A NOVEL CREATININE BIOSENSOR MODIFIED WITH PURE SILK FIBROIN BASED ON PENCIL GRAPHITE ELECTRODE (PGE)

A THESIS SUBMITTED TO THE GRADUATE

SCHOOL OF APPLIED SCIENCES

OF

NEAR EAST UNIVERSITY

By

UBONG LOIS ABIA

In Partial Fulfillment of the Requirements for
the Degree of Master of Science
in
Biomedical Engineering

NICOSIA, 2018

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Approval of Director of Graduate School of Applied Sciences

Prof. Dr. Nadire Çavuş

We certify this thesis is satisfactory for the award of the degree of Masters of Science in Biomedical Engineering

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I hereby declare the thesis titled: A novel creatinine biosensor modified with pure silk fibroin based on pencil graphite electrode (PGE) is an original work and has not been submitted whether in part or in full for a degree. I hereby declare that all the informations in this document has been obtained and presented in accordance with the academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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Signature:

Date:

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To My Parent…

ABSTRACT

Kidney disease is among the top leading causes of death today around the world, disturbances in renal functions cause the creatinine level to rise in the blood, and thus the accurate determination of creatinine level is very important in the diagnosis, monitoring, and evaluation of kidney diseases. Creatinine helps to evaluate the kidney function. In this work, a novel enzyme-based amperometric biosensor has been fabricated based on a pencil graphite electrode for the determination of creatinine by coating the electrode surface with creatininase, sarcosine oxidase enzyme modified with pure silk fibroin prepared from locally sourced cocoons.The buffer used was 5mM phosphate buffer pH 7.0. The effect of time, temperature were also investigated. Optimization study with 100µM of H_2O_2 concentration was carried out and the electrochemical behaviors of the biosensor were investigated using chronoamperometry techniques.

***Keywords*:** Biosensor; Creatinine; Sarcosine oxidase; Hydrogen peroxide; Pencil graphite electrode

ÖZET

Günümüzde böbrek hastalıkları dünyada en çok ölüme sebep olan hastalıklardan biridir, böbrek fonksiyonlarındaki rahatsızlıklar kandaki kreatinin miktarının artmasına sebep oluyor ve bu yüzden böbrek hastalıklarının tanısı, izlemi ve değerlendirilmesi için kreatinin miktarının doğru ölçümü şarttır. Kreatinin böbrek fonksiyonlarını değerlendirmeye yardımcı olur. Bu çalışmada, elektrodun yüzeyini kreatineaz, yerli ipek kozalardan elde edilmiş saf ipek fibroin ile modifiye edilmiş sarkozinoksideaz enzimi ile kaplayarak kreatinin ölçmek için kullanılan bir kalem grafit elektrotlu enzim bazlı ampermetre biyosensör üretilmiştir. Kullanılan tampon 5mM pH 7.0 fosfat tampon. Bu çalışmada, optimum süre ve sıcaklığın etkisi de incelenmiştir. 100µM H_2O_2 ile bir optimizasyon çalışması yapılmıştır ve biyosensörün elektrokimyasal davranışları kronoampermetre ölçüm teknikleri kullanılarak incelenmiştir.

Anahtar kelimeler: Biyosensör; kreatinin; Sarkozin Oksidaz; Hidrojen Peroksit; Kalem grafit elektrodu

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LISTS OF ABBREVIATIONS

µL: microliter

µM: Micromolar

ADP: Adenosine diphosphate

Ag/AgCl: Silver-silver chloride

ATP: Adenosine triphosphate

CA: Creatinine amidohydrolase

CI: Creatine amidinohydrolase

CIH: Creatinine

cm: Centimeter

g: Gram

GAA: Guanido acetic acid

GFR: Glomerular filtration rate

H_2O_2: Hydrogen peroxide

mA: milliamperes

Mins: Minutes

Mm: Millimeter

mM: Millimolar

mV/s: millivolts per second

PBS: Phosphate buffer saline

PGE: Pencil graphite electrode

RE: Reference electrode

Sec: Seconds

SO: Sarcosine oxidase

V: Voltage

WE: Working electrode

CHAPTER ONE
INTRODUCTION

Creatine a chemical made by the body. It is a compound gotten from an amino acid. Creatine supplies muscle tissues its energy, it acts as the source of energy. Creatinine is a dehydrogenated form of creatine and it is usually transported in the blood and filtered into the kidneys. (Anthony and Malcolm, 2000). The kidney then filters out the creatinine and gets rid of it in the urine. Creatinine is an important analyte in clinical diagnosis, it is a chemical waste product of creatine removed entirely from the body in the urine. Creatinine can be formed in the body by an irreversible and spontaneous conversion from creatine to creatine phosphate. It supplies high energy the phosphate bonds use for an ATP reformation during a muscular contraction, around 2% creatine found in the entire body is converted per day to form creatinine (Yadav, 2012). It is crucial to know the creatinine level or concentration in the blood and urine because it reveals the state of renal (kidney) functions, muscular disorders, thyroid malfunction, and the amount of muscle a person has (Chi-Hua et al., 2013).

Creatinine is a component of the human blood, it is a breakdown product of creatine phosphate in muscles. Everyday creatinine is produced in the body at a constant rate, although it relies on muscle mass. Creatinine is transported to the kidneys by the blood, through glomerular filtration and proximal tubular secretion, after which the creatinine is filtered into the urine and passed out. Because of this, the blood and urine creatinine levels are important proofs for the functioning of the kidneys (Yadav, 2012). The creatinine production may increase with increased dietary protein intake but may not be affected by sicknesses or disorders such as sepsis, trauma, or dehydration (Jacobsen et al., 1980). A little amount of creatinine indicates a decrease in muscle mass, and an inflammation of the kidney or certain muscular disorders can increase the creatinine level in the blood. Whilst the normal creatinine concentration ranges from 0.2-0.7 mg/dl for children, and for women it ranges from 0.5 to 1.2 mg/dl and 0.6 to 1.5 mg/dl for men, nevertheless it could be less or more which solely depends on the gender and age. A significantly elevated amount from the normal range means there is a problem (Mohabbati-Kalejahi et al., 2012; Yadav, 2012). Because of certain disorders and complications that could arise in the low or high level of creatinine, it is important to have a frequent monitoring of creatinine in the blood or serum when you go for a general routine checkup (Tiwari and Shukla, 2009).

To determine creatinine there has been a chemical spectrophotometric method based on Jaffe's reaction although this reaction is not specific for just creatinine alone because of many interferences. The Jaffe method relies on a reaction that occurs between the analyte and alkaline picrate which gives a yellow-red complex. Although accurate, this methods can be expensive and time-consuming (P .E. Erden et al., 2006; Anna et al., 2004). There are other clinical techniques used to analyze the amount of creatinine in the blood but biosensors have proven to be more advantageous because they are fast, accurate, cost-effective, environmentally friendly and reduces the complexity of clinical routine (Anthony and Malcolm, 2000). Successes of biosensors for creatinine detection has continually followed two pathways which are based on the amperometric or potentiometric detection. Amperometric detection has so far proven to be very reliable (Anthony and Malcolm, 2000). A lot of amperometric biosensors can be based on a tri-enzyme system that is the 3-enzyme systems that allows creatinine to be converted to creatine, creatine to sarcosine, sarcosine to glycine and finally to hydrogen peroxide. Creatine can be detected amperomerically.

Biosensors have rapidly grown in the last few decades and have a lot of applications in various areas. Biosensors are also called biological sensors in clinical detection of creatine or creatinine. A biosensor is a device that incorporates a biological sensing element and converts the biological response into an electrical signal by the use of a physical transducer (Parikha, 2016). These biological elements or materials could be cells, enzymes, nucleic acid, antigen- antibody, biological tissues, or microorganisms. Any of these transducers or detectors can be used (electrochemical, amperometric, potentiometric, optical, piezoelectric, mass-based) to convert the signals into a measurable response such as light absorption, current, temperature change, or potential which can now be recorded and used to manufacture a biosensor with high sensitivity and selectivity (Qureshi et al., 2012; Zhang and Liu, 2016; Su et al., 2011).

