes, four different modification strategies (1-4) were used. After surface modification with CNT (except method-4), 1 mg GOx (21.2 U) in 10 µl potassium phosphate buffer solution (50 mM, pH 7.0) and 1% glutaraldehyde (10 µl, in the same buffer) were spread over the surface and allowed to dry at ambient conditions for 1 hour.

1. *poly($SNSNH_2$)/GOx/CNT-1:* CNT (1 mg) was dissolved in 1 mL, 98 % ethanol and sonicated for 30 min then, 10 µl of CNT dispersion were spread over the polymer coated electrode and allowed to dry overnight.
2. *poly($SNSNH_2$)/GOx/CNT-2:* CNT (1 mg) was dissolved in 1 mL mineral oil and 10 µl of dispersion was directly added to the carbon paste by mixing with carbon powder and oil and then the electrode was coated with conducting polymer as described before.
3. *poly($SNSNH_2$)/GOX/CNT-3:* CNT (1 mg) was dissolved in 1 mL cetyltrimethylammonium bromide (CTAB; 10 mg/mL in water). The use of CTAB offers a quick and effective method to disperse carbon nanotubes [126]. The dispersion (10 µL) was spread over the polymer coated electrode and allowed to dry overnight.

4. *poly(SNSNH$_2$)/GOx/CNT-4:* 10 µL CNT dispersion (1 mg in 1 mL CTAB) was mixed with 1 mg GOx and the solution was spread over the electrode surface. Then, 1 % glutaraldehyde (10 µL) were spread over the surface and allowed to dry at ambient conditions for nearly 1 hour.

Daily prepared electrodes were used in all experimental steps. Control experiments were done by using GOx immobilized carbon paste electrodes coated with polymeric matrix in the absence of CNT *poly(SNSNH$_2$)/GOx*. A simple description of the proposed biosensing system was shown in Figure 11.

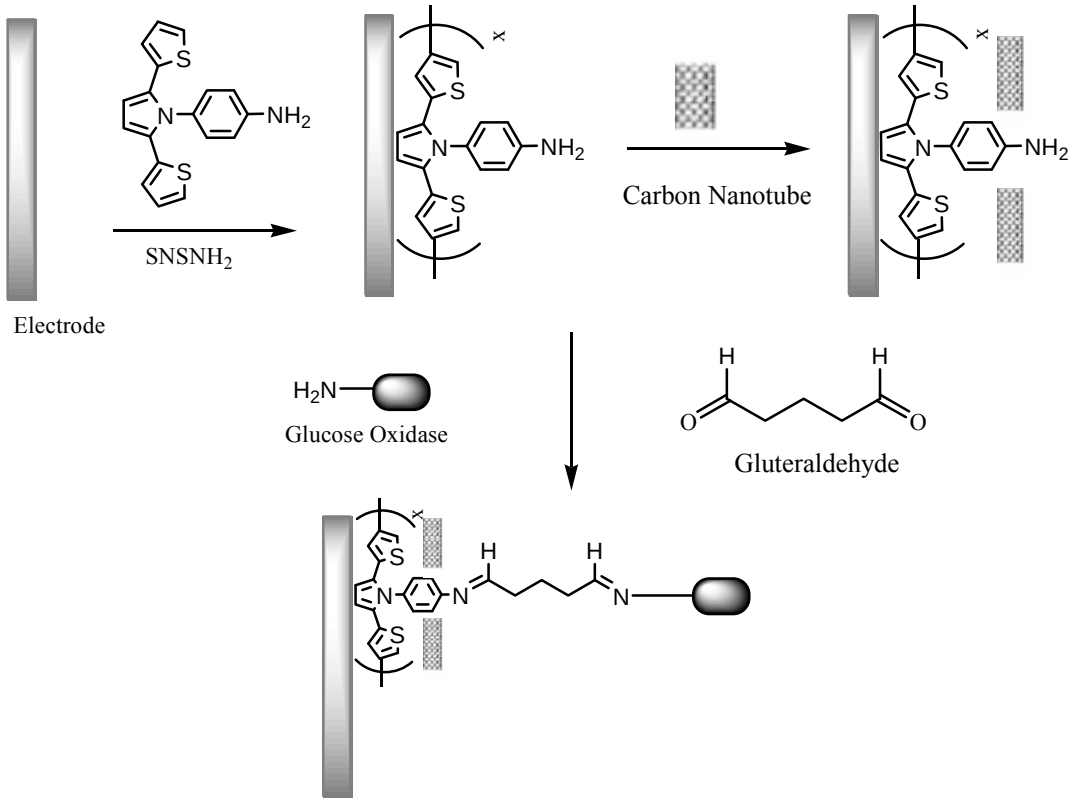


Figure 11. Description of the biosensing system *poly(SNSNH$_2$)*/GOx/CNT

2.3.9 Preparation of enzymatic ethanol biosensor based on *poly($SNSNH_2$)*, Alcohol Oxidase

"Conducting polymer was coated on the platinum electrode (0.16mm^2) surface through running 20 cycles by cyclic voltammetry. The polymerization was achieved in acetonitrile solution containing 5 mg/mL $SNSNH_2$ monomer, 0.1 M $NaClO_4$ and 0.1 M $LiClO_4$ at a scan rate of 0.5 Vs^{-1}" [117].

For the immobilization of alcohol oxidase, proper amounts of AOx and 1% glutaraldehyde in (10 µL) in potassium phosphate buffer solution (50 mM, pH 7.0) were spread over the surface of platinum electrode and allowed to dry at ambient conditions for nearly 1 hour.

2.3.9.1 Effect of Gold Nanoparticle on ethanol sensing

For the preparation of gold nanoparticle (AuNP) modified ethanol biosensors, 10nm gold nanoparticle colloidal solution was used. After electropolymerization of $SNSNH_2$, different electrodes including 0.5 µL, 1 µL, 1.5 µL and 2 µL AnNP with 1.5 µL AOX and 1µL glutaraldehyde. Ethanol biosensing responses were checked for 1 and 2mM ethanol.

2.3.9.2 Effect of Carbon Nanotubes on ethanol sensing

After electropolymerization of $SNSNH_2$, CNT which was dissolved in 1mL CTAB was spread over the electrode with different amounts (0.5 µL, 1 µL, 1.5 µL, ve 2µL). After adding glutaraldehyde and alcohol oxidase, electrodes allowed drying for 1 hour. Ethanol biosensing responses were checked for 1 and 2mM ethanol.

2.3.10 Amperometric response measurements

"All experiments were conducted at optimized conditions in the electrochemical cell with three electrode configuration, containing 10 mL of buffer using magnetic stirring with 210 rpm. After each measurement the electrode was washed with distilled water and kept in buffer. The electrode was initially equilibrated in buffer, and then the substrate was added to the electrochemical cell. The biosensor responses were registered as the current densities ($\mu A/cm^2$) or current (μA) by following the oxygen consumption at -0.7 V due to biological activity of the immobilized material. After every amperometric response measurements, the enzyme or microbial electrode was washed with distilled water and the buffer of the electrochemical cell was refreshed" [117,119].

2.3.11 pH optimization of the biosensors

Biosensing responses were checked in the pH of the potassium phosphate (pH 6.0-7.5, 50mM) and sodium acetate buffers (pH5.0-6.5, 50 mM) to see the effect of pH on the biosensors. The current density was adjusted as 100 % to the maximum response pH, and other values were calculated relative to this value.

2.3.12 Effect of biological material amount for biosensors

Biosensors containing different amount of biological material were prepared and their responses were checked to determine the appropriate biological material amount.

2.3.13 Effect of electropolymerization time

Effect of electropolymerization time of the polymer, which is directly correlated with the thickness of the polymer on the graphite electrode, was determined by preparing electrodes with 5, 10, 15, and 20 min of electropolymerization (referring to 50, 100, 150 and 200 scan numbers). Charges related with the scan number were also calculated for both conducting polymers.

2.3.14 Analytical characterization of biosensors

The analytical characteristics of the biosensors in terms of linear dynamic ranges and the equations were examined under optimized conditions. Calibration curves were plotted for current density versus substrate concentration (where y is the sensor response in terms of current density ($\mu A/cm^2$) and x is the substrate concentration in mM).

Repeatability of the biosensors was estimated by repetitive measurements with their substrates. Furthermore, the standard deviation and coefficient of the variation were calculated.

For some of the designed biosensors, substrate specificity towards mannose, galactose, xylose, methanol and ethanol were also tested.

2.3.15 Sample application using *poly(SNSNH$_2$)* and *poly(SNSNO$_2$)* based biosensors

The enzymatic biosensors were used to analyze glucose in gaseous fruit juices brand K and brand F. The microbial sensors were tested to analyze glucose in a fruit juice brand C and ethanol content in vodka brand M, whisky brand J samples. For using designed biosensors in sample applications, no sample pretreatment was required.

A commercial enzyme assay kit based on spectrophotometric Trinder reaction kit was used as the reference method for analysis of the glucose content. In the reaction kit, glucose oxidase oxides the glucose to D-gluconate with the formation of hydrogen peroxide. In the presence of peroxidase, a mixture of phenol and 4-aminoantipyrine is oxidized to a red quinine imine dye which is proportional to the glucose concentration in the sample [127]. In alcohol analysis, amount of ethanol detected in the sample by the proposed sensor was compared with the label value of the product.

CHAPTER 3

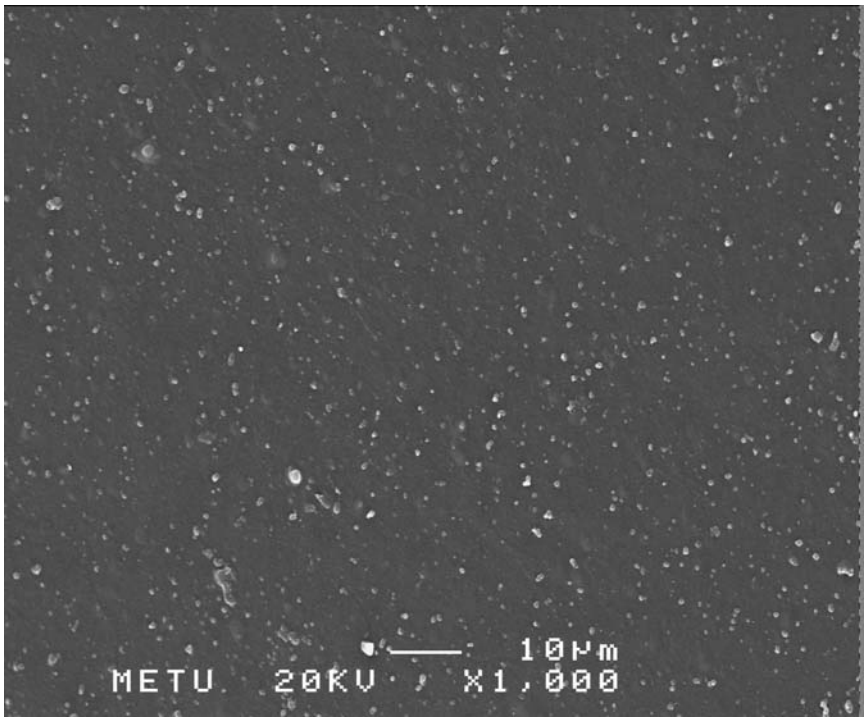
RESULTS AND DISCUSSION

3.1 Microbial biosensor based on *poly($SNSNH_2$)* and Gluconobacter Oxydans

3.1.1 Scanning electron microscopy images

The most precise information about the interaction between biological materials and immobilization matrices in the used systems can be obtained from the morphologies of microbial sensing surfaces. Scanning electron microscopy technique is utilized to screen the surface characteristics. Morphologies of matrices with and without cells were shown in Figures 12 a, b and c. Before analysis, the electrode surface was washed to remove unbound cells. It can be clearly seen from the micrographs that, the compact structure of *poly($SNSNH_2$)* provided a well-organized immobilization platform for the immobilization of cells. The ionic interactions between the cell surface and the amino groups in the polymer structure can also play role in joining the microorganisms on the polymer matrix [117].

a)



b)

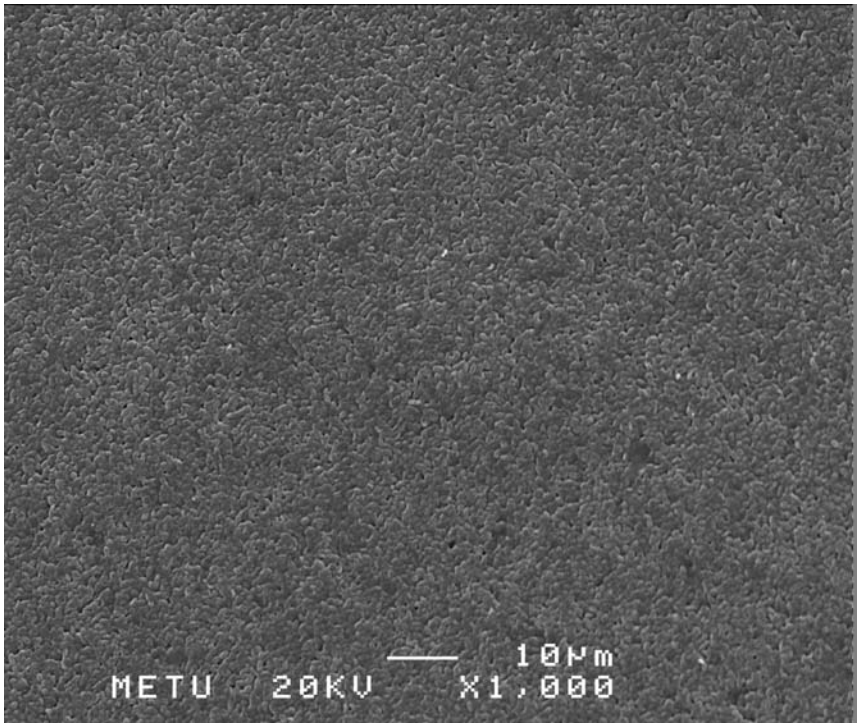


Figure 12. SEM images of (a) bare graphite b) *poly(SNSNH$_2$)* c) *poly(SNSNH$_2$)*/G.Oxydans

3.1.2 Effect of electropolymerization time

Electropolymerization has the advantages of the achievement of new properties using different supporting electrolytes or monomers and the control of the film thickness by regulating the amount of charge passed [128]. The most expedient electrochemical method for characterization is cyclic voltammetry. Cyclic voltammograms before and after the electropolymerization of $SNSNH_2$ on the graphite electrode were shown in Figure 13 [117].

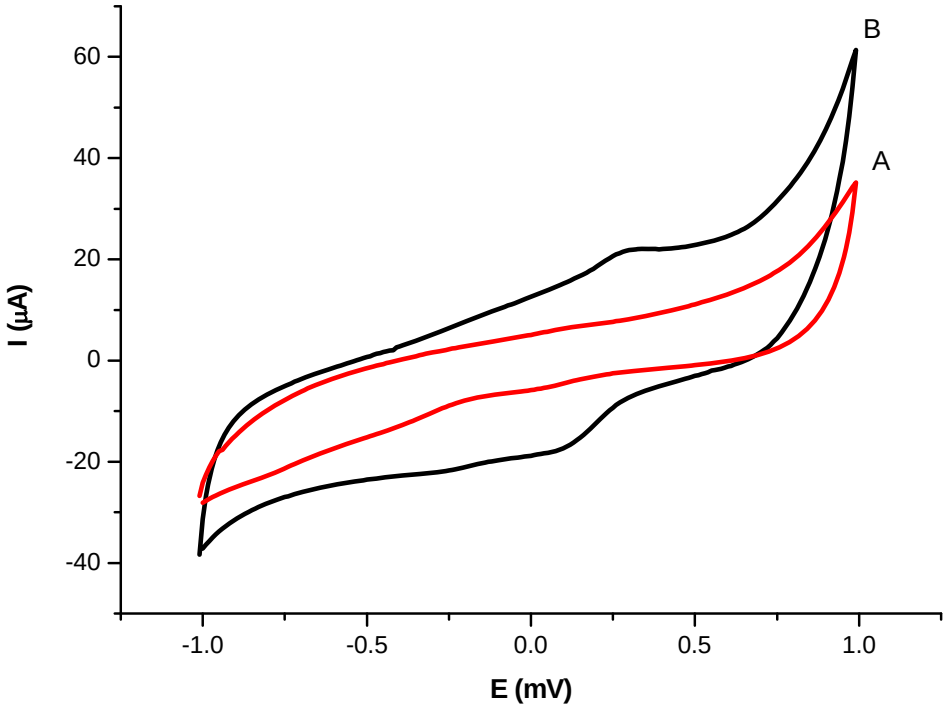


Figure 13. Cyclic voltammograms of (A) bare graphite electrode (B) after electrochemical polymerization of 100 scan $SNSNH_2$

Experimental Conditions: potassium phosphate buffer 50 mM, pH 6.5

Electropolymerization time is straightly correlated with the thickness of the polymer on the electrode. The thickness can be obtained with different number of scans can be measured in terms of charge. To optimize the thickness of electrode, four different electrodes were prepared with different charges as shown in Table 2 [117].