1.1 Creatine And Creatinine

Creatine is formed of glycine+arginine+methionine amino acid. Creatine is present in blood in the free form while in muscles it gains phosphate group from ATP by creatine kinase (CK) to give creatine phosphate - (creatine ~ p) or called phospho-creatine (PCr.) and the ATP is then converted into ADP.

Creatine~p is the main storage form of energy in muscles and is used during muscle contraction. When there is an intense physical strain, the ATP is rapidly reproduced from

ADP by the donation of a phosphate group which is from phospho-creatine (PCr). Creatinine is the anhydrous form and cyclic derivative of creatine. It is formed in muscles by non-enzymatic dehydration of creatine, usually excreted in the urine. Acts as one of the kidney's function test.

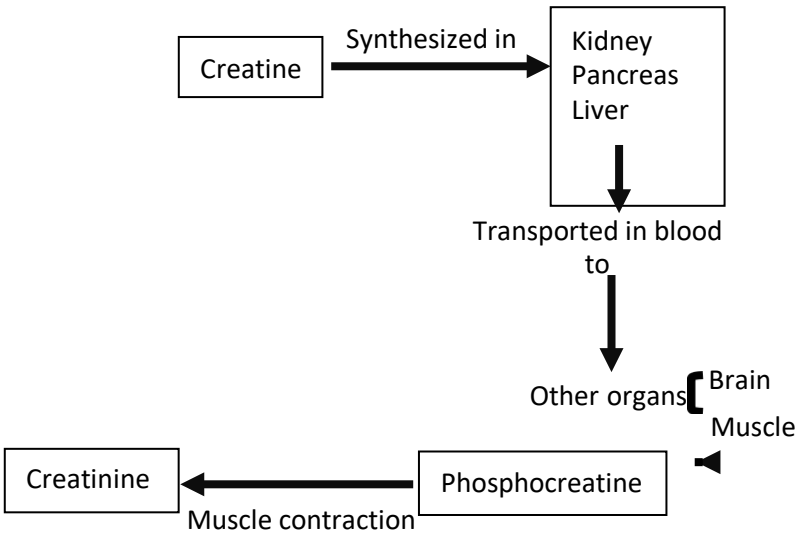


Figure 1.1: Formation of Creatinine

1.2 Clinical Significance of Creatinine Determination

Every day about 1% to 2% creatine produced by the muscles are irreversibly converted to creatinine. The creatinine level is higher in men than women because men usually may have a significant amount of skeletal muscle mass than women (Taylor, 1989).

Creatinine usually is filtered via glomerular filtration, proximal tubular secretion but creatinine is filtered from the blood chiefly by the kidneys. The creatinine in blood will increase if the filtration in the kidney is faulty or non-functional. Creatinine can be found in the blood and urine and the creatinine level can be used to calculate the creatinine clearance (CrCl), which corresponds closely with glomerular filtration rate, the rate at which blood flows through the kidneys.

The clearance rate is the kidneys' ability to handle creatinine. As the blood creatinine level increases higher and higher, the creatinine clearance and glomerular filtration rate reduce. Glomerular filtration rate is important because it is a measure of the kidney function, the

creatinine level in the blood alone can also be used to calculate the estimated amount of the glomerular filtration rate. In the presence of serious kidney impairment, there is hyper-secretion of creatinine which makes the creatinine clearance (CrCl) rate have a tendency to be excess over GFR (Shemesh et al., 1985). An increase in the creatinine does not always mean there is a real reduction in the GFR. For it to be high may be because of an increase in creatinine production and not necessarily because of a decrease in the kidney's function. When creatine supplements and a lot of protein is taken by muscle builders or athlete's to intensify their exercise performance this can increase creatinine by increasing the breakdown of the muscle. Other false factors can cause a rise in creatinine level which may not be associated with kidney impairment. Secretion of creatinine is carried out by the tubules and this can be blocked by several medications (some illnesses and medications can cause an increase) this then causes an increase in the level of creatinine (Samra and Abcar, 2012).

1.2.1 Serum Creatinine

Serum creatinine is the measure of creatinine in the bloodstream and it is a good indicator of the function of the kidneys, although this test may not be suitable to help detect early-stage of kidney diseases (Taylor,1989). To know how functional the kidneys are, to check and know the stage of kidney diseases, to know which treatment is best for the patient, the glomerular filtration rate test is done. The lower the GFR number is the likelihood of the kidneys not functioning well as they should. With the creatinine test, the GFR can be automatically calculated. There are algorithms available that help to estimate the creatinine level, the GFR, and parameters like the age, gender, body size, and race.

The normal range of GFR number is around 90 to 100. Generally, as the patient gets older the GFR decreases. The person does not necessarily need to have a kidney disease. According to the national kidney foundation, the following are the statistics for the ages with respect to the GFR number. Ages 70 and above have a GFR number to be 75. Ages 40 to 69 with GFR number between 99 and 85, and ages 20 to 39 have GFR number between 116 to 107. Nevertheless, it could be affected by age, race, weight, and height.

1.2.2 Urine Creatinine

For clinical diagnosis, it is quite important to know creatinine level in bodily fluids like urine, because it shows the kidney functions (Miura et al., 2013). A low amount of creatinine in the urine indicates that there is a low level of creatinine in the system. Looking at creatinine in the urine, calculating the creatinine clearance can be easy. By measuring the

concentrations of creatinine in the timed urine samples, that is urine passed within 24 hours is collected in a container and tested. If there are any abnormalities, it can be detected early enough and the result is gotten which indicates the amount of creatinine that passed through the kidneys into the urine (Bowers and Wong, 1980).

1.2.3 Creatinine Clearance

Creatinine clearance is crucial in estimating the glomerular filtration rate (GFR). The glomerular filtration rate is highly important because it gives information about the renal function. Creatinine clearance is usually estimated from the amount of creatinine in serum and urine. Any creatinine clearance number that ranges over 20-40 % of the normal shows us that there is a presence of a kidney disease or damage. Creatinine clearance can go above the normal range which is between 90 to110ml/min and if there is an increase, it is called hyper-filtration and a when there is a reasonable decrease in creatinine clearance it could indicate the possibility of a renal damage. By measuring a 24-hour urine collection, it measures the actual amount of creatinine removed. The GFR is a great indicator of the kidney function because it estimates to the doctor the number of nephrons that are functional. The production of creatinine is the same as the amount eliminated constantly by the body which is equivalent to the muscle mass. It can be estimated from a person's gender, age and weight. Urea measures the kidney function to some extent, but creatinine is a more precise, more specific measure of our kidney function than urea. Increase in the blood creatinine level indicates the presence of a kidney malfunction, low blood levels of creatinine also may indicate the presence of a disease associated with a decrease in muscle mass (Perrone et al., 1992). Although creatinine is a waste substance, it serves a vital diagnostic function. A high or even a low amount of creatinine in the blood is mostly accompanied by various renal diseases which may include;

1. **Glomerulonephritis:** A disease of both kidneys. An inflammation of the glomeruli, or small blood vessels in the kidneys which may be caused by several factors like diabetes, certain drugs or an infection from bacteria, virus or pathogens.

2. **Cardiovascular diseases:** Cardio means heart, cardiovascular diseases are associated with problems in the heart or blood vessels (veins and arteries). Cardiovascular diseases are mainly responsible for deaths in patients with renal failure.

3. **Acute Pancreatitis:** Creatinine clearance ratios can be used as an indicator of acute pancreatitis (Murray and Mackay, 1977).This is a serious inflammation of the pancreas which is very deadly even though treatments are carried out.