Table 2. Relation between electropolymerization times, number of scan and deposited charges

Electropolymerization Time (min)	***Number of Scan (cycles)***	***Deposited Charges (C)***
5	50	$9.5x10^{-4}$
10	100	$1.05x10^{-3}$
15	150	$7.90x10^{-4}$
20	200	$4.10x10^{-4}$

As shown in Figure 14 and Table 3, it is clear that the maximum biosensor activity was obtained when the working electrode was coated using $1.05x10^{-3}$ coulombs thickness after 10 min electropolymerization time. Longer deposition times caused the degradation and incompact microstructure [129]. Further experiments with $SNSNH_2$ were conducted using $1.05x10^{-3}$ coulombs (100 scan) thickness [117].

Table 3. **Effect of number of scans on the biosensing in terms of current and percentage.**

Number of Scans (cycles)	*Biosensing Response ($\mu A/cm^2$)*	*Biosensing Response (%)*
50	1.52	88.37
100	1.72	100
150	1.37	79.65
200	1.20	69.77

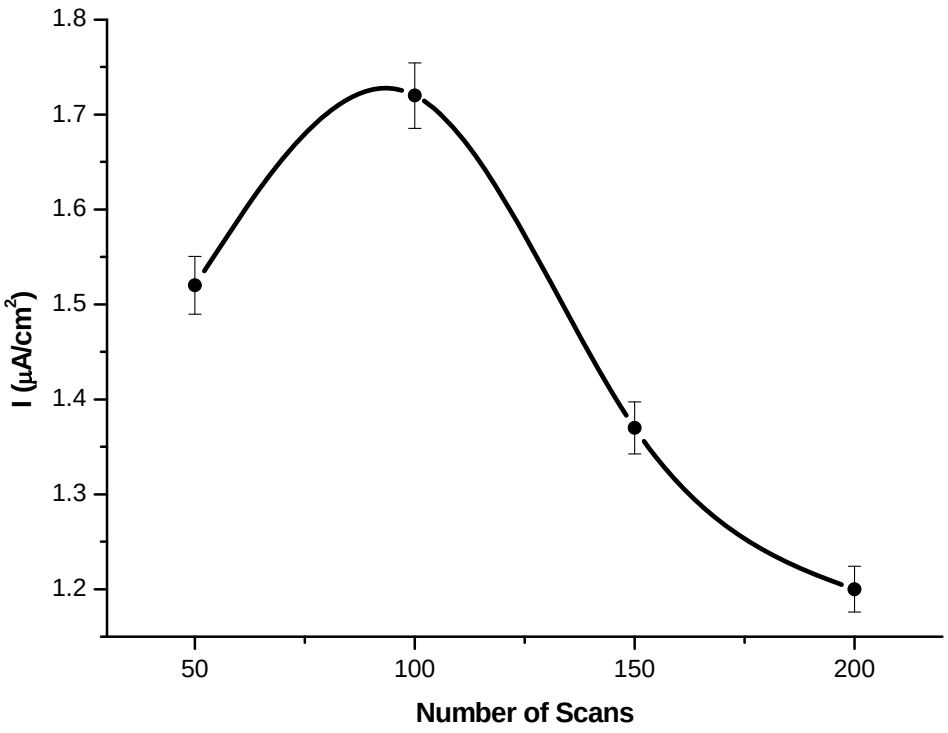


Figure 14. Effect of number of scan on the biosensor response

Experimental conditions: 25 µL *G.Oxydans*, potassium phosphate buffer (50 mM, pH 6.5), -0.7 V, 10 mM glucose.

3.1.3. Effect of *G.Oxydans* amount

To determine the suitable cell amount for biosensing, different biosensors containing 5 μL, 10 μL, 25 μL and 40 μL of *G.Oxydans* were prepared. The highest biosensing responses were obtained with 25 μL cell amount ($35x10^9$cell titer). When 5 μL *G.Oxydans* (with $7x10^9$ cell titer) was immobilized, the lowest biosensing response was obtained. In contrast, when cell amount was increased to 40 μL, a lower signal than that for 25 μL was obtained. This is a predictable outcome resulted from the diffusion problems due to the high cell density. For further experiments 25 μL *G.Oxydans* were immobilized on *poly(SNSNH$_2$)* matrix. (Figure 15 and Table 4) [117].

Table 4. Effect of cell amount and cell titer on biosensing response in terms of current and percentage.

Cell amount (μL)	*Cell titer (no unit)*	*Biosensing Response (μA/cm^2)*	*Biosensing Response (%)*
5	$7x10^9$	0.75	49.50
10	$14x10^9$	1.04	68.65
25	$35x10^9$	1.52	100
40	$40x10^9$	1.13	74.85

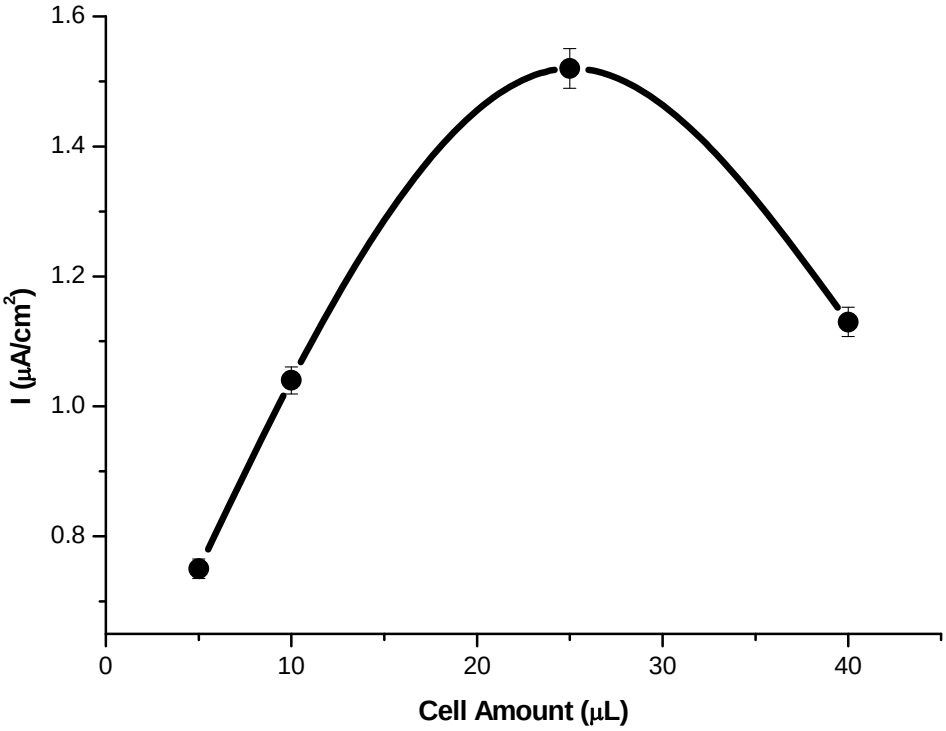


Figure 15. Effect of cell loading on the biosensor response

Experimental conditions: 100scan $SNSNH_2$ potassium phosphate buffer (50 mM, pH 6.5), -0.7 V, 10 mM glucose

3.1.4 Effect of pH

The effect of pH on biosensing response was optimized by adjusting the pH between 6.0 and 7.5 using phosphate buffer (50mM). The biosensing response of the microbial sensor towards 10 mM glucose at pH between 6.0 and 7.5 was shown in Figure 16 and Table 5. pH 6.5 was chosen as the optimum pH due to its maximum biosensing response. Further experiments were conducted with this pH value [117].

Table 5. Effect of pH on biosensing response in terms of current and percentage

pH	*Biosensor Response ($\mu A/cm^2$)*	*Biosensor Response (%)*
6	1.57	86.35
6.5	1.82	100
7	1.31	71.98
7.5	1.25	68.85

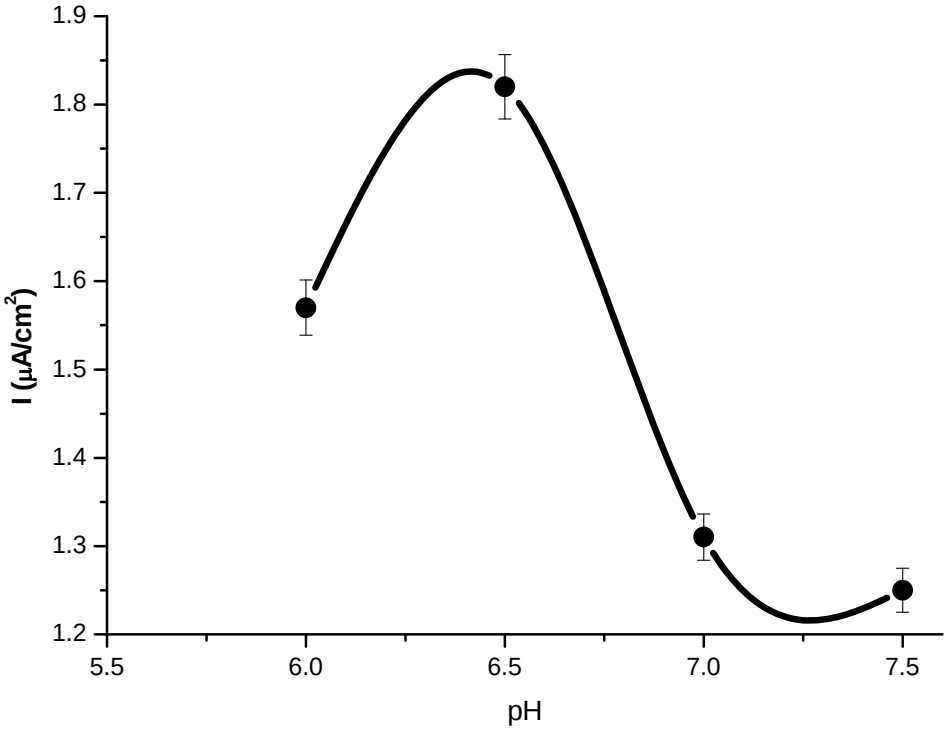


Figure 16. Effect of pH

Experimental conditions: 100 scan $SNSNH_2$, 25µL *G.Oxydans,* Potassium phosphate buffer (pH 6.0-7.5, 50 mM), -0.7 V, 10 mM glucose

3.1.5 Analytical approach for *poly($SNSNH_2$)* and *Gluconobacter Oxydans* based biosensor

Microbial sensor was characterized analytically under optimized conditions. For the *poly($SNSNH_2$)* based microbial sensor, a linear calibration graph was obtained for current density versus substrate concentration between 0.1 and 2.5 mM glucose. A linear relation was defined by the equation of y=0.349x+0.406, (R^2 =0.941) where y is the sensor response in current density ($\mu A/cm^2$) and the x is the substrate concentration in mM.

Repeatability of the microbial sensor was tested for 2.3 mM glucose, (n=4) and the standard deviation and coefficient of variation were calculated as ±0.067 mM and 2.9 % respectively [117].

3.1.6. Analytical approach for *poly($SNSNH_2$)* and *Gluconobacter Oxydans* based Au modified biosensor

Calibration curve for the gold nanoparticle modified system was examined under optimized conditions. A linear relation for glucose substrate was found between 0.05-1.5 mM with the equation y = 1.214x + 0.235, (R^2=0.995) and a response time of 130 s. When these data is compared with the non AuNP microbial sensor, higher current responses and sensitivity were obtained [117].

As shown in Figure 17, when the AuNPs were added to the biosensing system, higher responses were obtained as a result of existence of metal nanoparticles due to contribution of the facilitated electron transfer between the oxidative enzymes in microbe and the electrode surface. It is also possible that high surface area due to the AuNP on the polymer matrix can provide immobilizing of highest cell amount causing higher biosensor response [130].

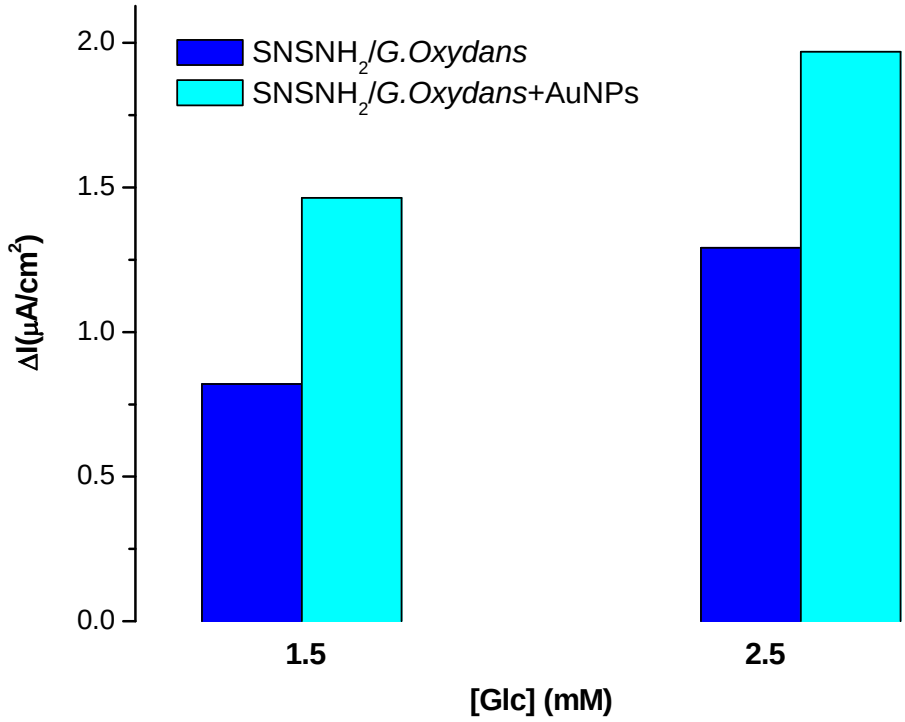


Figure 17. Biosensing responses of *poly(SNSNH$_2$)*/ *G. Oxydans* and *poly(SNSNH$_2$)*/ *G. Oxydans*/ Au NPs towards 1.5 and 2.5 mM Glucose

Experimental conditions: 100 scan $SNSNH_2$, 25µL *G.Oxydans,* Potassium phosphate buffer (pH 6.0-7.5, 50 mM), -0.7 V, 10 mM glucose

To see the conducting polymer effect on biosensing response a calibration curve was also obtained for the microbial electrode where there is no coating with *poly(SNSNH$_2$)*. For this system, irreproducible and lower biosensing responses were observed. This reveals that the conducting polymer provides a good immobilization platform for *G. Oxydans* on the electrode surface where they can attach and endure during the operational conditions (Figure 18) [117].

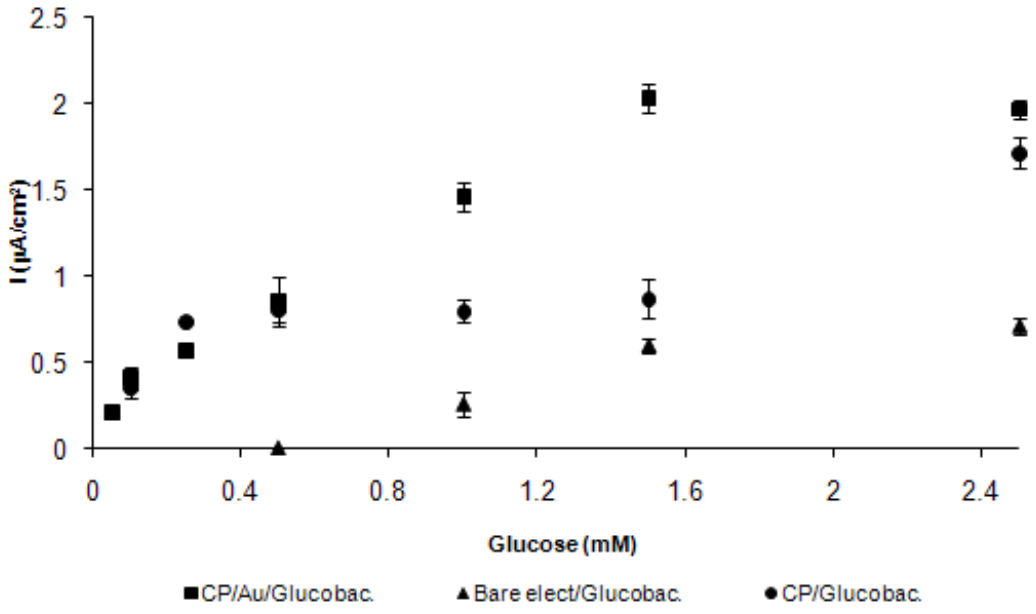


Figure 18. Calibration curves for microbial biosensor *poly(SNSNH$_2$)/ G.Oxydans, poly(SNSNH$_2$)/G.Oxydans*/AuNP and *G.Oxydans*

Experimental conditions: 100 scan $SNSNH_2$, 25µL *G.Oxydans,* Potassium phosphate buffer (pH 6.5, 50 mM), -0.7 V

Substrate specificity of the microbial biosensor towards mannose, galactose, xylose, methanol and ethanol were tested. There was no response for mannose, galactose, xylose and methanol whereas microbial sensor gives response to ethanol in the range of 0.1 mM-5.0 mM with equation y=0.163x+0.425 (R^2=0.978). It can be resulted that it is possible to make selective ethanol analysis in the presence of methanol [117].

The operational stability of microbial sensors was also determined at optimum conditions towards 1.5 mM glucose. After 5 hours and 22 measurements 11% decrease was seen in biosensing response [117].

3.1.7 Detection of glucose in real samples with microbial biosensor

Microbial biosensor was used for glucose detection in fruit juice and ethanol detection vodka and whisky samples. A spectrophotometric method was utilized as the reference method. Samples were used as the substrates instead of glucose and ethanol. Under optimized conditions, biosensing responses towards samples were recorded and the corresponding data were calculated from glucose and ethanol calibration curves. For the glucose analysis, values obtained from the calibration curves were compared with values gained by the spectrophotometric method. The glucose amount in fruit juice was determined as 17.64 g/L ± 1.89 by microbial sensor and calculated as 18.19 g/L ± 2.77 by the spectrophotometric method [117].

For the ethanol analysis, data were compared with the ethanol label values. The alcohol amount in whisky sample was determined as 46.6±5.71 % by the microbial biosensor. Alcohol amount in vodka sample was determined as 35±7.55 % by the microbial biosensor (Table 6) [117].

Table 6. Detection of glucose and alcohol with the biosensor

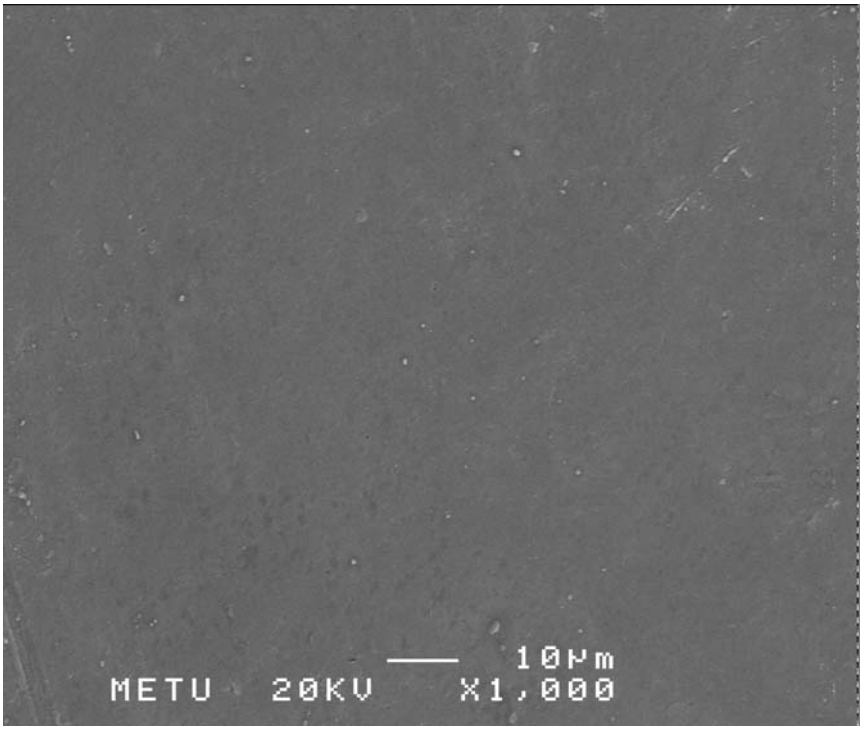
Sample	***Biosensor***	***Label Value***
Fruit Juice	17.64 g/L ± 1.89	18.19 g/L ± 2.77
Vodka	35±7.55 %	40 %
Whisky	46.6 ±5.71 %	43 %

3.2 Microbial biosensor based on *poly(SNSNO$_2$)*, *Gluconobacter Oxydans* and *Pseudomans Fluorescens*

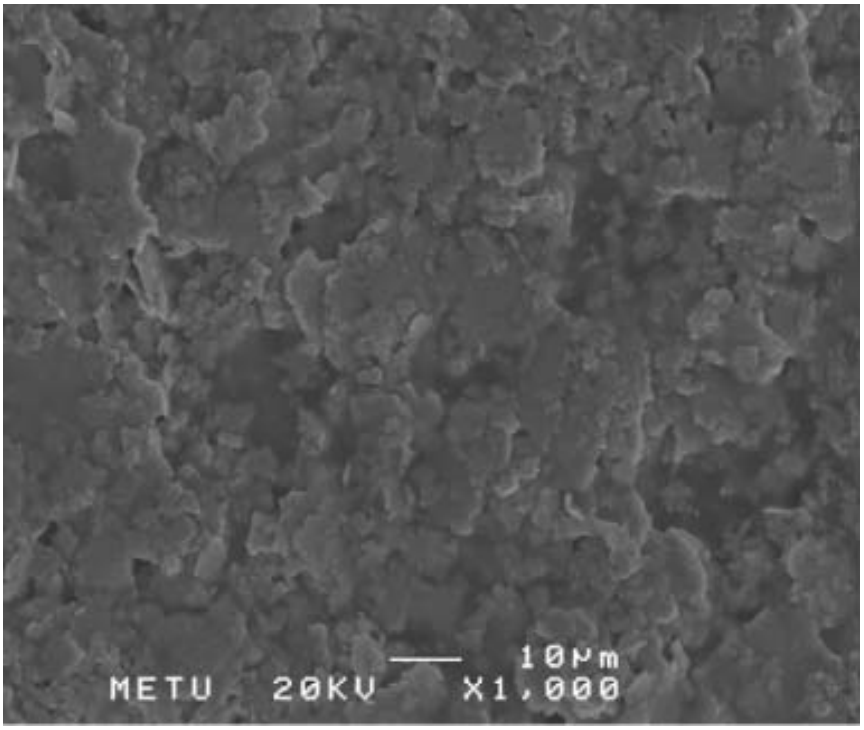
3.2.1 Scanning electron microscopy image of the biosensor

Morphologies of matrices were observed and shown in Figures 19 a, b, c and d. When it is compared with bare graphite and *poly(SNSNO$_2$)* coated graphite, it can be clearly seen that *poly(SNSNO$_2$)* provides more porous surface which enables cells to attach to the surface and provides an efficient platform for intact cells so cells could keep their metabolic activities [119].

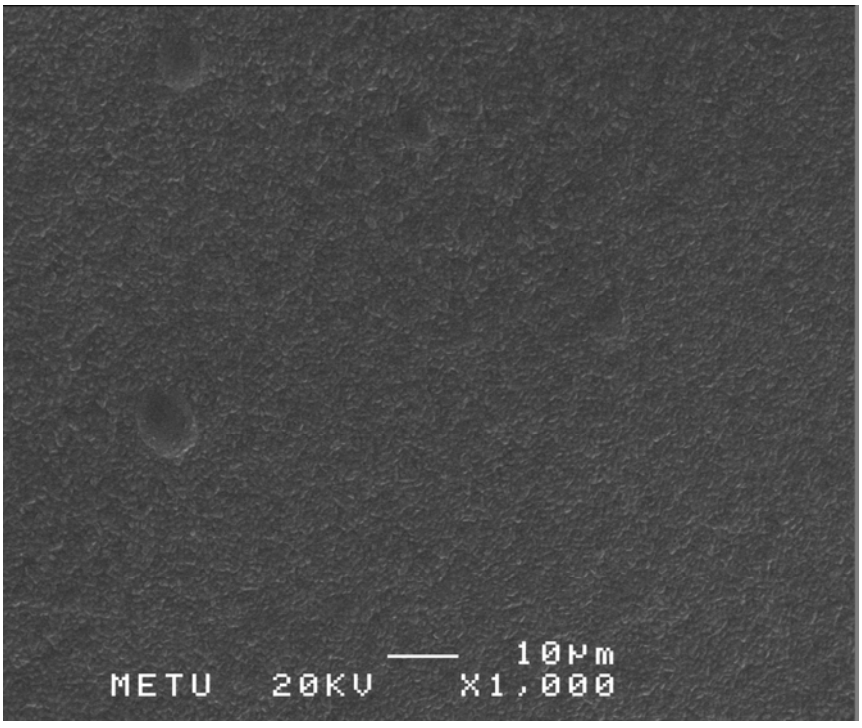
a)



b)



c)



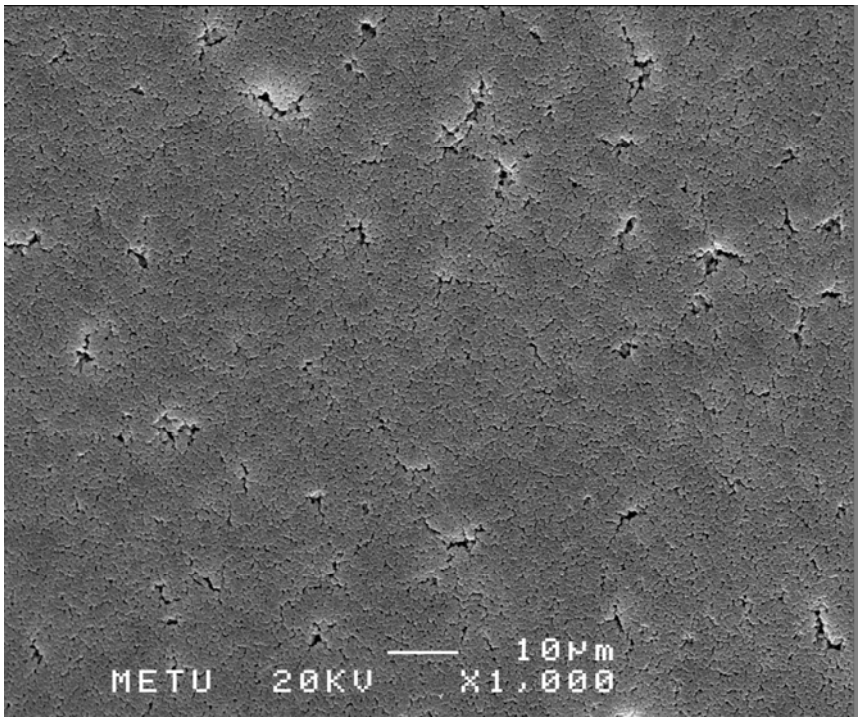
d) 

Figure 19. SEM images of a) bare graphite electrode b) *poly(SNSNO$_2$)* c) *Gluconobacter oxydans* cells d) *Pseudomonas fluorescens* on *poly(SNSNO$_2$)* modified graphite electrodes

3.2.2. Effect of Electropolymerization time on biosensing response

Electropolymerization improves homogeneous film formation on the electrode surface, regardless of electrodes shape or size. Moreover, electropolymerization can be carried out with large electrode surfaces with a control of thickness. The film thickness can easily be controlled with the measurement of the total charge during the deposition of conducting polymer [119]. Cyclic voltammograms of bare electrode and after the electropolymerization of $SNSNO_2$ on the graphite electrode were shown in Figure 20.

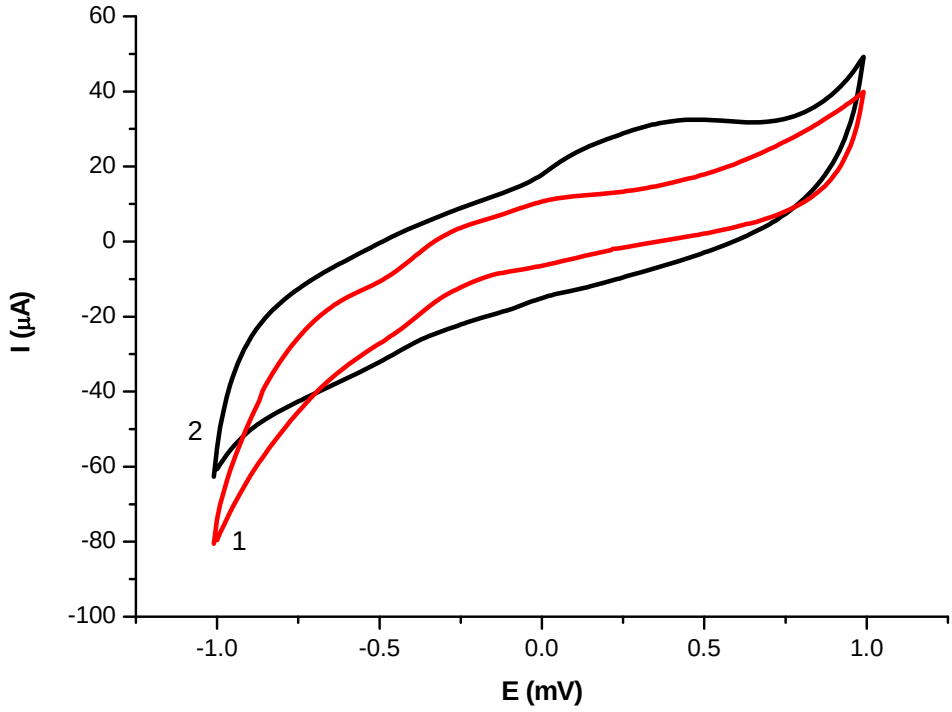


Figure 20. Cyclic voltammogramms (1) bare graphite electrode (2) after electrochemical polymerization of $SNSNO_2$.

Experimental conditions: 100 scan $SNSNO_2$, potassium phosphate buffer pH 6.5, 50 mM

Electrochemical polymerization was carried out by cycling the potential. Both the rate of growth and the quality of the conducting polymer films produced affected from the electropolymerization time [131]. The total charges involved in the film formation and the scan numbers were measured after 5, 10, 15, and 20 min of electropolymerization, as stated in Table 7.

Table 7. Relation between electropolymerization times, number of scan and deposited charges.

Electropolymerization Time (min)	*Number of Scan (cycles)*	*Deposited Charges (C)*
5	50	$2.75x10^{-4}$
10	100	$4.92x10^{-4}$
15	150	$5.21x10^{-4}$
20	200	$2.16x10^{-3}$

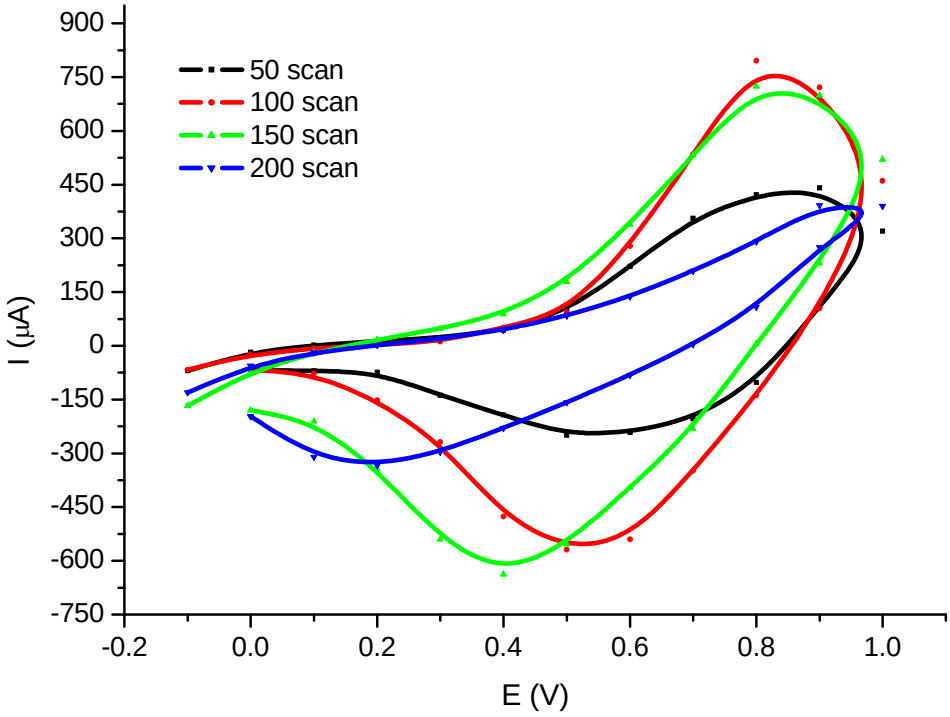


Figure 21. Cyclic voltammograms $SNSNO_2$ with different number of scans

As shown in Figure 21, 22 and Table 8 maximum activities were obtained when the working electrode was coated using $4.92x10^{-4}$ Coulombs. For both *poly($SNSNO_2$)/G. oxydans* and *poly($SNSNO_2$)/P. Fluorescens* biosensors the most convenient thickness was obtained with 10 min electropolymerization time. After 10 min, incompact matrix structure develops and causes lower current signals [119].