4. **Nephrectomy:** nephron means kidney, ectomy means removal. The surgical removal of a kidney is termed nephrectomy. In cases of kidney cancers or any other kidney diseases that need the removal of the kidney. Bilateral nephrectomy is the removal of both kidneys at the same time. After bilateral nephrectomy, the creatinine of the blood increases very fast. This procedure could also be used to remove healthy kidneys from a donor (living or deceased).

5. **Preeclampsia:** Preeclampsia is a condition common with pregnancy, in which women experience hypertension (high blood pressure). It is called 'pregnancy-induced hypertension' where a lot of proteins are found in the urine of the patient and this could be a sign of a possible kidney disease. Although the increase or rise in the blood pressure is the only sign we can observe that is associated with this disease, there is a damage done to the maternal endothelium and the kidneys.

1.3 Metabolism of Creatinine

Creatine synthesis occurs primarily in the liver. Creatinine begins as arginine transforms to glycine to then form guanido-acetic acid (GAA) (glycocyamine), the entire reaction is done in the kidneys mainly but also in the small intestine and pancreas. After the guanido-acetic acid has been transported to the liver, it is methylated by S-adenosyl methionine (SAM) and creatine is formed and released. Creatine can be carried by the muscles and several tissues. In a reaction catalyzed by creatine kinase, a phosphate group is introduced to the muscle creatine to form phosphate creatine. Our body creatine is contained in the muscles, blood, kidney, and several body fluids. The excretion of urinary creatine is mostly lesser than 100 mg in 24hr. With a high pH and low temperature the creatine level is increased, but for creatinine, the increment is based on a high level of temperature and also the presence of an acidic medium. Creatinine has a membrane permeable nature and it diffuses into the blood, out of the tissues and then excreted in the urine (Wyss and Kaddurah-Daouk, 2000).

1.4 Methods Which Have Been Used To Determine Creatinine

Jaffé method was one of the first methods to be used for the determination of creatinine (1886), nevertheless, newer methods like enzymatic methods have been employed now. This method experienced a lot of challenges, one of which was interferences (Suzuki, 1994). Other

methods for creatinine determination in urine, blood, and serum have emerged through the years. These methods including, chemical colorimetric methods, enzymatic colorimetric methods, gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), capillary electrophoresis, molecular imprinted polymer methods and optical methods using UV-absorbance technique. Many of these methods have proven suitable for several applications but they have encountered limitations such as specificity, sensitivity and possibly reliability. Other complications associated with some of these methods are: they could be time-consuming, complex, delicate, and bulky and are usually not cost effective. Some may require expertise's handling the methods and so biosensors are novel methods used. Recently, there are several biosensors developed for the detection of creatinine.

1.4.1 Colorimetric Methods

Jaffe reaction method involves alkaline sodium picrate. In an alkaline environment, creatinine reacts with picric acid to form a red-orange color at 510 nm using a spectrophotometry it absorbs. This colour can be formed at a particular rate which is proportional to the concentration of creatinine found in the sample (Yadav, 2012).

$$\text{Creatinine} + \text{SodiumPicrate} \xrightarrow{\text{Alkali}} \text{Creatinine - Picrate complex (red-orange)}$$

Max Jaffe reaction method has been known for over a century. In 1914, Folin experienced an analytical breakthrough where he improved the reaction of the Jaffe method for detecting the total creatinine in blood, milk and exudates, the preliminary precipitation with picric acid. The disadvantage of Jaffe method was that the reaction of picric acid used under all his conditions was that it was not specific for creatinine. Jaffe stated glucose and also creatinine solution reacted with the reagents used and gave similar colors (Jaffe, 1886). Several drugs and compounds have shown to interfere with this method (Weber and Zanten, 1991).

1.4.2 Creatinine Biosensors Based On Dissolved Oxygen Meter

Dissolved oxygen creatinine biosensor has been developed which used the 3-enzymes system. CA, CI and SO enzymes were immobilized onto the electrode surface (Suzuki et al., 2001). Coupling these enzymes together allows the transformation of creatinine. Creatinine at the beginning of the reaction is followed by the consumption of oxygen in the third reaction.

Creatinine is converted into hydrogen peroxide by the aid of the enzymes, which can then be measured. The oxygen probe detects the concentration of oxygen.

$$\text{Creatinine} + H_2O \xrightarrow{\text{creatinine amidohydrolase}} \text{Creatine}$$

$$\text{Creatine} + H_2O \xrightarrow{\text{creatine amidinohydrolase}} \text{Urea} + \text{Sarcosine}$$

$$\text{Sarcosine} + O_2 + H_2O \xrightarrow{\text{sarcosine oxidase}} \text{Glycine} + HCHO + H_2O_2$$

By Immobilizing nitrifying bacteria and creatininase onto the membrane the sensing part of the electrode of the DO meter, serum creatinine can be determined (Kubo et al., 1983). The creatininase hydrolyzes creatinine to N-methylhydantoin and ammonium ion, when the ammonia is produced, it is then oxidized to nitrite and nitrate by nitrifying bacteria. A reaction occurs between the bacteria, and oxygen is consumed and that reduction of oxygen is detected by an oxygen electrode (Kubo et al., 1983).

1.4.3 Potentiometric Creatinine Biosensor

Potentiometric biosensors work on the principle that on the working electrode, the charge potential is measured when no current flows between the working and reference electrode, that is the potential difference between the working electrode and reference is measured. It is a non-faradaic electrode process. Urea and ammonia can be measured using this method and several designs as the electrode can be used for the biosensor design like wires, macroelectrodes, thick films, thin films can be used (Pundir et al., 2013). This method has several advantages like; simplicity, low interference from creatine, require one enzyme, and other disadvantages like; low enzyme stability, low sensitivity of the gas-sensing electrode, and low limit of detection. Because of these drawbacks, other methods have being developed and employed.

1.4.4 Nanomaterials based Creatinine Biosensor

Using nanomaterials creatinine biosensors have being fabricated, and this method possesses several advantages over other methods because of its high sensitivity and specificity. Nanomaterials like nanoparticles (NPs), Fe3O4 NPs, carbon nanotubes (CNTs), and ZnO are used lately help to improve the performance of several sensing systems. There is a high surface area-to-volume ratio of NPs which helps increase molecular interactions and the bio-recognition site (Pundir et al., 2013).

1.5 Chemical Reaction of Creatine to Creatinine

Creatine is carried by the blood via active tissues such as skeletal muscles and brain, through an active transport system. A chemical reaction occurs and this process then converts creatine to form phosphocreatine. This conversion is catalyzed by creatine kinase. During this reaction, that is when the creatinine is formed spontaneously (Allen, 2012). Attached to a creatine molecule are phosphocreatine molecules which are produced as phosphate groups. In the muscle, they tend to behave as storage for ATP (rich energy molecules), to aid the conversion of ADP to ATP, phosphocreatine serves as a phosphate donor and helps supply energy when a contraction occurs. As ATP in the cells run out, the phosphate group is lost, that was produced from phosphocreatine after it undergoes a reaction, it is then transformed back to creatine. A phosphate group is found from phosphocreatine which binds to a molecule called ADP which is then converted back to ATP. It is an extra energy supplier to cells. Creatinine is produced by the biological process mentioned above. Whenever there is a rise in creatine, phosphocreatine will increase also.

1.5.1 Chemical Structure of Creatine

Creatine is a natural substance made by the body, a nitrogenous organic acid that supplies energy to muscle cells. Chemical names of creatine are; Creatine; 57-00-1; Creatin; Kreatin; Krebiozon; N-amidinosarcosine. The molecular formula of creatine is $C_4H_9N_3O_2$, the molar mass is 131.135 g/ mol, Biological half-life: 3 hours, it is also soluble in water.

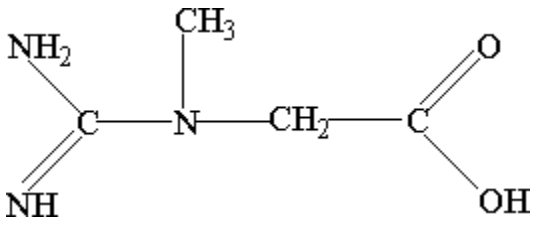


Figure 1.2: Chemical Structure of Creatine

1.5.2 Chemical Structure of Creatinine

Creatinine is a waste substance produced by the body after the metabolism of creatine. The chemical names are as follows; Creatinine;60-27-5;2-Imino-1-methylimidazolidin-4-one;1Methylglycocyamidine; 1-Methylhydantoin-2-imide. The molecular formula of creatine is $C_4H_7N_3O$, the molar mass of creatinine is 113.12 g/mol, the biological half-life of creatinine is 3.85 hours in a normal male adult that ranges between ages 20 to 39 years (Chiou&Hsu,1975).

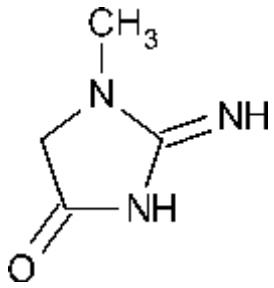


Figure 1.3: Chemical Structure of Creatinine

1.5.3 Functions of Creatine and Creatinine

As the creatine is stored in the muscle cells they gradually help to produce adenosine triphosphate. Creatinine is a component of the muscle tissue which is excreted by the kidney. Serum level is used to as an indicator to evaluate the kidneys function. When the blood creatinine levels increase it means there could be a damage to the nephrons.

1.5.4 Differences Between Creatine And Creatinine

1. Creatine's structure is usually linear whereas creatinine occurs as a cyclic structure.
2. Creatine is required by the body while creatinine is not needed by the body.
3. Creatine is from amino acids. After the breakdown of creatine phosphate creatinine is formed.
4. Creatine molar mass is about 131.13 g mol^{-1}, while for creatinine the molar mass is 113.12 g mol^{-1}.
5. Creatinine is a compound with the molecular formula $C_4H_7N_3O$
6. Creatine is an organic nitrogenous acid whilst creatinine is not.

7. Creatine is usually stored in the muscles and increases the muscle mass while the production of creatinine reduces the muscle mass.
8. Creatine is taken as a supplement to gain strength and build body cell mass.
9. Creatinine is an excretory product removed from the body via the kidneys through the urine.
10. Creatine is the main indicator of muscle injury and creatinine is a main indicator of checking the kidneys functions.

1.6 Biosensor

Biosensors are analytical devices that combines a biological element with a physicochemical detector to sense or detect specific analytes of interest.

There are three main components in a biosensor. A biological receptor, a transducer, and a signal processor. A bioreceptor usually consists of an immobilized biological component, which detects the specific target molecule or analyte. Common bioreceptors are antibodies, nucleic acids, cells, enzymes, DNA or microorganisms (Kahn and Plaxco, 2010). The interaction between the sample analyte and the receptor is detected by the transducer. Transducers are converters, they convert physical quantities to electrical signals or vice versa. These converters change the biochemical signal into an electrical signal. Usually, a reaction will occur between the analyte and bioreceptor which causes changes (Veeradasan and Uda, 2014). The signal processing system is where the electrical signal is amplified. Which will eventually be sent to a microprocessor or microelectronics for data processing. The measured signal is produced and displayed on the digital screen. Providing specificity, sensitivity, fast, real-time, accurate results are the most crucial aspect of biosensors. Depending on the transduction process, biosensors are divided into; piezoelectric, electrochemical, optical, thermal biosensors. Electrochemical biosensors stand out amongst all other biosensors because they are widely used, and also they are globally commercialized electronic devices (Dzyadevych et al., 2008). Electrochemical sensors have very high selectivity and sensitivity also because of their high compatibility, and low cost with modern miniaturization and microfabrication technologies which have recently been incorporated are now more available (Xueji et al., 2008). Electrochemical biosensors have three electrodes system; a reference electrode, a working electrode, and a counter or auxiliary electrode. Today, biosensors are used for different applications from clinical (patient monitoring, the point of care) to non-clinical (environmental monitoring, and industrial processes).

Biosensors has an edge over other analytical methods because of the following;

- Its simplicity of operation
- Its miniaturization and fabrication ease

- Sensitivity and specificity
- Speed or rapidness of response
- Point of care device
- Can be easily interfaced with microprocessor or computer for data processing and control

1.6.1 Block Diagram of a Biosensor

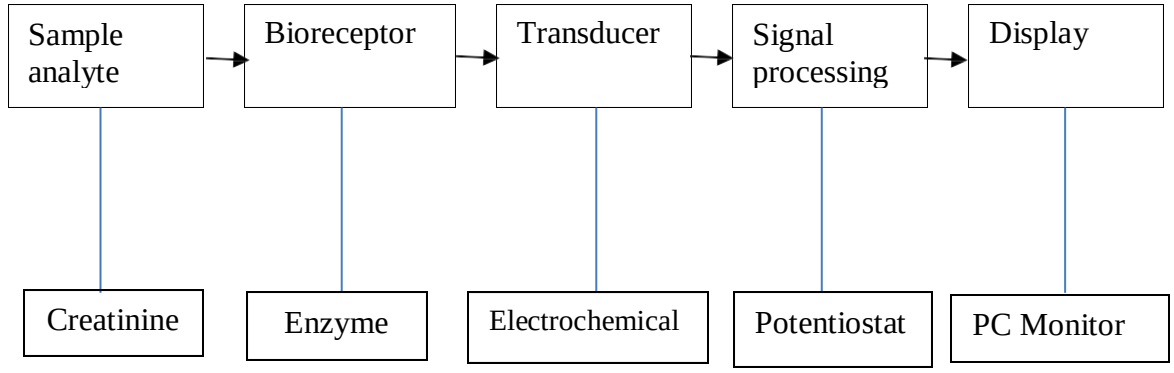


Figure 1.4: Block Diagram of a Biosensor

1.6.2 Classification of Biosensors

Biosensors are classified based on either the biological mechanism or transduction process (Veeradasan and Uda, 2014). Different categories of the biosensor are shown below;

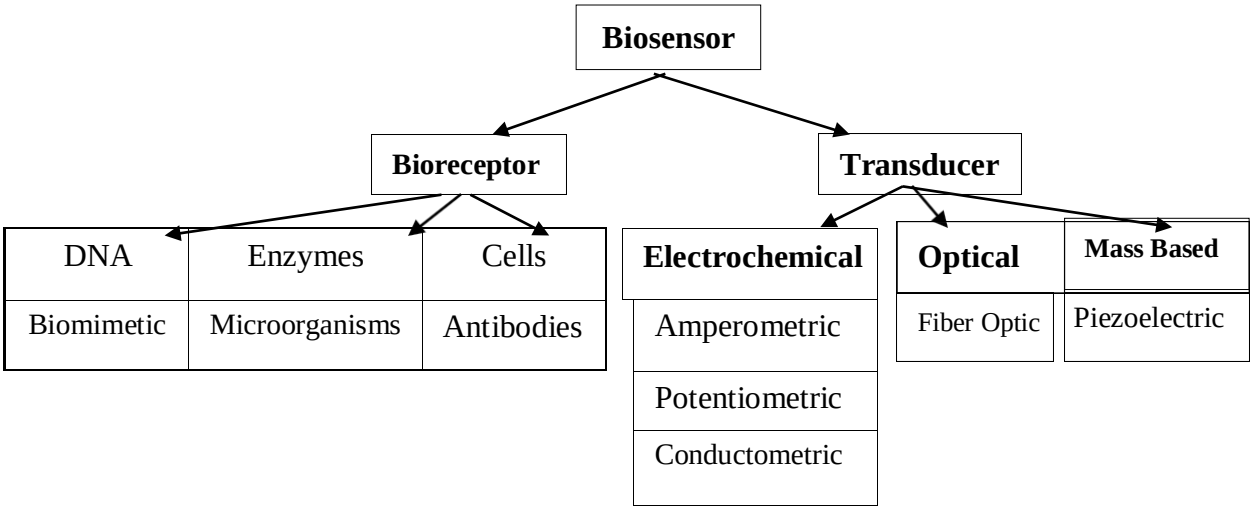


Figure 1.5: Classification of Biosensors

1.6.3 Amperometric Biosensors

During an amperometric measurement, it is based on electron movements. The working electrode has a stable known potential as changes occurs on current. The current is observed in relation to the concentration of the analyte used (Wang, 2006; Bard, 2001). Amperometric biosensor works on a principle that when a potential is applied to the working electrode, current is produced.