Table 8. Effect of number of scans on biosensing response for *poly($SNSNO_2$)/G. oxydans* and *poly($SNSNO_2$)/P. Fluorescens biosensors*

Number of Scans (cycles)	***Biosensing Response ($\mu A/cm^2$)***	***Biosensing Response (%)***
50	0.70	38.77
100	1.80	100.00
150	0.72	38.92
200	0.30	16.90

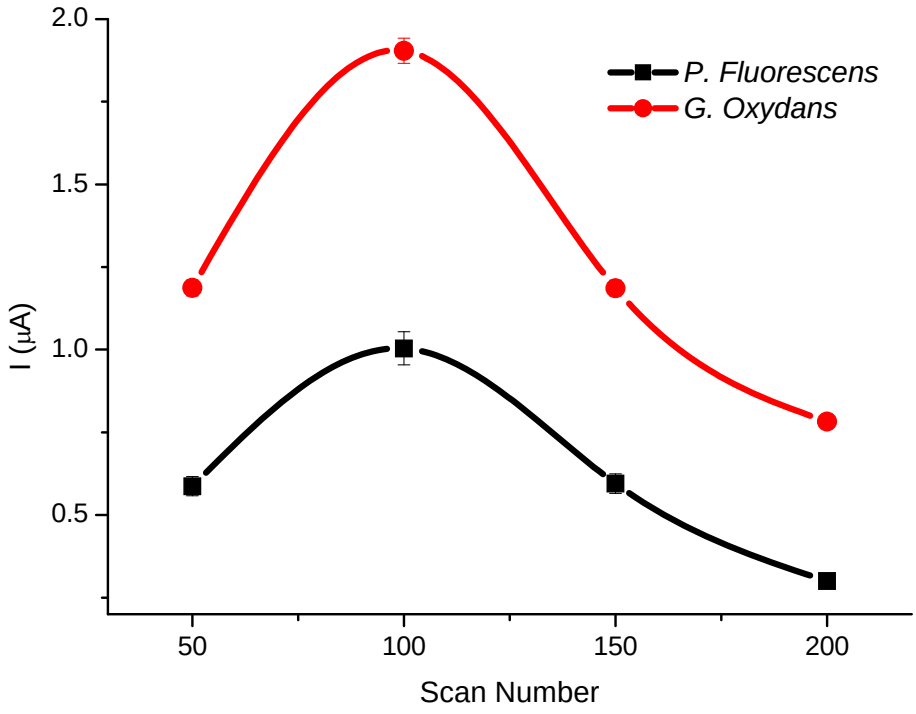


Figure 22. Effect of number of scan on biosensing responses for *poly(SNSNO$_2$)/G. oxydans* and *poly(SNSNO$_2$)/P. Fluorescens* biosensors

Experimental conditions: 50mM pH 7 potassium phosphate buffer, -0.7 V, 0.5 mM glucose.

3.2.3 Effect of pH on biosensing response for *poly(SNSNO$_2$)/G. oxydans* and *poly(SNSNO$_2$)/P. Fluorescens* biosensors

The effect of pH on the electrode response examined for glucose in 50 mM potassium phosphate buffer pH values between 6.5–8.0. For *G. oxydans* pH 7.0 and *P. fluorescens* pH 7.5 was chosen as optimum pH values because maximum biosensor responses were observed at these pHs. Further studies were carried out with these values (Figure 23) [119].

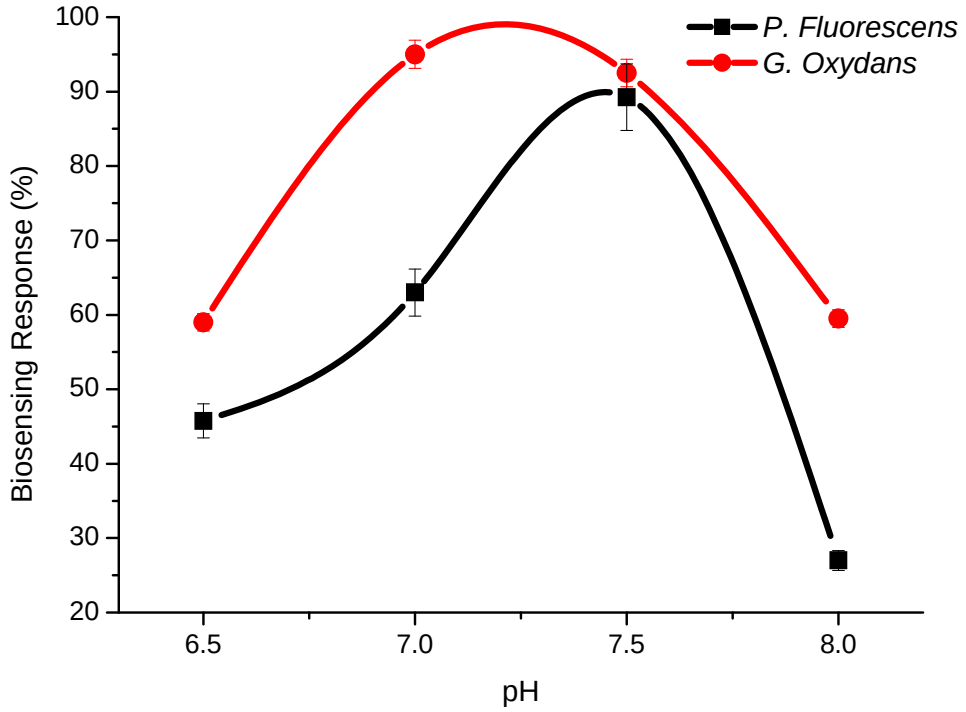


Figure 23. Effect of pH on biosensing response for *poly(SNSNO$_2$)/G. oxydans* and *poly(SNSNO$_2$)/P. Fluorescens biosensors*

Experimental conditions: 100 scan $SNSNO_2$, 6.5-8.0: Potassium phosphate buffer (50 mM), -0.7 V, 0.5 mM glucose

3.2.4 Analytical characterization of *poly(SNSNO$_2$)* and *Gluconobacter Oxydans and Pseudomonans Fluorescens* based biosensors

The microbial sensors were analytical characterized under optimized conditions using glucose as the substrate in 50 mM potassium phosphate buffer pH 7.0 for *G. oxydans* and pH 7.5 for *P. fluorescens*. Calibration curves were obtained from current density versus substrate concentration graphs.

For the *G. oxydans* based biosensor, a linear relationship was observed between 0.25 and 4.0 mM glucose with the equation y=0.359x+0.420 (R^2=0.985). *P. fluorescens* sensor exhibited a narrow linearity between 0.2-1.0 mM glucose with an equation y=3.241x-0.251 (R^2=0.992). For both systems, current signals remained constant at higher concentrations of glucose, showing that the systems reached to saturation [119].

The repeatability of the microbial sensors was estimated with 0.8mM glucose for both biosensors (n=5). The standard deviations and variation coefficients were calculated as 0.815±0.034 and 4.2 % for *G. oxydans* based and 0.539±0.006 and 1.0 % for *P. fluorescens* based biosensors [119].

In order to examine the operational stability of both microbial biosensors, electrodes were immersed in the reaction cell containing the buffer solution at the optimized conditions. After 5 hours *G. oxydans* based system lost only 6.0 % of its activity whereas *P. fluorescens* sensor lost only 3.0 % of its activity after 3.5 hours [119].

Substrate specificity of the proposed microbial biosensors under optimized conditions to various substrates such as mannose, galactose, xylose, methanol and ethanol were checked (Figure 24). *G. oxydans* based biosensor showed noticeable response only towards ethanol. Therefore, *G. oxydans* based sensor was calibrated against ethanol. In the range of 0.1 mM-5.0 mM given by the equation y = 0.163x +0.425 (R^2=0.978). Since the system has no response to methanol, it could be promising to make selective ethanol testing in the presence of methanol. Also, the microbial biosensor may be used for glucose and ethanol analyses in the same sample after chromatographic separation. No responses were detected for mannose, methanol for *G. oxydans* based biosensor whereas *P. fluorescens* based biosensor revealed response towards galactose and xylose [119].

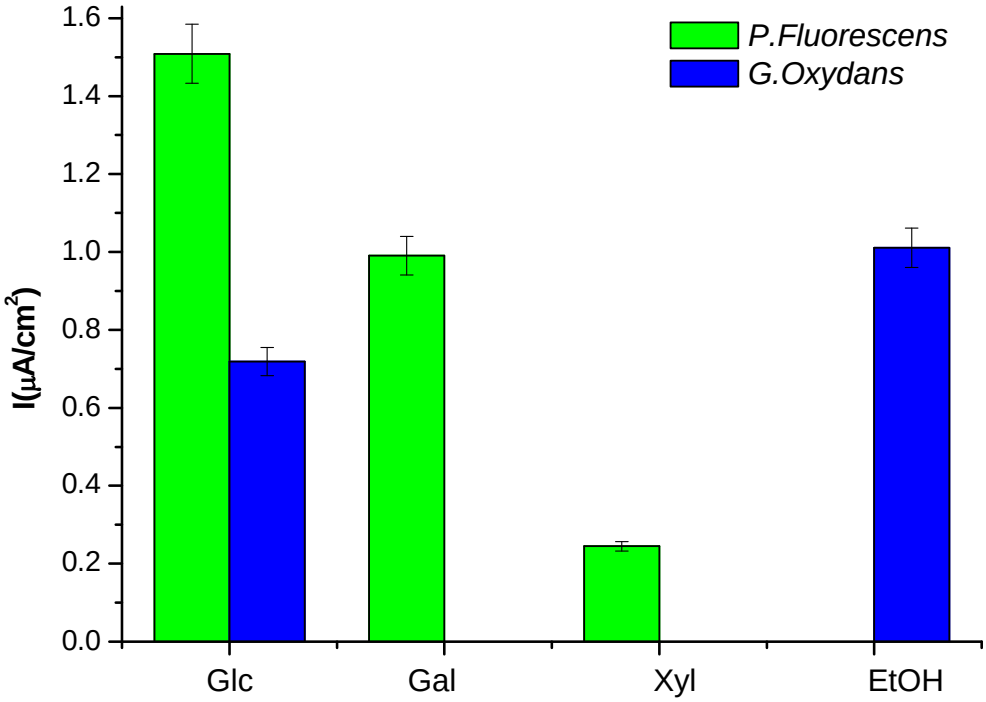
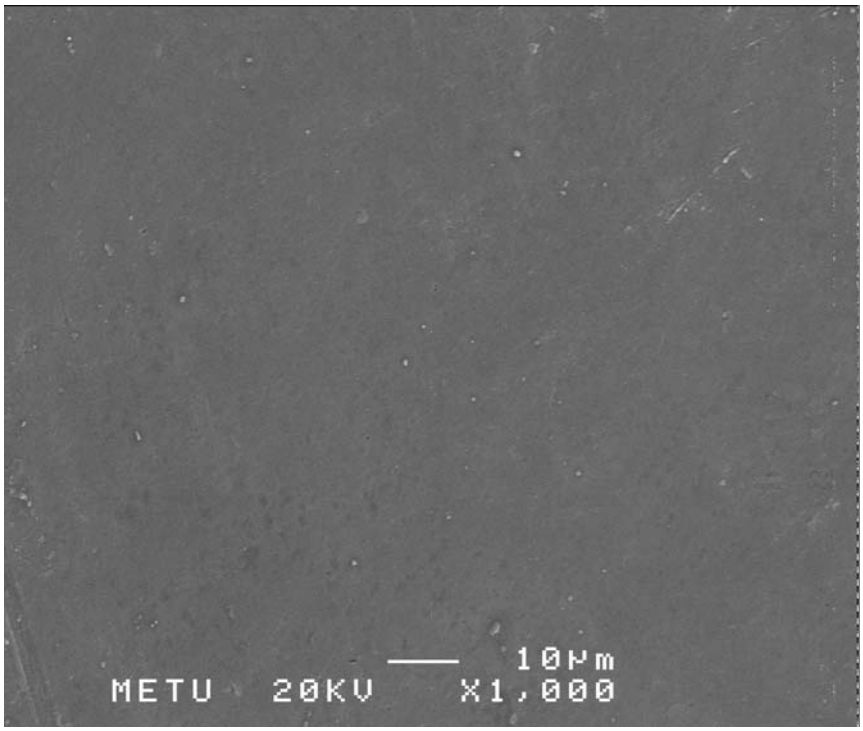


Figure 24.Substrate specificity of the *poly(SNSNO$_2$)/G. oxydans and poly(SNSNO$_2$)/P. fluorescens* biosensors

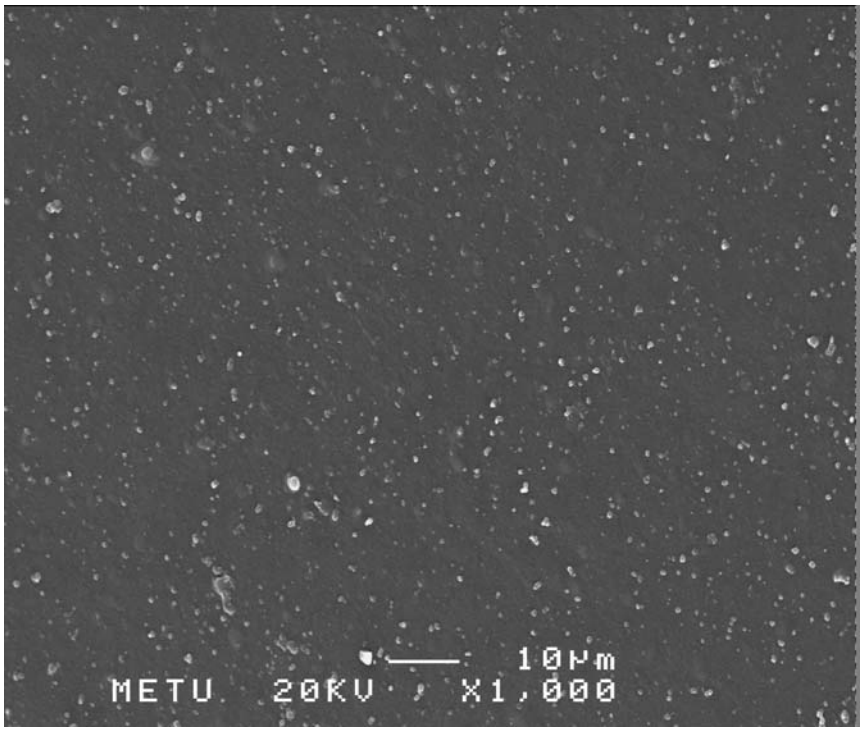
Experimental conditions: potassium phosphate buffer, 50 mM, pH 7.0 for *G. oxydans*; pH 7.5 for *P. fluorescens*, −0.7 V, substrate concentrations: 0.5 mM

3.3 Enzymatic glucose biosensor based on *poly(SNSNH$_2$)*, Glucose oxidase, Gold Nanoparticles

3.3.1 Scanning Microscopy images of the biosensor

a) 

b)



c)

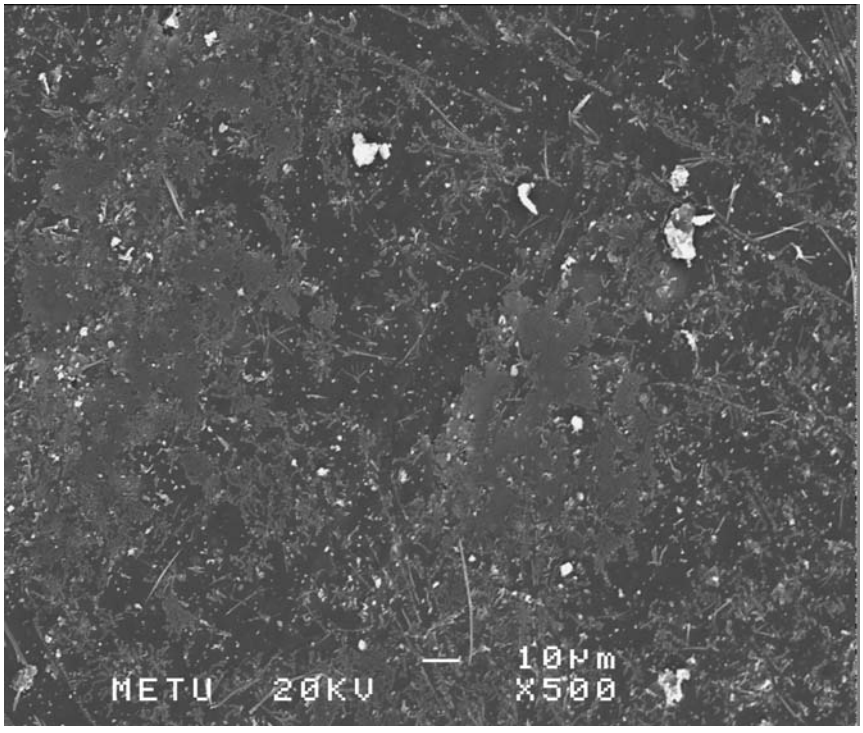


Figure 25. SEM images of a) bare graphite electrode b) *poly(SNSNH$_2$)* c) *poly(SNSNH$_2$),* Glucose oxidase, Gold Nanoparticles

3.3.1 Atomic Force Microscopy image of the biosensor

Atomic force microscopy (AFM) was used for the surface characterization of electrodes as shown in Figure 26 and gold nanoparticles can be easily seen from the AFM.