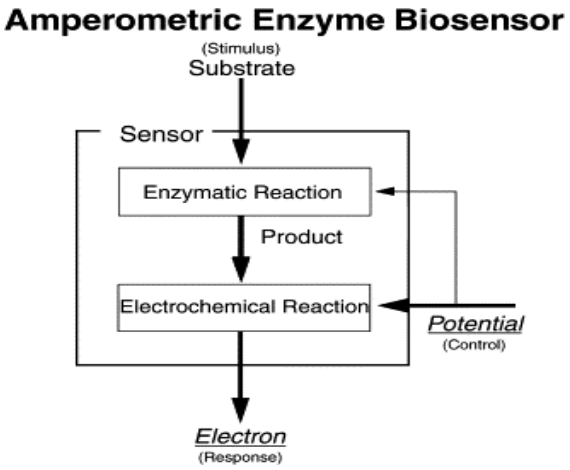


Figure 1.6: Amperometric Enzyme Biosensor
http://www.sciencedirect.com/science/article/pii/S0009898103002419

1.7 Enzymes As Receptors For Creatinine Biosensors

Enzymes as biological recognition molecules are mostly used for biosensor designs. Enzymes are proteins which speed up chemical reactions. Enzymes acts on molecules called substrates, which are then converted to a product. The nature of enzymes is that they possess both hydrophilic and hydrophobic amino acids (proteins). There are a few complexities working with enzymes, so it is necessary to understand their nature. Retaining their activity is an example, and there are strategies that can be employed to retain or improve the activity of enzymes (Talbert and Goddard, 2012).

1.7.1 Enzyme-Based Biosensors

Enzyme biosensors are one of the latest biosensor designs but the enzymes used here need to be immobilized onto an electrode surface for the measurement of specified analyte. Because of its vast clinical applications, recent developments are now aimed at improving the quality of immobilization, activity, and enzyme stability. For creatinine biosensors, creatinase, creatininase, and sarcosine oxidase are mainly used (Wang, 2006).

1.7.2 Creatininase

Creatininase is an enzyme used for creatine and creatinine sensors. It is a member of the urease-related amidohydrolases family. A breakdown occurs as creatininase reacts with water (hydrolysis)where creatinine is converted to creatine which is afterward metabolized to urea and sarcosine by creatinase. Sarcosine is then oxidized with sarcosine oxidase to form formaldehyde, glycine and hydrogen peroxide. Creatinine and H_2O are the two substrates of this enzyme, and its product is creatine. This enzyme usually causes a reaction to begin.

1.7.3 Sarcosine Oxidase (SO)

Sarcosine oxidase (SO) is an enzyme which has always had a significant role to play in enzymatic method (Suzuki, 1994). Coupling sarcosine oxidase with creatinase and creatininase can be used to construct a biosensor for the enzymatic detection of creatinine. In several microorganisms, sarcosine is metabolized to glycine. The reaction is as follows; $\text{Sarcosine} + O_2 + H_2O \rightarrow \text{Glycine} + \text{Formaldehyde} + H_2O_2$. Sarcosine oxidase is usually used this day as part of a multi-enzyme cascade so that when immobilized can construct amperometric biosensors (Monošík et al., 2012).

1.8 Electrodes Used In Electrochemical Biosensors

The electrochemical biosensor has 3 different types of electrodes used for the system setup.

I. **Reference electrode:** The reference electrode is used as a reference for measurement, mostly by other electrodes. A reference electrode is usually stable and has an accurate electrode potential. At a constant buffer concentration, a high stability of the electrode potential can be reached by using a redox system.

II. **Working electrode:** The working electrode is the electrode in an electrochemical system on which main reactions of interest occur. The working electrode is mostly used with the auxiliary electrode and the reference electrode in a 3 electrode system. In the working electrode, the reaction can be anodic or cathodic, it depends on the reaction that occurs at the surface (Kumar and Neelam 2016).

III. **Auxiliary electrode: Also known as the counter electrode.** A platinum wire is mostly used and this is due to the fact that platinum wire is an inert material. The working electrode is not affected by its behaviors

.

1.8.1 Pencil Graphite Electrode

Pencil graphite can be used as an electrode material because of certain properties it possesses but they have to be modified with certain electrochemical catalysts for them to be used as a good and senstive sensor (Skrzypczynska et al.,2018; Dilgin et al.,2012). Pencil graphites compose mainly of graphites, but also some amount of wax and clay. The graphite has layers, one individual layer is commonly called graphene. Pencil graphite leads are used lately as disposable pencil graphite electrodes, in 1996 pencil leads were used as new electrodes for abrasive stripping voltammetry (Blum, 1996). As the years passed on many other applications of PGE has being employed (Pourbeyram and Mehdizadeh,2016; Kawde et al., 2015; Karimi- Maleh et al., 2015). Pencil graphites electrode are easily available, affordable and have a good sensitivity and conductivity (Stradolini et al., 2018). Other attractive properties of the pencil graphite electrodes are they are capable of working successfully, low background current, a renewable electrode surface, anodic potential range, are inert, are disposable, and are quite easy to use (Ozsoz et al.,2003; David et al.,2017).

1.9 Silk Fibroin

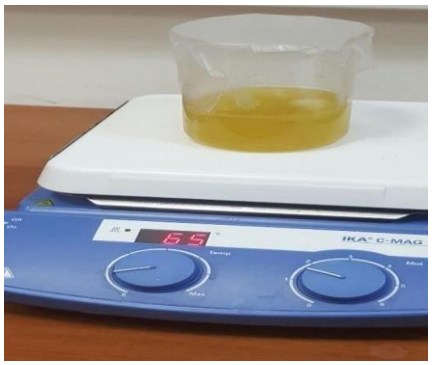
Silk fibroin is a final product of cocoons which is extracted from silkworms (bombyx mori). Silk is a protein-based biomaterial. Silk used as a biomaterial is commonly produced from Bombyx mori. Purifying silk fibers is carried out by an easy degumming procedure to remove the sericin contained in it. It biocompatibility and biodegradability has met all the suitable biological properties and has made it a good biomaterial used these days. The synthesis of pure silk fibroin follows a routine process; Firstly, the cocoons gotten from the Bombyx-mori (silkworm) have to be cut (for measurement purposes and to allow the degumming process to be faster) into smaller bits and cleaned to remove any unwanted dirt and impurities. Degumming is the process of removing the sericin (a glue protein and a sticky substance) produced by the silkworm that holds the strands of silk together. This is done by adding 1g of cocoons and 100ml of Na_2CO_3 into a conical flask which is then

electro-spurned with a magnetic stirrer for three hours in three different rounds, the electro spurned fibers are washed with deionized water after each round in other to completely remove sericin. The 0.1mol sodium carbonate (Na_2CO_3) can be prepared by adding 5.3g Na_2CO_3 into a beaker containing 500ml of deionized water. After the degumming process, fibers are obtained and washed. The fibers are then allowed to dry overnight. The dried fibers are picked in other to obtain strands of fibers that are not bonded together. The next step is the dissolution process- In this step, a strong electrolyte solution is prepared and the picked fibers are put with a certain amount of the solution.6g of picked fibers into 100ml of strong electrolyte solution for about 3 hours. Preparation of electrolyte solution: 29.15ml Ethanol, 27.75ml Calcium Chloride ($CaCl_2$), 36ml deionized water are mixed to form the strong electrolyte solution. Dialysis is the final step which is done in the span of 3 days. We added the solution into a semi- permeable membrane and we put it into a deionized water in a large beaker. Placed it on a magnetic stirrer for 1 hour for the first day and allowed to sit in the water bath overnight, the other 2 days it undergoes 3hours each day with a change of water after each round. No heat applied. After the whole procedure, the pure silk fibroin solution was obtained.

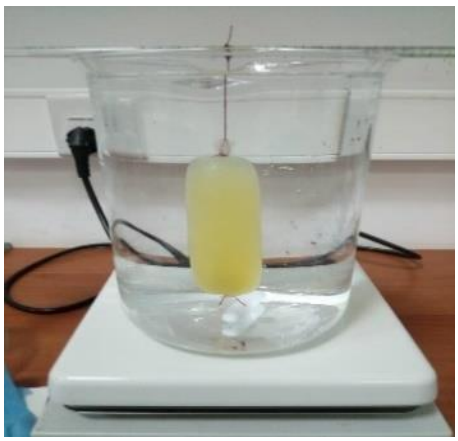
a) Coocons from Bombyx Mori

b) Cleaned and Cut Cocoons

c) Degumming



d) Dissolution



e) Dialysis

f) Pure Silk Fibroin

Figure 1.7: The Purification of Silk Fibroin

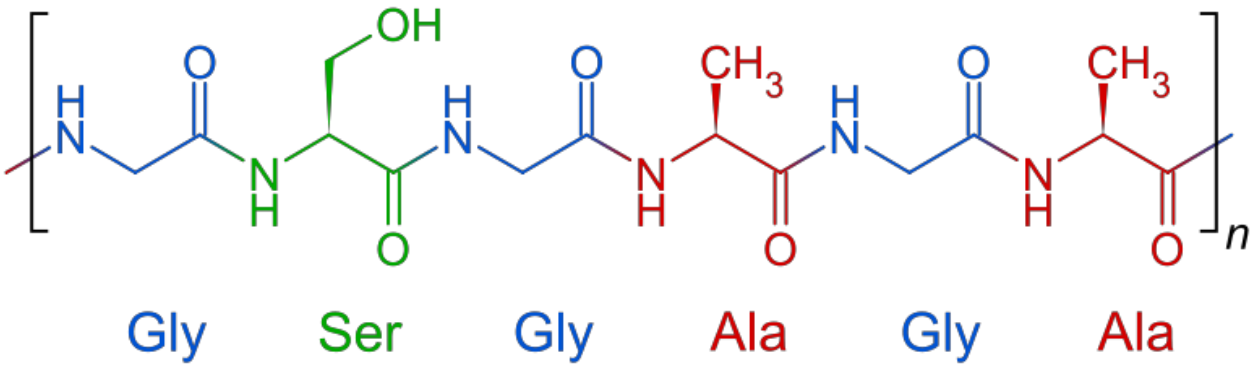


Figure 1.8: The Primary structure of Silk Fibroin

Silk is known as a widely used biomaterial because of its mechanical, chemical and thrombogenic properties. Silk used for biomaterials has silk fibroin protein as a component. This fibrous protein composes of a parallel beta- sheet structure, which is also composed of high amino acid with about 43% of glycine, 30% of alanine, and 12% of serine. This structure with several composition allows it to possess unique tensile strength and stability (Nikhom et al., 2012).

1.9.1 Silk Fibroin acts as a support for Enzyme Immobilization

Silk fibroin is a protein that has an edge over many other natural proteins because of properties it possesses such as low toxicity, its biocompatibility and high nutrient, its low cost and ease of preparation. It has proven to serve as an enzyme immobilize support for biosensors. Silk fibroin and enzymes have been used in various biosensor applications for the

determinations of hydrogen peroxide, glucose, and uric acid where the enzyme stock solution can be mixed with the silk fibroin (Zhang, 1998).

1.10 Cross-Linking

Crosslink is a term used to define a bond which connects one or more polymer chain (macromolecule) to another. Crosslinking is using crosslinks or cross-linkers to improve the physical or mechanical property of the polymer, used for protein- protein interactions. There are several crosslinking methods and reagents. It is carried out by a multifunctional reagent. Two reactive groups are reactive towards other groups (sulfhydryl and amines) are contained in cross-linkers, and create chemical covalent bonds that are between molecules (Mattson et al., 1993). Crosslinking is a simple process and its biomolecules chemically bind strongly which is an extra advantage.

There are several physical and chemical enzyme immobilization techniques used for the construction of biosensors namely; entrapment, adsorption, covalent bonding, and crosslinking (Devaraja Gouda., 2000).

In biosensor design and applications the most common enzyme immobilization method is cross-linking. The crosslinking agents used was tri-ethylene glycol dimethacrylate (tri-EGDMA). EGDMA is a diester which is formed by condensing an ethylene glycol to two equivalents methacrylic acid.

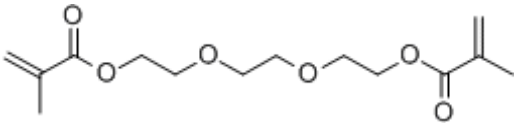


Figure 1.9: Structure of Tri-EGDMA

1.11 Objectives of the Study

In this study, the objective is to construct a novel biosensor approach to determine creatinine by immobilizing enzymes onto the surface of a pencil graphite electrode (PGE) modified with pure silk fibroin prepared from locally sourced cocoons. Certain parameters were investigated to check the performance of the electrode surface.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Materials

The amperometric measurements were done using a potentiostat-Galvanostat autolab model, connected to a 3-electrode system. The working electrode WE used is the pencil graphite electrode, the reference electrode is Ag/AgCl, and the auxiliary or counter electrode is the platinum wire that was used. Platinum wire exhibits low current in electrolytes solution. The mechanical pencil used as a holder for the pencil graphite was purchased from a local bookshop model 0.5mm Pentel P205, forceps were used to hold the graphites and placed into the mechanical pencil tip. The pencil graphite used was also purchased from a local bookshop having a diameter of 0.5mm HB model by Tombow. A phosphate buffer solution was used which was prepared by mixing KH_2PO_4 and Na_2HPO_4 with distilled water. NaOH or HCl were added to obtain the appropriate pH. The pH values were measured using pH meter WTW pH7110 inoLab model. PBS used was having a pH of 7.0 at 5mM.

Creatininase (C3172-1KU from microorganism) and sarcosine oxidase (from Bacillus sp.) enzymes were purchased from Sigma. Sarcosine oxidase from Bacillus sp. which is used in this study has a yellowish amorphous powder appearance, lyophilized powder, with activity 25-50 units/mg solid. For sarcosine oxidase to be used within the conditions of clinical assays, it must be highly pure and stable.

Cocoons were purchased from büyük Han used to prepare the pure silk fibroin. Sodium carbonate (Na_2CO_3) was used for the process of degumming, calcium chloride ($CaCl_2$) was purchased from Sigma Aldrich which was used along with ethanol and de-ionized water in the dissolution process. Used in the dialysis of the dissolved pure silk fibroin is the dialysis-tubing cellulose membrane gotten from Sigma Aldrich dialysis-tubing cellulose membrane was. Tri (ethylene glycol) dimethacrylate was used as a cross linker for the silk fibroin.