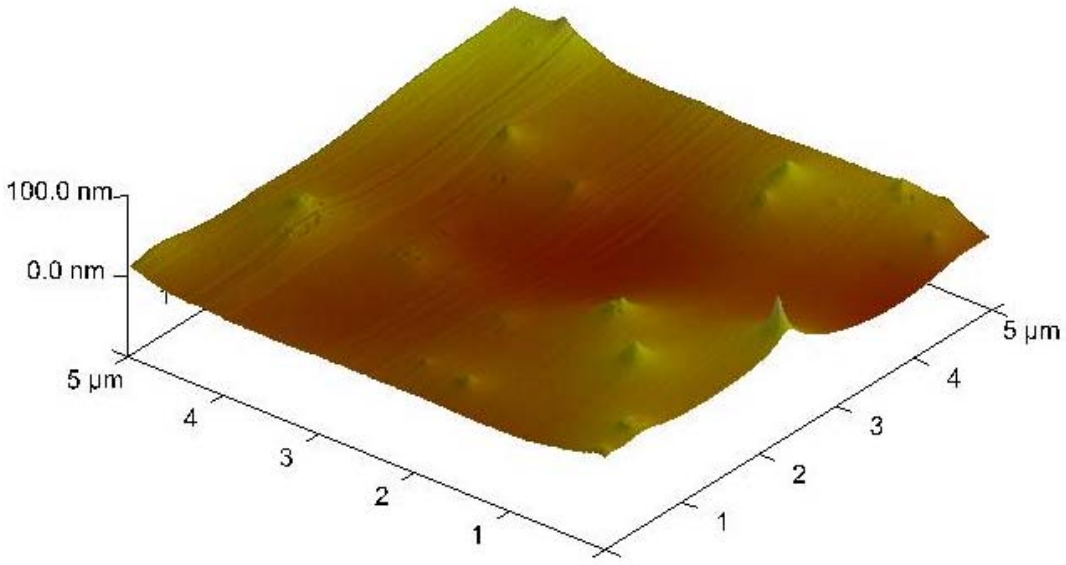


Figure 26. **AFM image of the *poly(SNSNH$_2$)/GOx/AuNP* biosensor**

3.3.2 Optimization of Gold Nanoparticle amount

The amount of nanomaterials on the surface is the most critical parameter that directly affects biocatalytic activity of the enzyme as well as the immobilization yield and the way how to contact with the electroactive surface. Since CP of $SNSNH_2$ has free amino groups, it is easy to achieve protein immobilization.

In order to examine the effect of AuNP amount on the response, different enzyme electrodes including 1.0 %, 0.5 %, 0.1 % and 0.05 % AuNP (in 10 nm size) were constructed and maximum response was obtained when 0.1% AuNP was used. While no effect was observed with 0.05% AuNP, 1.0% AuNP caused the lowest signals. This is an expected result that as the might be due to the improper surface structure which is not allowed to a good communication between the active site of the enzyme and the matrix. On the other hand, inappropriate roughness might cause inefficient immobilization yield that leads poorer response signals. Hence, 0.1% amount of AuNP was chosen as the optimum and used for further experimental steps as shown in Figure 27 and Table 9.

In addition, since a better contact between the biomolecules and the electroactive surface can be provided by AuNP modification in the structure, higher response signals were obtained depending on the NP and amount in compared with non-modified surfaces.

Table 9. Biosensing response of *poly(SNSNH$_2$)/GOx/AuNP* for 2 mM glucose

Au Amount (%)	***Biosensor Response ($\mu A/cm^2$)***	***Biosensor Response (%)***
0	1.19	47.15
0.05	1.13	44.77
0.1	2.52	100
0.5	1.95	77.46
1	0.26	10.30

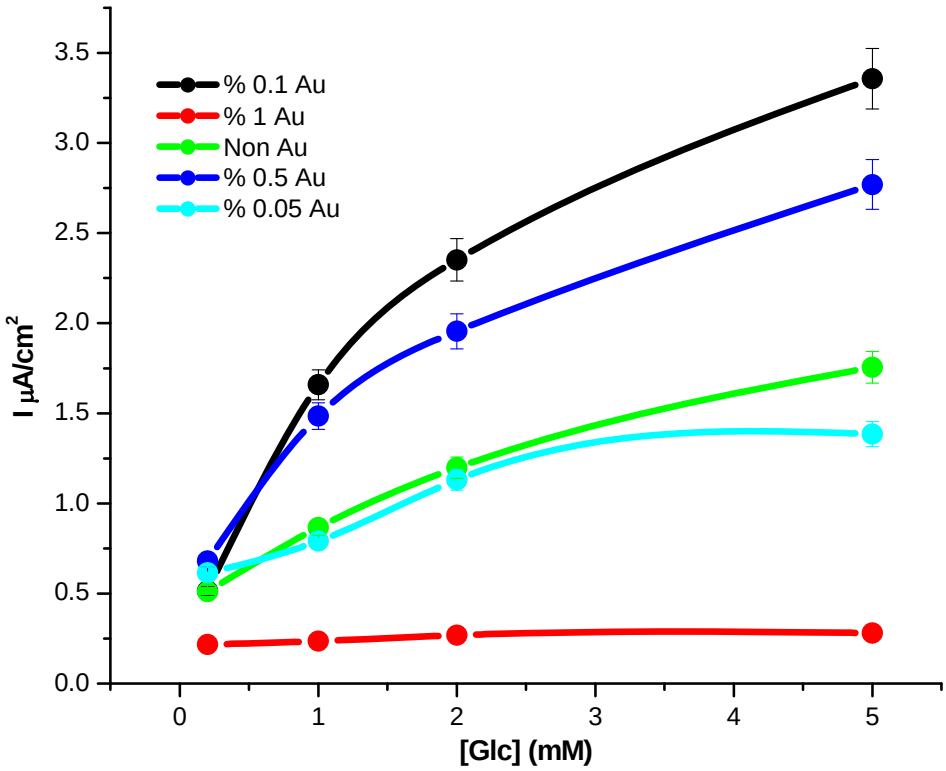


Figure 27. Effect of AuNP amount on the *poly(SNSNH$_2$)/GOx/AuNP* biosensor

Experimental conditions: 1.05×10^{-3} C $SNSNH_2$, 21.2U GOx, pH 5.5 sodium acetate buffer (50 mM),-0.7 V.

3.3.3 Effect of pH on *poly(SNSNH$_2$)*/AuNP/GOx biosensor

The effect of pH on biosensor was determined by adjusting the pH of the potassium phosphate and sodium acetate buffers (50 mM) between 5.0 and 6.5. As shown in Figure 28 and Table 10, the maximum current response was obtained at pH 5.5, therefore optimum pH was assigned as pH 5.5 and it is used for further experiments.

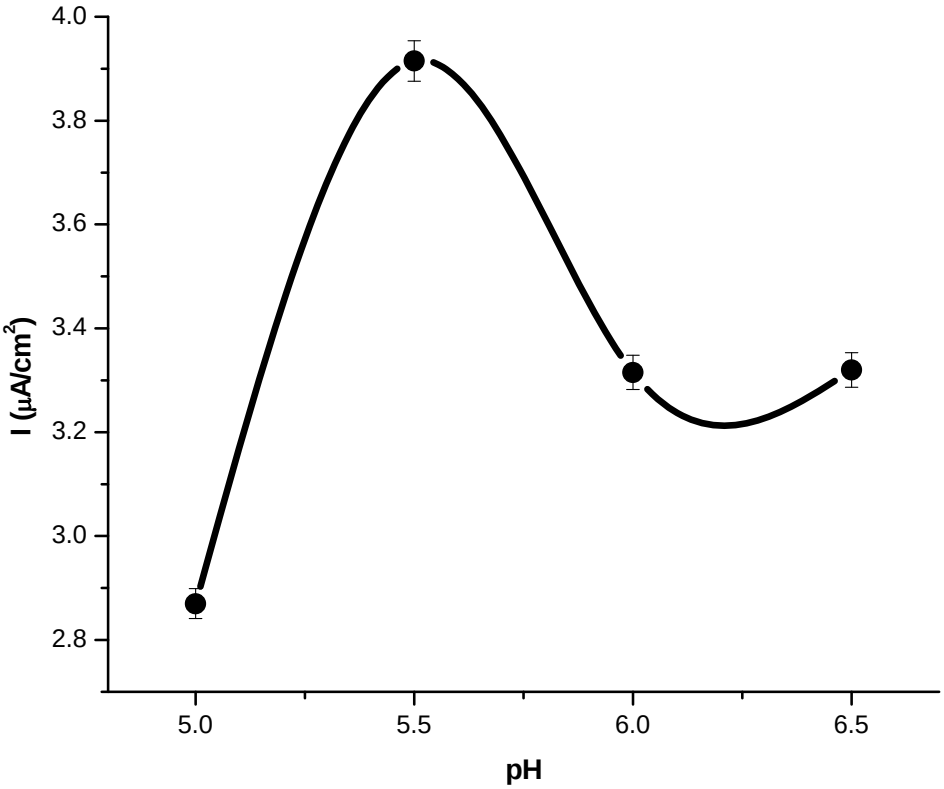


Figure 28. Effect of pH on *poly(SNSNH$_2$)/GOx/AuNP* biosensor

Experimental conditions: 1.05×10−3 C $SNSNH_2$, 0.1% AuNP, 21.2 U GOx, pH 5-5.5 sodium acetate buffer (50 mM), pH 6-6.5 potassium phosphate buffer (50 mM), -0.7 V.

Table 10. pH Effect on biosensor response

pH	*Biosensor Response ($\mu A/cm^2$)*	*Biosensor Response (%)*
5	2.87	73.30
5.5	3.91	100
6	3.32	84.80
6.5	3.31	84.67

3.3.4. Effect of enzyme loading on *poly(SNSNH$_2$)*/AuNP/GOx biosensor

To determine the appropriate GOx amount, different biosensors containing 0.5, 1.0 and 2.0 mg enzymes which equal 10.1, 21.2 and 42.4 U were prepared. Maximum response was obtained by 1mg of GOx. When the amount of GOx increased as twice, lower signals than that of 1 mg was obtained since high density of biological material on electrode causes diffusion problems and therefore lower current responses obtained. Therefore 1 mg enzyme loading with 21.2 U was used for the further experiments. (Figure 29 and Table 11).

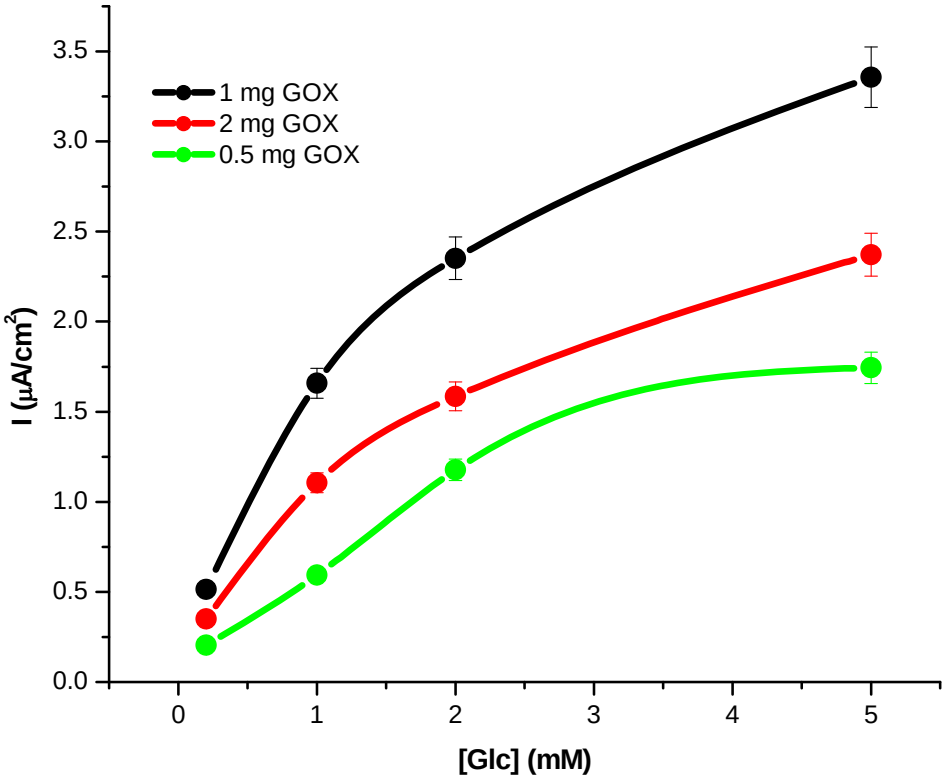


Figure 29. Enzyme loading on the *poly(SNSNH$_2$)/GOx/AuNP* biosensor

Experimental conditions: 1.05×10^{-3} C $SNSNH_2$, 0.1 %, 10 nm AuNP, 21.2 U GOx, pH 5.5 sodium acetate buffer (50 mM), -0.7 V

Table 11. Effect of enzyme amount on *poly(SNSNH$_2$)*/GOx/AuNP *with* 2mM glucose

Enzyme amount (mg)	*Biosensor Response ($\mu A/cm^2$)*	*Biosensor Response (%)*
0.5	1.18	50.06
1	2.35	100
2	1.59	67.39

3.3.5 Analytical Characterization of *poly(SNSNH$_2$)*/AuNP/GOx biosensor

Linear dynamic ranges and the equations were obtained to characterize the proposed *poly(SNSNH$_2$)*/AuNP/GOx biosensor analytically at the optimized electrode configuration and conditions (in sodium acetate buffer, 50 mM, pH 5.5). The linear relation was observed using glucose as the in the range of 0.005-0.2 mM and defined by the equation of y=1.597x+0.264 (R^2= 0.993) (Figure 30).

Repeatability of the *poly(SNSNH$_2$)*/AuNP/GOx biosensor was tested for 0.125 mM glucose (n= 6) and standard deviation (S.D) and coefficient of variation (CV) were calculated as ±0.0164 and 7.0 %.

The stability of the designed biosensor is usually the deciding factor in the determination of the lifetime of enzyme-based biosensors. Stability of the biosensor was tested for 6 hours at operational conditions and it is found that the lost its 16 % activity upon 25 use.

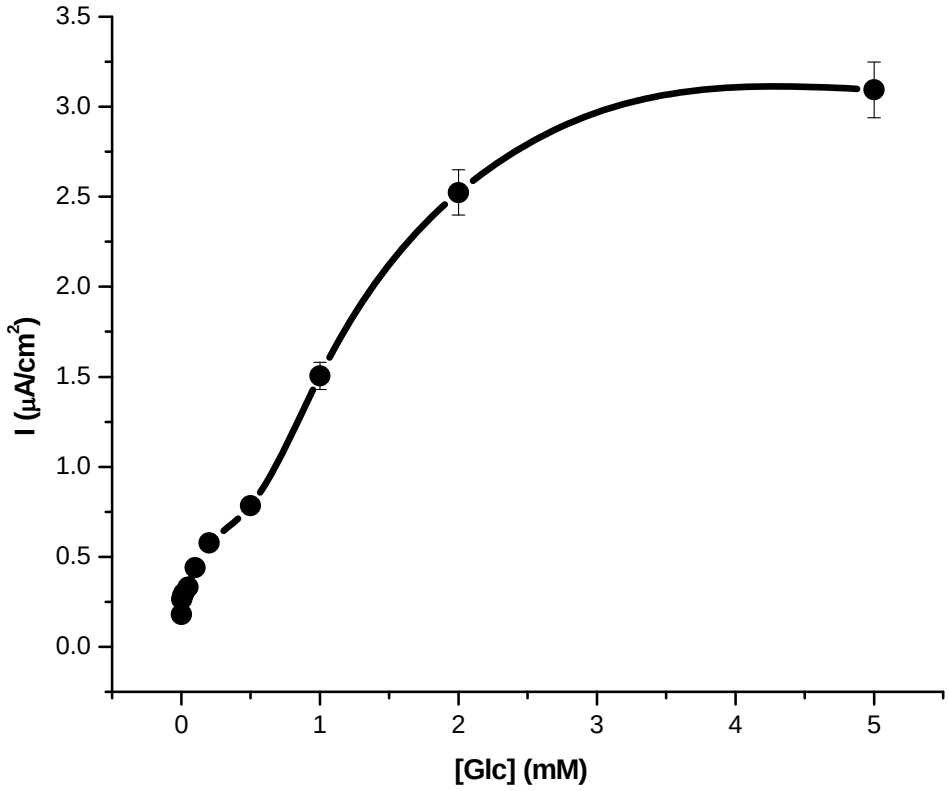


Figure 30. Calibration graph for *poly(SNSNH$_2$)/GOx/AuNP* biosensor

Experimental conditions: 1.05×10^{-3} C $SNSNH_2$, 0.1 %, 10 nm AuNP, 21.2 U GOx, pH 5.5 sodium acetate buffer (50 mM), -0.7 V.

3.3.6 Glucose detection in real samples with the designed biosensor

The glucose amount in brand C was determined as 16.185 g/L ±0.262 by the biosensor, and calculated as 13.975 g/L ±0.86 by the spectrophotometric method. The glucose amount in brand F was determined as 11.95 g/L ± 1.343 by the biosensor, and calculated as 11.42 g/L ± 0.72 by the spectrophotometric method. As can be seen from the data, similar results were obtained for both methods (Table 12).