Figure 2.1: Ag/AgCl electrode

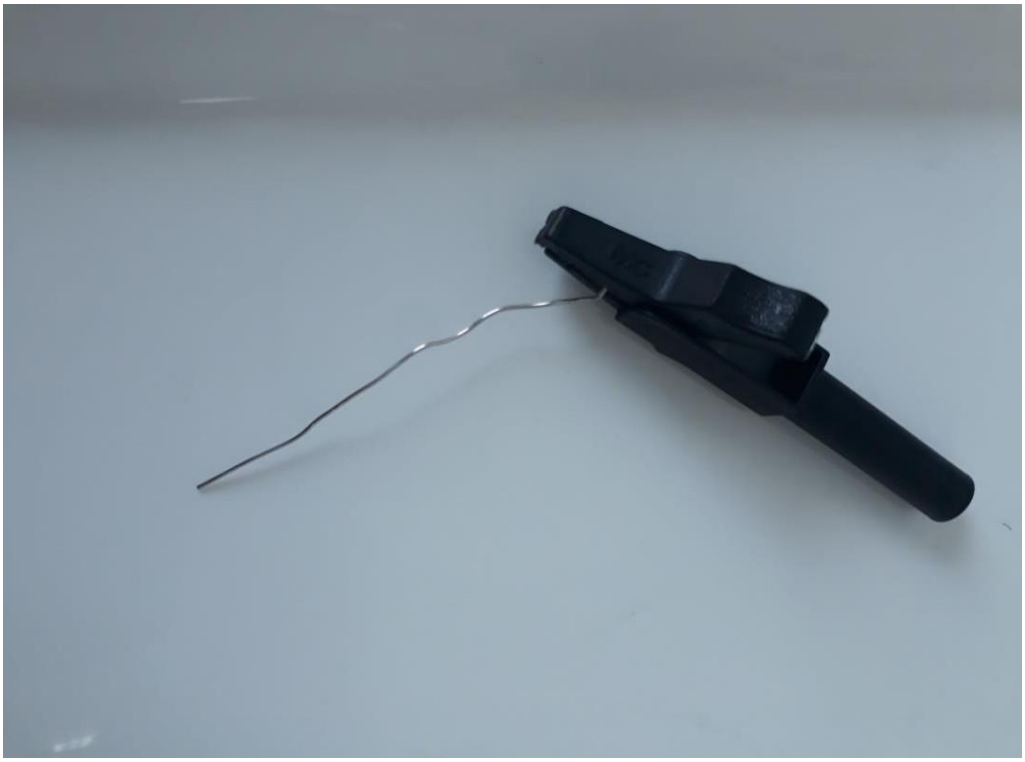


Figure 2.2: Platinum wire electrode

Figure 2.3: PGE, Mechanical Pencil & Forcep

2.2 Method

The enzymes were prepared and mixed with pure silk fibroin solution, samples were placed in eppendorf tubes and the pencil graphite electrodes were put inside and allowed for 1hour for the enzymes to immobilize onto the surface of the graphite electrodes, then was rinsed with distilled water. The graphite enzyme electrodes were put in the mechanical pencil and connected to the WE after which was dipped in the cell containing PBS. All other connections were made and measurements are taken, measuring the response of the electrode in terms of current when particular voltages were applied. The buffer solution used for the chronoamperometric measurements was the phosphate buffer saline. Chronoamperometry measurements of the enzyme electrode, optimization studies with 100µM of H_2O_2 concentration was carried out and the electrochemical behavior of the biosensor was investigated using chronoamperometry techniques.

2.3 Preparation Of Creatininase And Sarcosine Oxidase Enzymes With Pure Silk Fibroin

0.0994g creatininase and 0.0026g of sarcosine oxidase were weighted using an analytical weighing balance which was mixed together in a 25ml beaker with 0.04g of pure silk fibroin solution. Subsequently, a 5ml phosphate buffer was added to the stock solution above. For crosslinking, Tri-EGDMA was used together with PBS. When not in use, solutions were kept in the refrigerator at 4°C.

2.4 Preparation of the Electrodes

PGE is used as the working electrodes. Electrical conductivity was achieved by connecting a metallic wire to the 2 ends of the pencil by soldering. The bare PGEs were electrochemically pre-treated to clean the surface by applying chronoamperometry technique with potential 1.4v for 30s. 1.5cm of the electrode tip was inserted into the buffer solution after the immobilization of the enzyme.

2.5 Amperometric Creatinine Biosensor

Pure silk fibroin was added to the mixture containing creatininase, sarcosine oxidase, and PBS which was immobilized on the surface of the working electrode for 1 hour. The modified PGE was connected to a 3-electrode electrochemical cell system. The PGE as the working electrode, Ag/AgCl as reference electrode and Pt wire as auxiliary or counter electrode. All electrodes were connected to Autolab Potentiostat/Galvanostat and a computer using the Nova 2.1.1 software. When a potential was applied the electrode responses were measured in regards to the current in mA. During the reaction creatinine is broken down by the applied potential between the WE and the CE to generate H_2O_2, electrons are released which passes through the WE to the device which can be detected amperometrically. This principle is based on the hydrogen peroxide oxidation as the creatinine reacts with the enzymes, the current produced when a voltage is applied, is proportional to the amount of creatinine. Many amperometric creatinine biosensors are based on a 3-enzyme method, which converts creatinine to creatine, creatine to sarcosine and sarcosine to glycine and finally, hydrogen peroxide (H_2O_2) is lastly released. To obtain a creatinine biosensor, a chronoamperometric hydrogen peroxide technique was used. Below is a catalyzed reaction of the enzymes;

$$\text{Creatinine} + H_2O \xrightarrow{\text{creatinine amidohydrolase}} \text{Creatine}$$

$$\text{Creatine} + H_2O \xrightarrow{\text{creatine amidinohydrolase}} \text{Urea} + \text{Sarcosine}$$

$$\text{Sarcosine} + O_2 + H_2O \xrightarrow{\text{sarcosine oxidase}} \text{Glycine} + HCHO + H_2O_2$$

Figure 2.4: AutolabPotentiostat/Galvanostat connected to a 3-electrode electrochemical cell.

Figure 2.5: AutolabPotentiostat/Galvanostat instrument for electrochemical techniques with a display monitor and system unit

CHAPTER THREE

RESULT AND DISCUSSION

3.1 Cleaning Surface of PGE

The bare PGEs were electrochemically pre-treated to clean the surface by applying chronoamperometry technique with potential 1.4v for the 30s. About 1.5cm of the electrode tip would be inserted into the electrolyte solution in the cell. An output signal is obtained which indicates the readiness of the electrode.