Table 12. Glucose biosensing in two fruit juices with *poly(SNSNH$_2$)/GOx/AuNP*

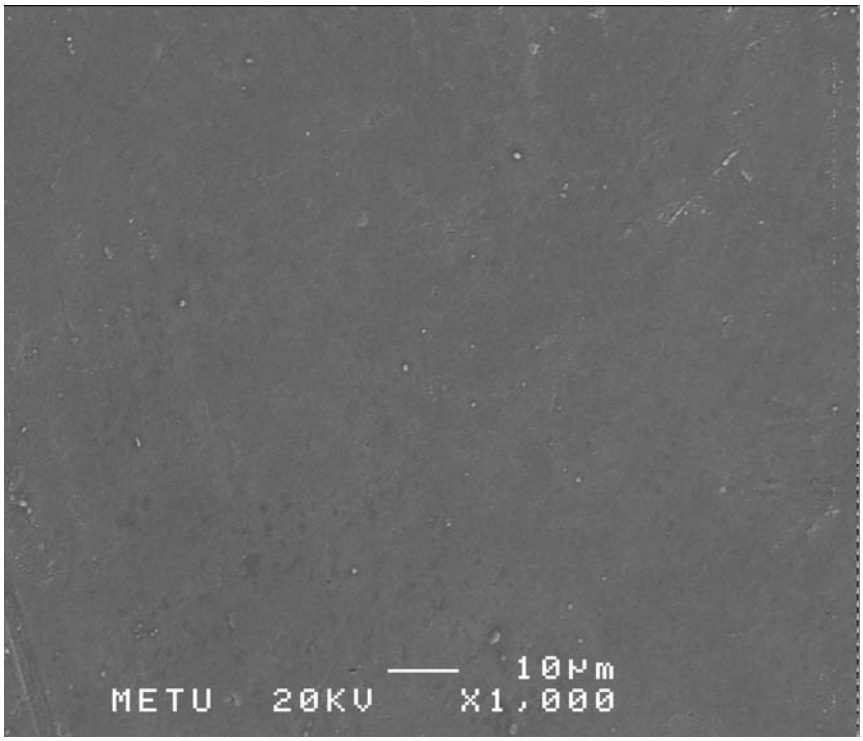
	Biosensor	***Spectrophotometric method***
Brand C	16.185 g/L ±0.262	13.975 g/L ±0.86
Brand F	11.95 g/L ± 1.343	11.42 g/L ± 0.72

3.4 Enzymatic glucose biosensor based on *poly(SNSNH$_2$)*, Carbon nanotubes and Glucose oxidase

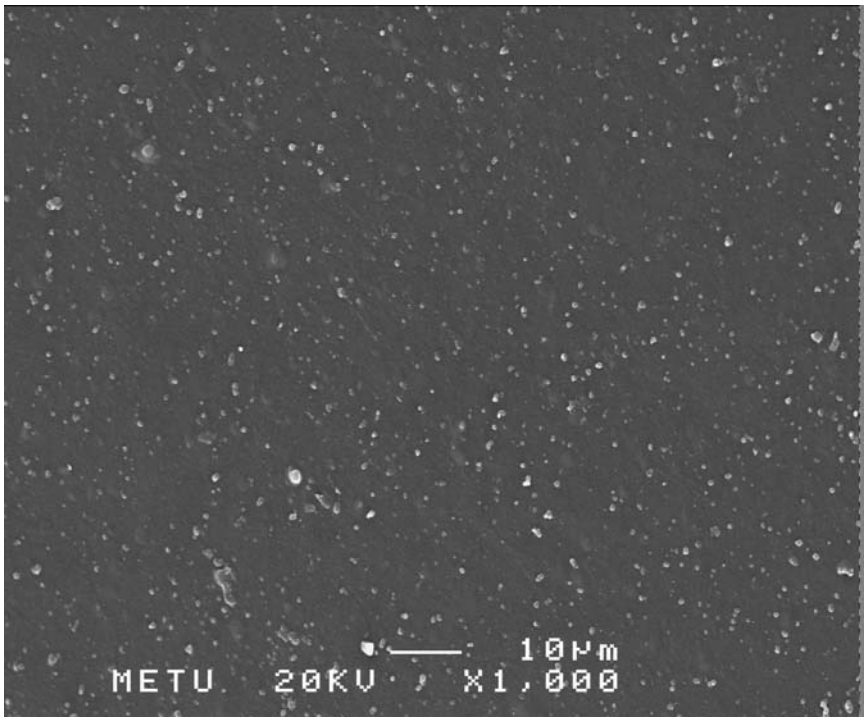
3.4.1 Scanning electron microscopy images

Scanning electron microscopy (SEM) was used for the surface characterization of electrodes and SEM image revealing the morphology of *poly(SNSNH$_2$)*/ GOx/CNT is given in Figure 31. It can be clearly seen that CNT modification was achieved.

a)



b)



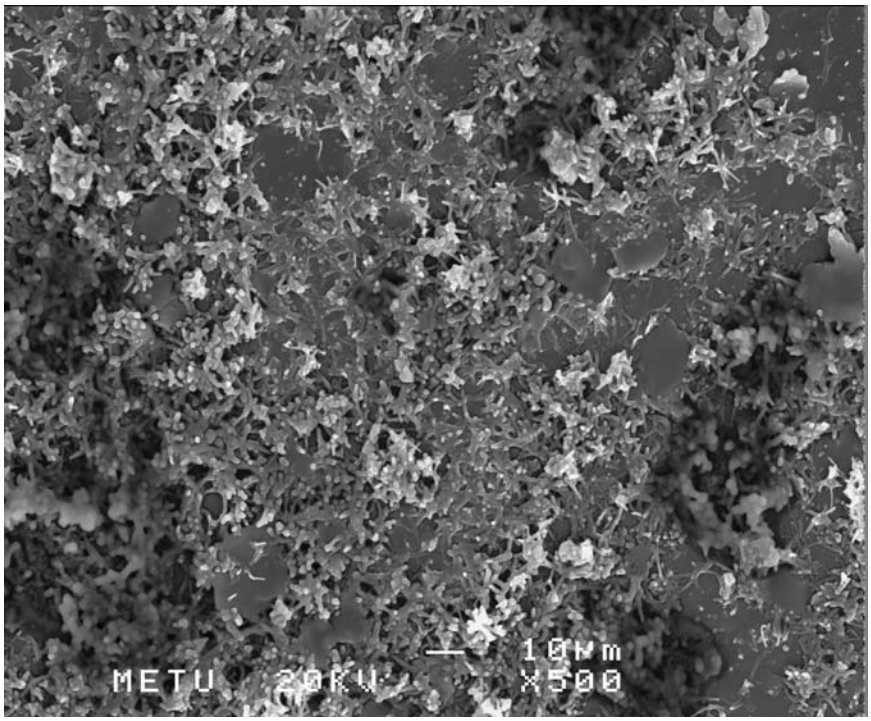
c) 

Figure 31. SEM image of a) bare graphite b) *poly(SNSNH$_2$)* c) *poly(SNSNH$_2$)*/GOx/CNT biosensor

3.4.2 Effect of the way of modification glucose biosensor with carbon nanotubes

In order to examine the effect of different strategies to modify the surface with CNT, four different GOx/conducting polymer of $SNSNH_2$ based electrodes were prepared. Biosensing responses towards 0.2 mM and 0.5 mM glucose were compared with each other as well as with the non-modified *poly(SNSNH$_2$)*/GOx electrode (Figure 32). According to the data, ethanol seems to be more efficient dispersing agent; providing the most suitable surface since maximum sensor performance was achieved by *poly(SNSNH$_2$)*/GOx/CNT-1. Hence, the other three methods were excluded from further investigation.

Moreover, compare to the lowest response for the non-modified electrode, the response of *poly($SNSNH_2$)*/GOx/CNT-1electrode was greatly enhanced. Similar observations were obtained in a previous work where the immobilization of horseradish peroxidase onto electropolymerized polyaniline films doped with CNTs. It was reported that the large response current was not only due to the presence of CNTs, but also the synergistic effect because of the efficient interaction of conducting polymer with the nanostructures. This facilitates the charge transfer and increases the overall conductivity and thus, improved the electrode performance [132].

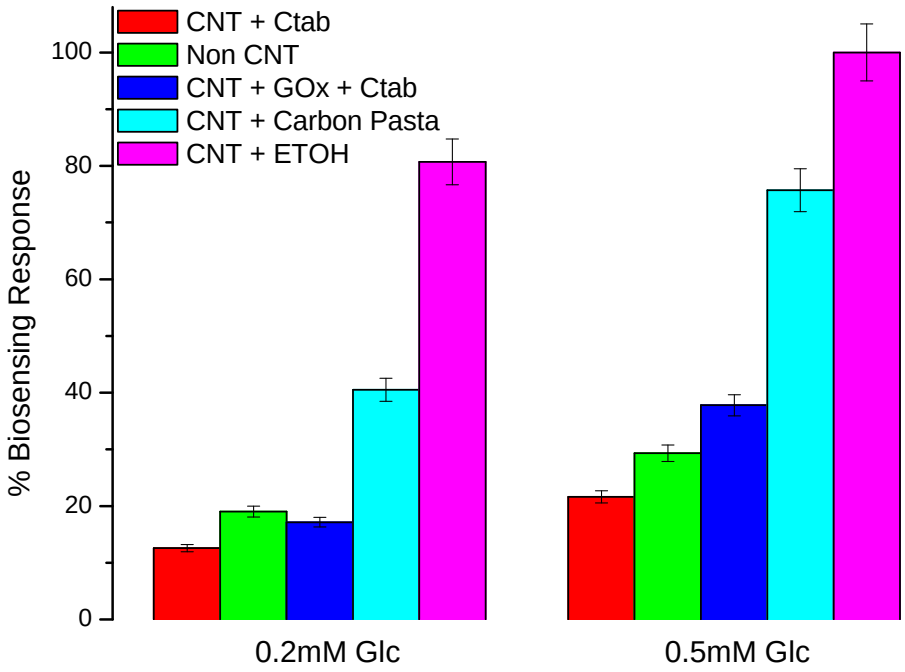


Figure 32. Comparison of biosensing response towards 0.2 mM and 0.5 mM Glc for CNT modified *poly($SNSNH_2$)/GOx* biosensors

Experimental conditions: 1.05×10^{-3} C $SNSNH_2$, 21.2 U GOx, 50 mM pH 5.5 sodium acetate buffer, applied potential –0.7 V.

3.4.3 Effect of carbon nanotubes amount on glucose biosensing

Since CNTs have a large surface area and high surface energy, these structures can strongly adsorb enzyme molecules [15]. However, it is clear that to obtain higher biosensor performance, CNT amount should be properly adjusted to avoid the improper matrix structure for the biomolecules immobilization as well as to provide high stability.

The response of the biosensor is greatly affected by the amount of CNTs on the electrode surface. This can be managed by controlling the CNT amount in ethanol. *poly($SNSNH_2$)*/GOx/CNT biosensors prepared from electrodes modified with different amounts of CNT dispersion (5, 10, 15 µL) were used to detect glucose, and the results were shown in Figure 33 and Table 13.

When the highest CNT amount (15 µL) was used, improper matrix formation may occur by blocking the functional groups on the matrix causing the entrapment of low amounts of enzyme. Furthermore, the active side of the enzyme may not be available because of the improper orientation of biomolecules due to aggregation on the electrode. Hence, lower responses (compare to the one with 10 µL and almost same responses with 5 µL) were obtained. Thus, further experiments were performed with the electrode containing 10 µL CNT dispersion.

Table 13. Effect of CNT amount on *poly($SNSNH_2$)/GOx/CNT* biosensor

Amount of CNT (µL)	***Biosensor Response ($\mu A/cm^2$)***	***Biosensor Response (%)***
5	10.22	72.59
10	14.08	100
15	9.67	68.69

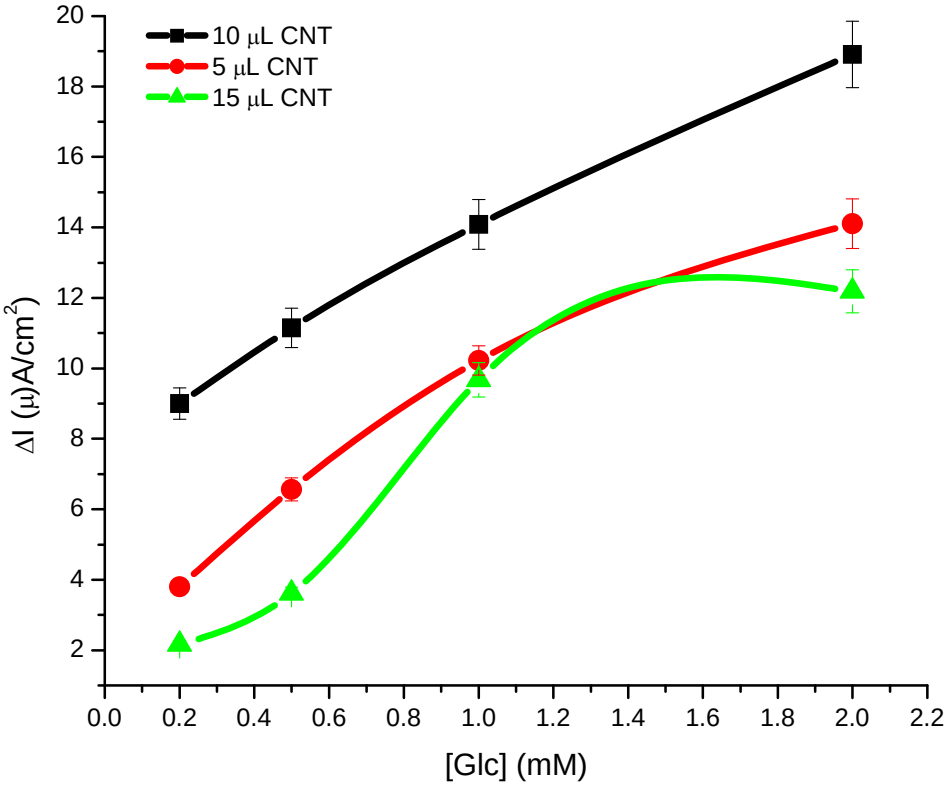


Figure 33. Optimization of CNT amount in *poly(SNSNH$_2$)/GOx/CNT* biosensor

Experimental conditions: 1.05×10^{-3} C $SNSNH_2$, 21.2 U GOx, 50 mM pH 5.5 sodium acetate buffer, applied potential –0.7 V.

3.4.4 Effect of pH on *poly(SNSNH$_2$)*/CNT/GOx biosensor

As shown in Figure 34 and Table 14, the maximum biosensing current response was obtained at pH 5.5. Further experiments were conducted at this optimum pH value.

Table 14. Effect of pH on *poly(SNSNH$_2$)/GOx/CNT* biosensor

pH	*Biosensor Response ($\mu A/cm^2$)*	*Biosensor Response (%)*
4	5.93	72.90
4.5	6.02	74.10
5	7.42	91.40
5.5	8.12	100.00
6	6.87	84.60

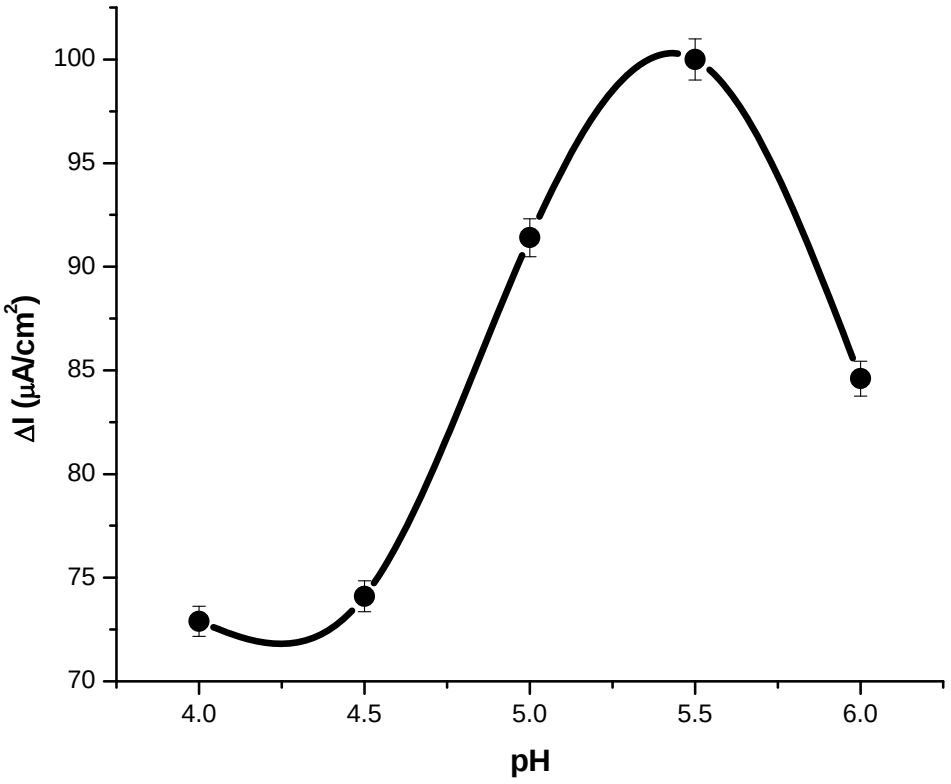


Figure 34. **Effect of pH on *poly(SNSNH$_2$)/GOx/CNT* biosensor**

Experimental conditions: 1.05×10^{-3} C $SNSNH_2$, 10 µL CNT, 21.2 U GOx, sodium acetate buffer (50 mM; pH 4- 5.5) and potassium phosphate buffer (50 mM, pH 6), 1 mM glucose, applied potential –0.7 V.

3.4.5 Analytical Characterization

Analytical characterization of the biosensor was achieved by obtaining linear dynamic ranges and the equations at the optimized electrode configuration and conditions (in sodium acetate buffer, 50 mM, pH 5.5). The linear relation was observed in the range of 0.1-2.0 mM glucose and defined by the equation; $y = 8.582x + 2.945$ ($R^2 = 0.994$).

Repeatability of the *poly(SNSNH$_2$)*/GOx/CNT biosensor was tested for 1 mM glucose (n= 7). The standard deviation and coefficient of variation were calculated as ±0.052 mM and 4.0 %, respectively.

Stability of a biosensor is usually the deciding factor in the determination of the lifetime of enzyme-based biosensors. In order to compare the effect of CNT insertion into the bioactive layer, stability of both *poly(SNSNH$_2$)*/GOx/CNT and *poly(SNSNH$_2$)*/GOx biosensors was tested for 6 hours and 25 measurements were carried out in the presence of 1 mM glucose at operational conditions. While 23% decrease was observed for *poly(SNSNH$_2$)*/GOx, *poly(SNSNH$_2$)*/GOx/CNT was lost only 12% of its activity. This enhanced stability is attributed to the presence of optimized CNT amount.

3.4.6 Glucose analysis in commercial samples

The *poly(SNSNH$_2$)*/GOx/CNT-1 biosensor was used for glucose analysis in various brands of commercial beverages and the data were compared with those obtained from the spectrophotometric method based on Trinder reaction. The comparison of the results obtained from both systems was summarized in Table 15. It is clear that the use of the proposed biosensor provides very similar results obtained with the reference method.

Table 15. Glucose biosensing in two fruit juices with *poly(SNSNH$_2$)/GOx/CNT* biosensor

	Biosensor (g/L)	***Spectrophotometric method (g/L)***
Brand C	15.302 g/L ±0.61	13.975 g/L ±0.86
Brand F	13.095 g/L ±1.19	11.42 g/L ± 0.72

In order to compare AuNP and CNT effect on *poly(SNSNH$_2$)* biosensor, a calibration curve with four data were drawn. It is clearly seen from the Figure 35 CNTs increases biosensing responses much more than AuNPs. When both CNTs and AuNPs were mixed on the surface of *poly(SNSNH$_2$)* electrode prepared a mixed trend was obtained. When graphite electrode was used instead of carbon paste electrode, due to smaller surface area of graphite electrode, lower biosensing responses were obtained (Figure 36).

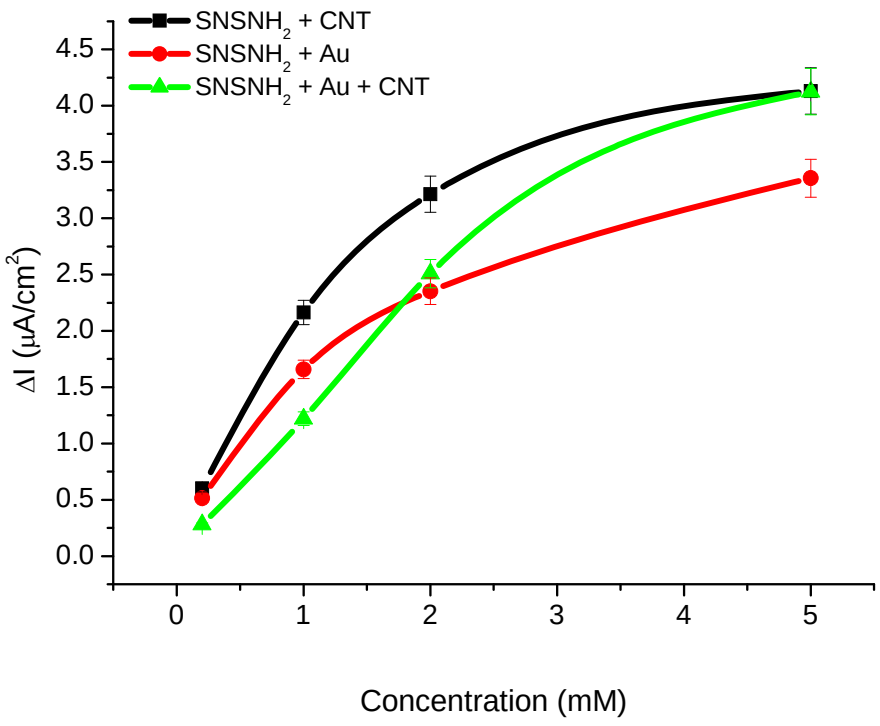


Figure 35. Comparison of AuNPs, CNTs AuNP+CNT modified poly($SNSNH_2$) modified biosensors

Experimental conditions: 1.05×10^{-3} C $SNSNH_2$, 10 µL CNTs, % 0.1 AuNPs, 21.2 U GOx, sodium acetate buffer (50 mM; 5.5) 1 mM glucose, applied potential –0.7 V

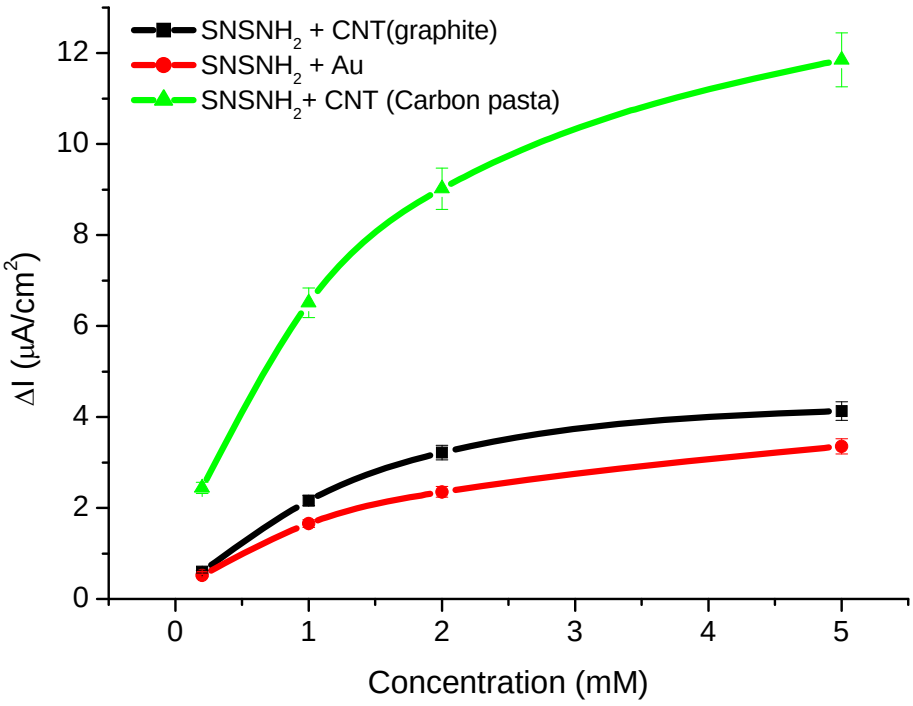


Figure 36. Comparison of AuNPs, CNTs modified poly($SNSNH_2$) modified biosensors

Experimental conditions: 1.05×10^{-3} C $SNSNH_2$, 10 µL CNTs, % 0.1 AuNPs, graphite and carbon paste electrodes, 21.2 U GOx, sodium acetate buffer (50 mM; 5.5) 1 mM glucose, applied potential –0.7 V

3.5 Enzymatic ethanol biosensor based on *poly($SNSNH_2$)*, and Alcohol oxidase

3.5.1 pH optimization

The maximum current response was obtained at pH 7 phosphate buffer as shown in Table 16 and Figure 37. For further experiments, pH 7 phosphate buffer was used as optimum pH.

Table 16 Effect of pH on *poly($SNSNH_2$)/AOx* biosensing response

pH	*Biosensor Response ($\mu A/cm^2$)*	*Biosensor Response (%)*
6	0.31	62.63
6.5	0.37	74.75
7	0.49	100.00
7.5	0.34	68.69

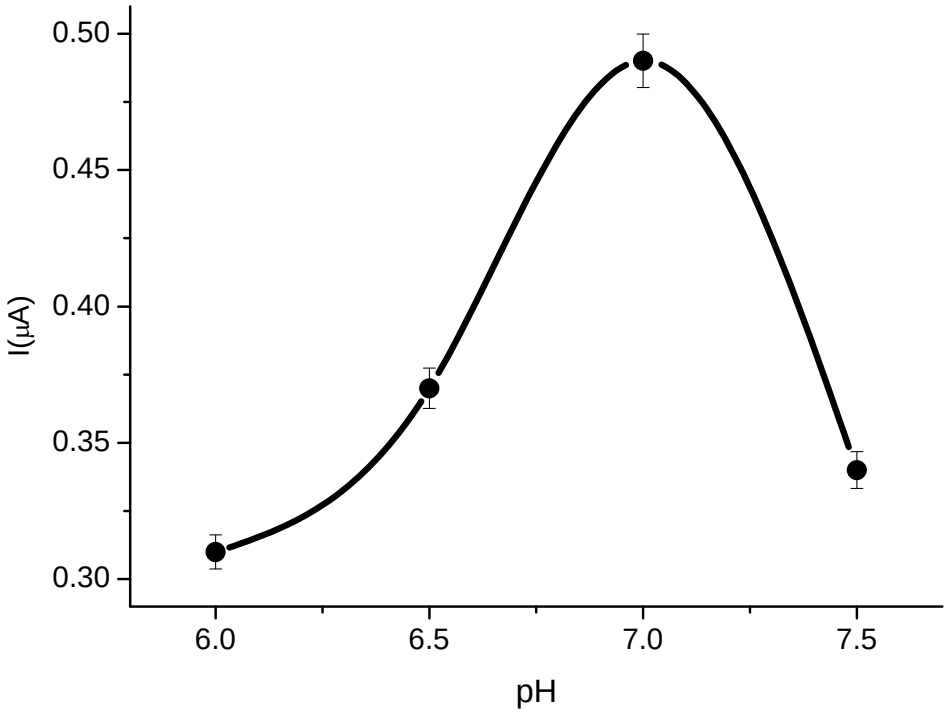


Figure 37. Effect of pH on *poly(SNSNH$_2$)/AOx* biosensor

Experimental conditions: 20 scan $SNSNH_2$, 1 μL AOx, potassium phosphate buffer (1 mM; pH 6-7.5), 1 mM ethanol, -0.7 V.

3.5.2 Effect of polymer thickness on *poly(SNSNH$_2$)/*AOx biosensor

After 15, 20, and 50 scans of $SNSNH_2$ electropolymerization, the biosensor response was checked for 2mM ethanol. Scan numbers and the corresponding biosensor responses were stated in Figure 38 and Table 17. When the platinum electrode surface was coated with 20 scans $SNSNH_2$, optimum biosensor response was obtained. Therefore, 20 number of scans was used in further experiments.

Table 17. Relations between numbers of scans and biosensor responses

Electropolymerization Time (min)	*Number of Scans (cycles)*	*Biosensor Response (µA)*
1.5	15	0.26
2	20	0.50
5	50	0.53

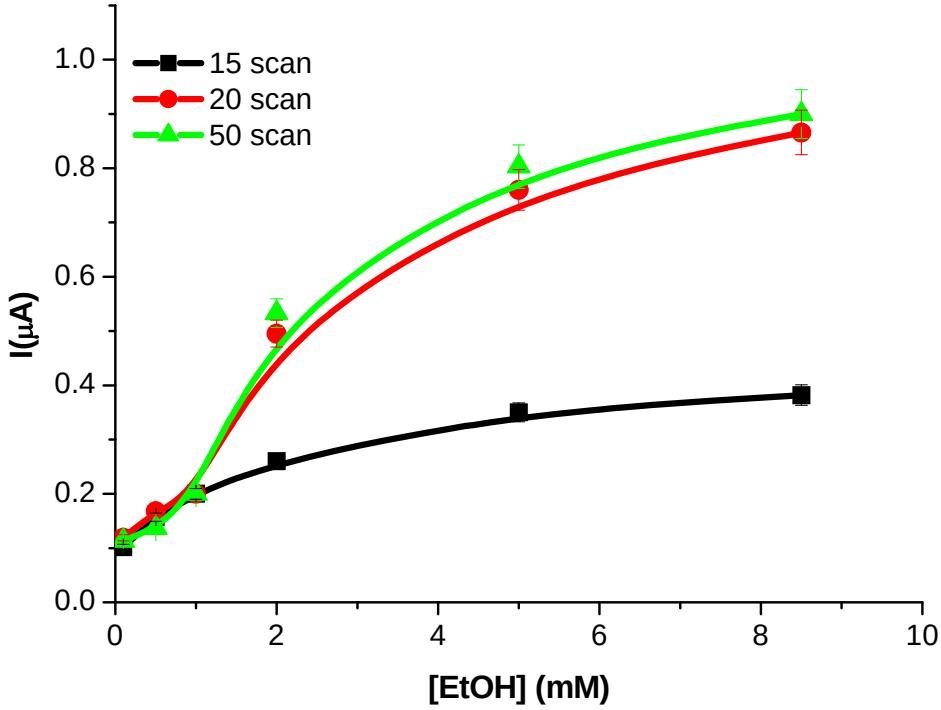


Figure 38. Effect of thickness on *poly(SNSNH$_2$)/AOx* biosensor

Experimental conditions: 1 µL AOx, potassium phosphate buffer (1 mM; pH 7), 1 mM ethanol, -0.7 V.

3.5.3 Effect of enzyme loading on biosensing

Different amount of alcohol oxidase (0.5 µL, 1 µL, 1.5 µL, 2 µL) were mixed with 1 % glutaraldehyde, spread over the *poly($SNSNH_2$)* coated platinum electrode and allowed to dry 1 hour. Each enzyme electrodes were tested for 1mM ethanol. 1.5 µL (1.56 U, 0.055 mg protein) was found as optimum enzyme amount for ethanol biosensing. For further experiments this amount was used for immobilizing alcohol oxidase (Figure 39, Table 18).

Table 18. Effect of enzyme loading on *poly($SNSNH_2$)/AOx* biosensing response

pH	*Biosensor Response ($\mu A/cm^2$)*	*Biosensor Response (%)*
0.5	0.23	27.05
1	0.50	58.23
1.5	0.85	100
2	0.81	95.29

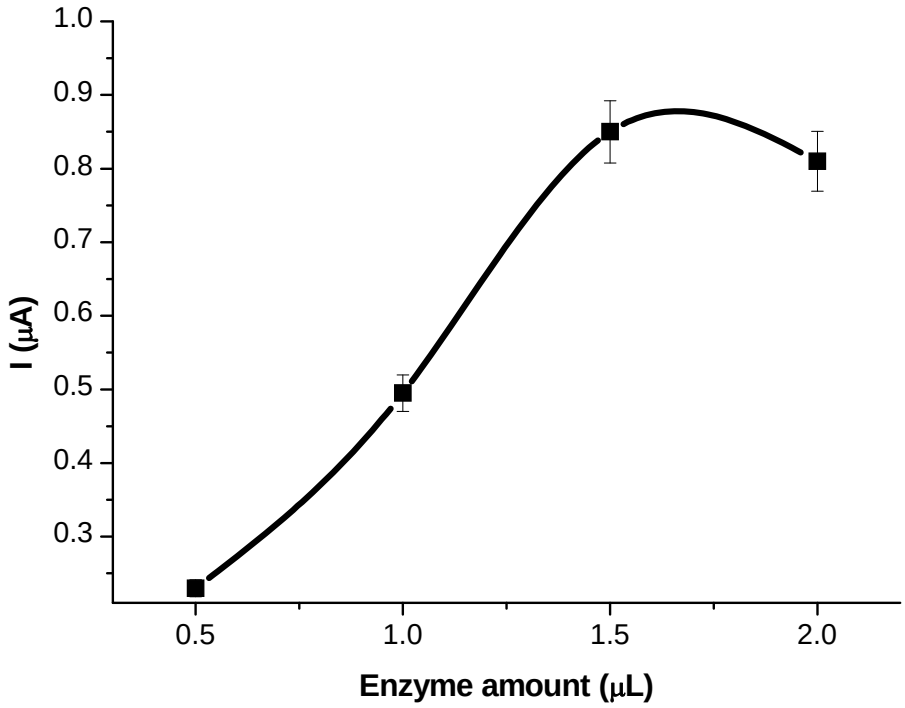


Figure 39. **Effect of enzyme loading on *poly(SNSNH$_2$)/AOx* biosensor**

Experimental conditions: 20 scan $SNSNH_2$, potassium phosphate buffer (1 mM; pH 6-7.5), 1 mM ethanol, -0.7 V.

3.5.4 Analytical characterization

Analytical characterization of the biosensor was performed under optimized conditions; pH 7 phosphate buffer, 20 scans $SNSNH_2$, 1.5 μL alcohol oxidase (1.56 U, 0.055 mg protein). Linear analytical range was obtained between 0.1-5 mM ethanol with the equation y=0.1415x+0.1353 (R^2= 0.959). (Figure 40)

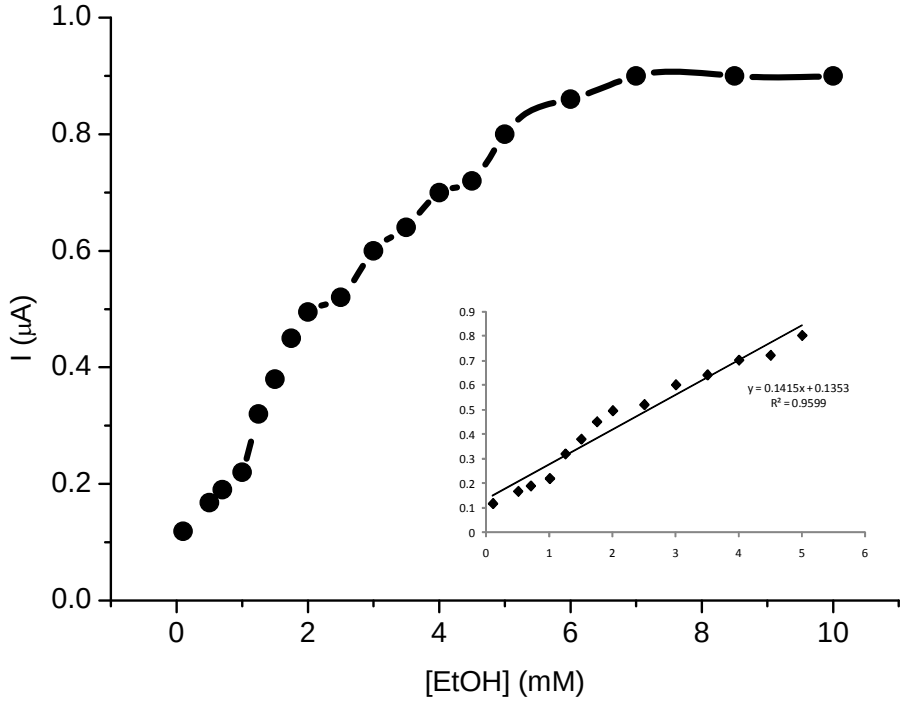


Figure 40. Analytical characterization of *poly(SNSNH$_2$)/AOx* biosensor

Experimental conditions: 20 scans $SNSNH_2$, potassium phosphate buffer (1 mM; pH 6-7.5), 1.5 µL alcohol oxidase (1.56 U, 0.055 mg protein), -0.7 V.

Repeatability of the *poly(SNSNH$_2$)*/AOx biosensor was tested for 2 mM glucose (n= 4). The standard deviation and coefficient of variation were calculated as ±0.015 mM and 3.1 % respectively.

Stability of *poly(SNSNH$_2$)*/AOx biosensor was tested for 6 hours and 25 measurements were carried out in the presence of 2 mM ethanol at operational conditions. *poly(SNSNH$_2$)*/AOx biosensor lost only 7 % of its activity.

Moreover at optimized conditions 2mM ethanol was added to system continuously. Increase in current upon edition of ethanol can be clearly seen from the Figure 41.

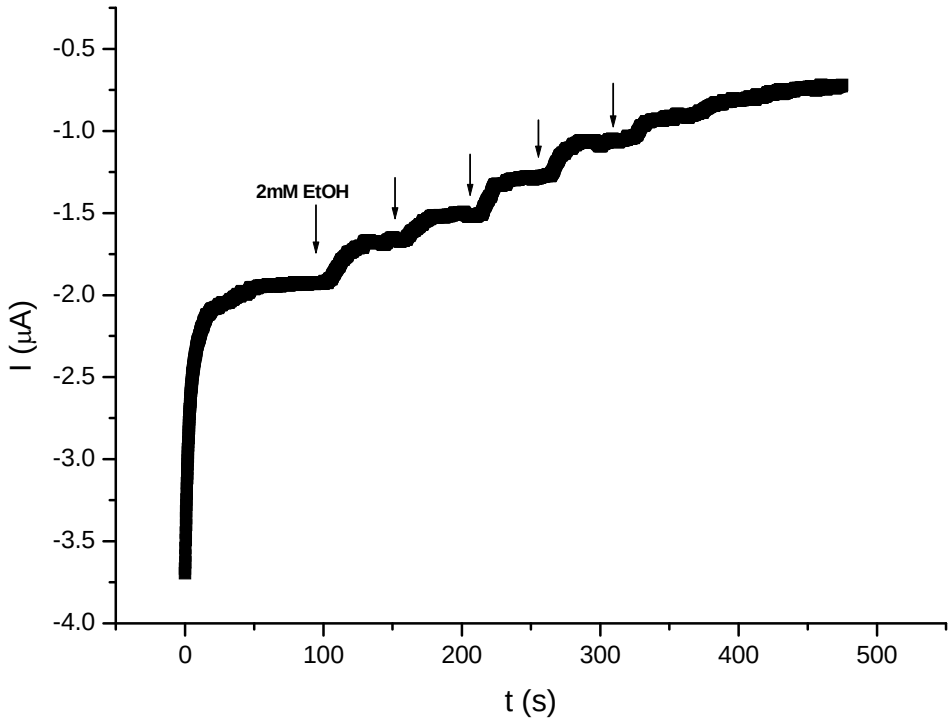


Figure 41. Continuous addition of 2mM Ethanol to *poly(SNSNH$_2$)/AOx* biosensing system

Experimental conditions: 20 scans $SNSNH_2$, potassium phosphate buffer (1 mM; pH 7), 1.5 μL alcohol oxidase (1.56 U, 0.055 mg protein), -0.7 V.

3.5.5 Ethanol analysis in commercial samples

The *poly(SNSNH$_2$)*/AOx biosensor was used for ethanol analysis in vodka and whisky. The data were compared with label values. The comparison of the results obtained from both systems was summarized in Table 19. Clearly, with the proposed biosensor very similar results obtained with label values.

Table 19. Ethanol biosensing in vodka and whisky

	Biosensor (%)	*Label value (%)*
Vodka	39.4 ± 2.69	40
Whisky	41.3 ± 1.80	40

3.5.6 Modification of the *poly($SNSNH_2$)*/AOx biosensor with Gold Nanoparticles

After deposition of 20 scans $SNSNH_2$ by cyclic voltammetry on platinum electrode, with 0.5µL, 1µL, 1.5µL, 2µL gold nanoparticles were added onto the electrode. Biosensing response was checked towards 1 mM and 2mM ethanol. Maximum biosensing response was obtained when 1µL AuNP added to the electrode. (Figure 42).

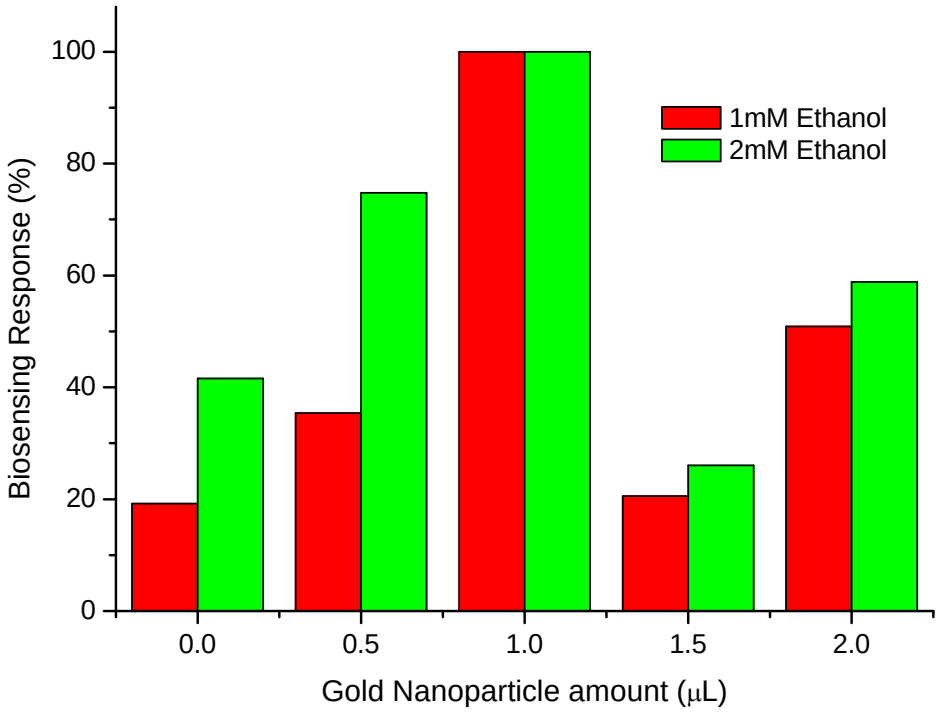


Figure 42. Gold Nanoparticles effect on *poly(SNSNH$_2$)/AOx* ethanol biosensor

Experimental conditions: 20 scans $SNSNH_2$, potassium phosphate buffer (1 mM; pH 7), 1.5 μL alcohol oxidase (1.56 U, 0.055 protein), -0.7 V.

3.5.7 Modification of the *poly(SNSNH$_2$)*/AOx biosensor with Carbon Nanotubes

Ethanol biosensing responses were checked for 1 and 2mM ethanol for CNT modified electrodes. Maximum biosensing response was obtained when 1.5 CNT was added to biosensor. (Figure 43).

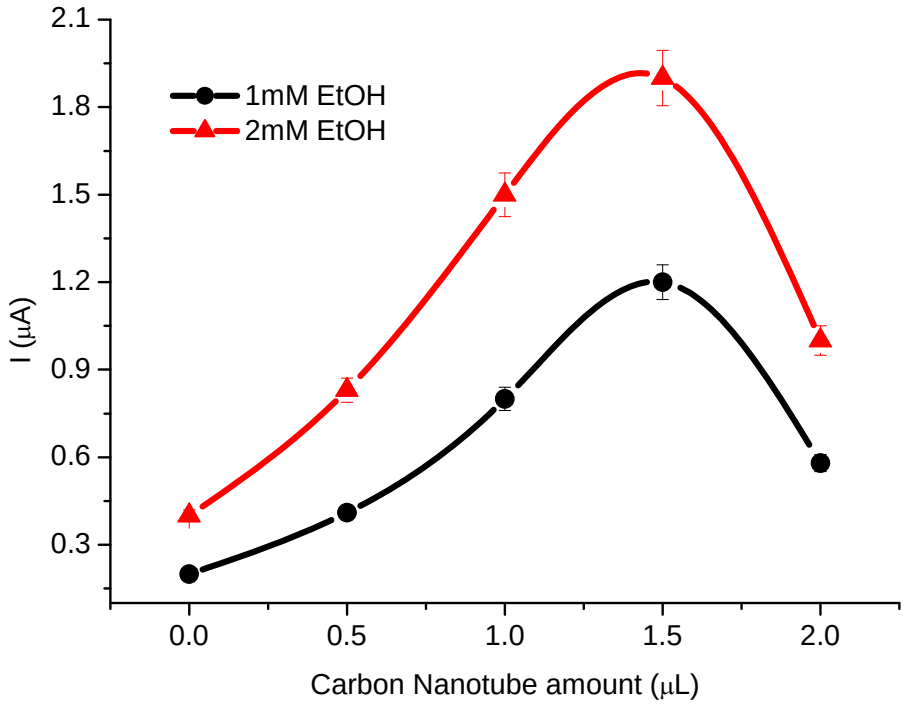


Figure 43. Carbon nanotube effect on *poly(SNSNH$_2$)/AOx* ethanol biosensor

Experimental conditions: 20 scans $SNSNH_2$, potassium phosphate buffer (1 mM; pH 7), 1.5 μL alcohol oxidase (1.56 U, 0.055 protein), -0.7 V.

CHAPTER IV

CONCLUSION

In this thesis, six different biosensors based on conducting polymers of 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine ($SNSNH_2$) and 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H*-pyrrole ($SNSNO_2$) were prepared. Electrochemical technique was used for polymerization of conducting polymers and two different immobilization techniques were used for immobilizing enzyme or microbial, into the conducting polymer matrices. The proposed biosensors were characterized and optimized. Optimum pH, thickness, enzyme amount were determined and linearity, repeatability, operational stability experiments were performed. Carbon nanotubes and gold nanoparticles were also added to the biosensing system to see the effect of nanoparticles. The biosensors also used for ethanol and/or glucose biosensing in commercial samples.

In the first part of thesis a biosensor was designed by immobilizing *Gluconobacter oxydans* in $SNSNH_2$ matrix on graphite electrode. The biosensor preparation method was a two-step procedure where the cells were immobilized by adsorption on the surface after the electropolymerization step. Use of dialysis membrane to cover the surface after immobilization turned out to be a way of conserving the bioactive surface during the operation. The preparation is simple and not time consuming. Besides, systems proposed showed good linearity and repeatability as well as high operational stability. Glucose amount in fruit juice and ethanol amount in vodka and whisky were determined. This work is published in *Sensors and Actuators B: Chemical*.

In the second part of thesis, second biosensor was designed with electrochemical polymerization of 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H*-pyrrole were achieved via cyclic voltammetry on graphite electrode. Afterwards, *Pseudomonas fluorescens* and *Gluconobacter oxydans* were immobilized successfully on the conductive matrix. The proposed biosensor showed a good linear range, and repeatability as well as high operational stability. This work is published in *Bioelectrochemistry*.

In the third part, gold nanoparticle effect was researched on *poly(SNSNH$_2$)*/glucose oxidase biosensor. Covalent binding of glucose oxidase was achieved to SNSNH$_2$ by the help of glutaraldehyde on graphite electrodes. Nanoparticles amount, optimum pH was determined for both biosensors. After analytical characterization, glucose amount in two fruit juices were determined with *poly(SNSNH$_2$)*/GOx/AuNP. *Poly(SNSNH$_2$)*AuNP/GOx biosensor provides easy preparation and it is useful as a biosensor for glucose detection.

Proposed system was designed using AuNP as the appropriate microenvironment to efficiently immobilize the biomolecule on the surface of CP containing functional NH$_2$ groups. It is known that the immobilization approaches require preserving the stability and biological activity of the biomolecules as well as their orientation, distribution, and proximity to the electrode surface and reaction substrate. Due to the unique properties of AuNPs, nanostructured electrodes have been shown to be a versatile tool to construct biosensors that can accomplish such requirements. Especially, advantages in biocompatibility, stability, sensitivity, possibility of electrocatalysis, and easiness of sensor preparation can be claimed thanks to AuNPs. It can be concluded that the combination of CP and AuNP cause the immobilized enzyme to have higher bioactivity which results fast, stable and sensitive responses to the substrate.

In the fourth part, CNTs were added to *poly($SNSNH_2$)*/GOx system. The biosensor system optimized and characterized. Addition of CNT dispersion on the electropolymerized $SNSNH_2$ provided attractive matrix properties with unique and versatile properties for the biomolecule immobilization.

Covalent binding of the redox enzyme onto the CNT modified polymeric material yielded a nanobiocomposite structure. This may serve as a potential candidate for biocatalytic nanoscale-systems based on glucose oxidation reaction in various biotechnological applications and biological analysis.

In the last part, biosensor was designed with immobilizing alcohol oxidase on platinum electrode via crosslinking with glutaraldehyde. The proposed biosensor was characterized and optimized in terms of thickness, enzyme loading, pH, AuNPs, CNTs, linear range, repeatability and operational stability.

Conducting polymers have concerned much attention due to providing suitable matrices for biological materials. Numerous numbers of papers on the advantages of using CPs for novel catalytic surfaces have been published. The use of conducting polymers with particular properties with the immobilized biological systems enables to develop novel biomicroelectronic devices. In future, biosensors based on CPs would be gradually more miniaturized due to the flexibility of electrodeposition with in micro or nano order. Moreover, it can be possible to obtain microbial or enzymatic sensors in required scope with the appropriate immobilization method.

Table 20. *poly(SNSNH$_2$)* and *poly(SNSNO$_2$)* based biosensors designed in this thesis

Matrice	Electrode Type	pH	Linear Range	Linear equation	R^2	Repeatability	Operational stability
poly(SNSNH$_2$)/ G.Oxydans	Graphite	6.5	0.1-2.5mM	y=0.349x+0.406	0.941	for 2.3 mM glucose, (n=4) S.D. ±0.067 mM C.V. 2.9 %	11%decrease after 5h 22 exp.
poly(SNSNH$_2$)/ AuNPs/G.Oxydans	Graphite	6.5	0.05-1.5 mM	y = 1.214x + 0.235	0.995	for 1.1 mM glucose, (n=4) S.D. ±0.064 mM C.V. 5.8 %	9 % decrease after 5h 22 exp.
poly(SNSNO$_2$)/ G.Oxydans	Graphite	7	0.25-4 mM	y=0.359x+ 0.420	0.985	for 0.8 mM glucose, (n=5) S.D. ±0..34mM C.V. 4.2 %	6% decrease after 5h
poly(SNSNO$_2$) /P.Fluorescens	Graphite	7.5	0.2-1mM	y=3.241x-0.251	0.992	for 0.8 mM glucose, (n=5) S.D. ±0.006 mM C.V. 1 %	3% decrease after 3.5h
Poly(SNSNH$_2$)/ AuNP/GOx	Carbon paste	5.5	0.005-0.2mM	y=1.597x+0.264	0.993	for 0.125 mM glucose, (n=6) S.D. ±0.0164 mM C.V. 7 %	16%decrease after 6h 25 exp.
Poly(SNSNH$_2$)/ AOx	Platinum	7	0.1-5 mM	y = 0.141x+0.35	0.959	for 2mM glucose, (n=5) S.D. ±0.015mM C.V. 3.1 %	7 %decrease after 7h 23 exp.

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