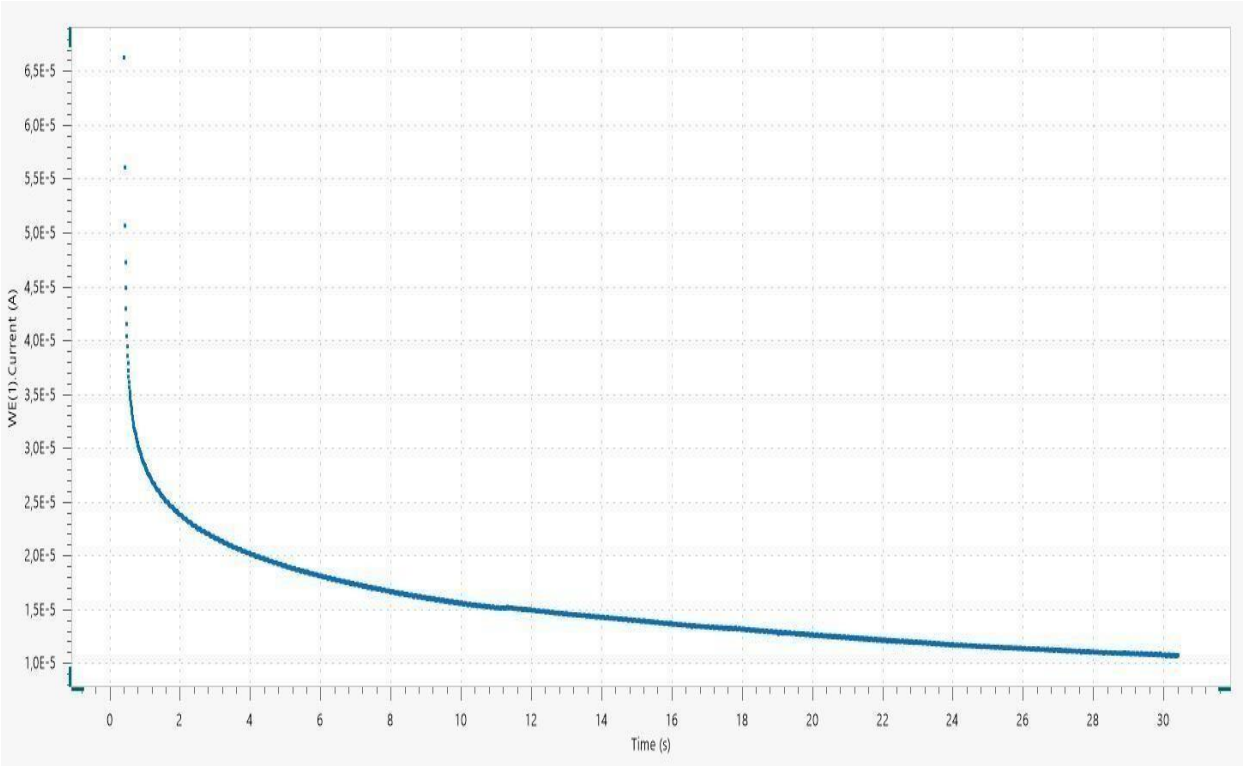


Figure 3.1: Output signal in Chronoamperometry

3.2 Chronoamperometric Determination of Hydrogen Peroxide

This is an important technique used in electrochemistry. In the presence of hydrogen peroxide, it is possible to measure the current response of the PGE. In chronoamperometry, the voltage is kept constant as the current is monitored within a specified time period. As 100µM H_2O_2 is added to the buffer solution, the steady-state of the current is increased as time increases. We got a staircase diagram indicating a good response. In figures 3.2 and 3.3 is an amperometric response of 100µM H_2O_2 at 0.6V during a 10 minutes experimement. Figure 3.4 for an interference study, ascorbic acid was added since it gives an intereference signal and a good H_2O_2 response was observed nevertheless.

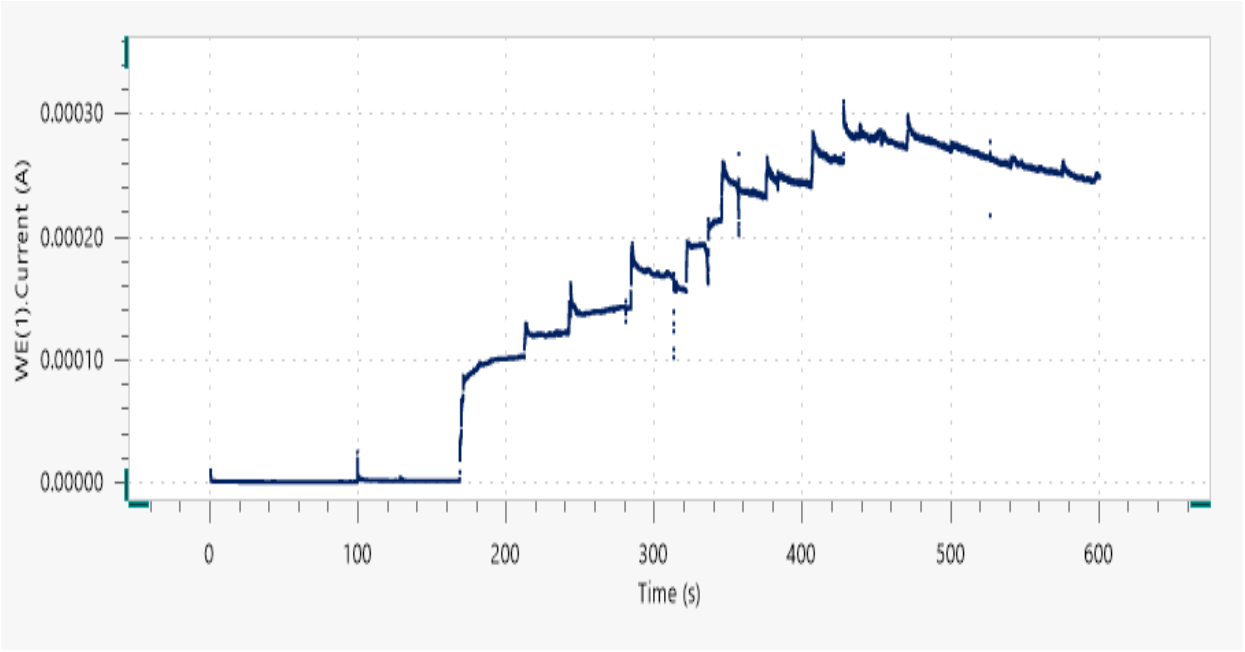


Figure 3.2: Chronoamperogram response of PGE in pH 7.0 PBS 5mM at 0.6V within 600secs, by successive addition of 100 µM H_2O_2

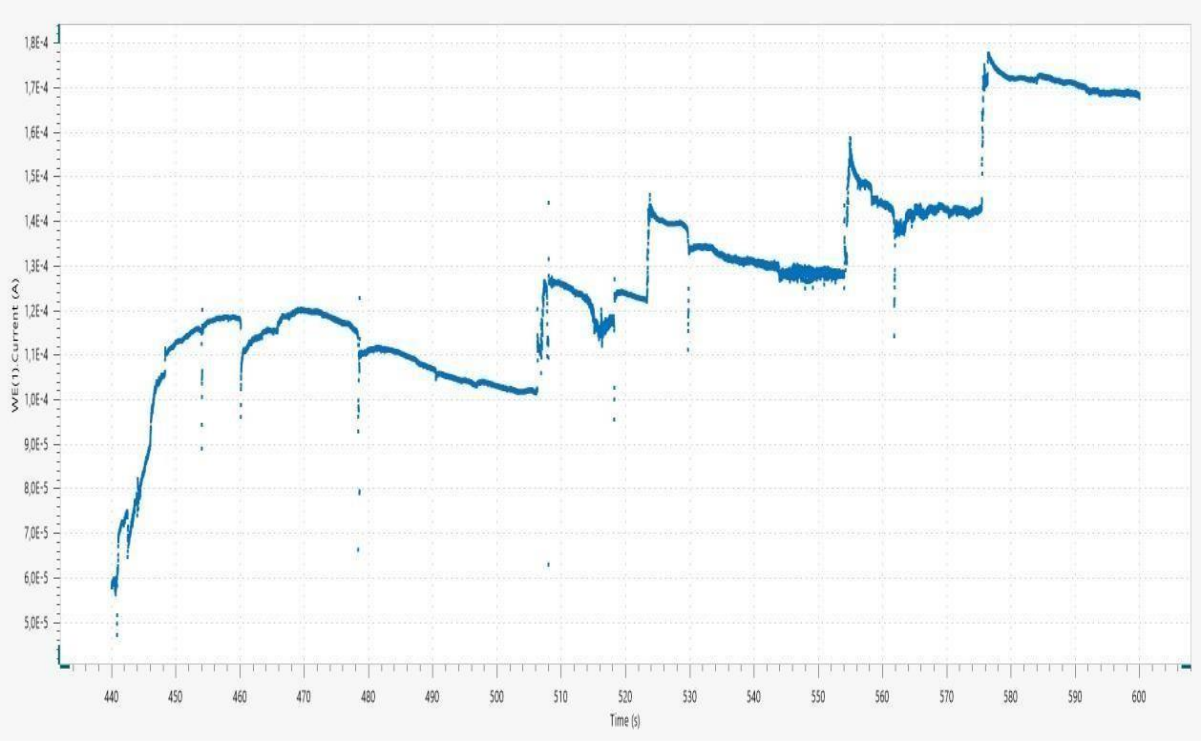


Figure 3.3: Chronoamperogram response of PGE in pH 7.0 PBS 5mM at 0.6V by successive addition of 100 µM H_2O_2

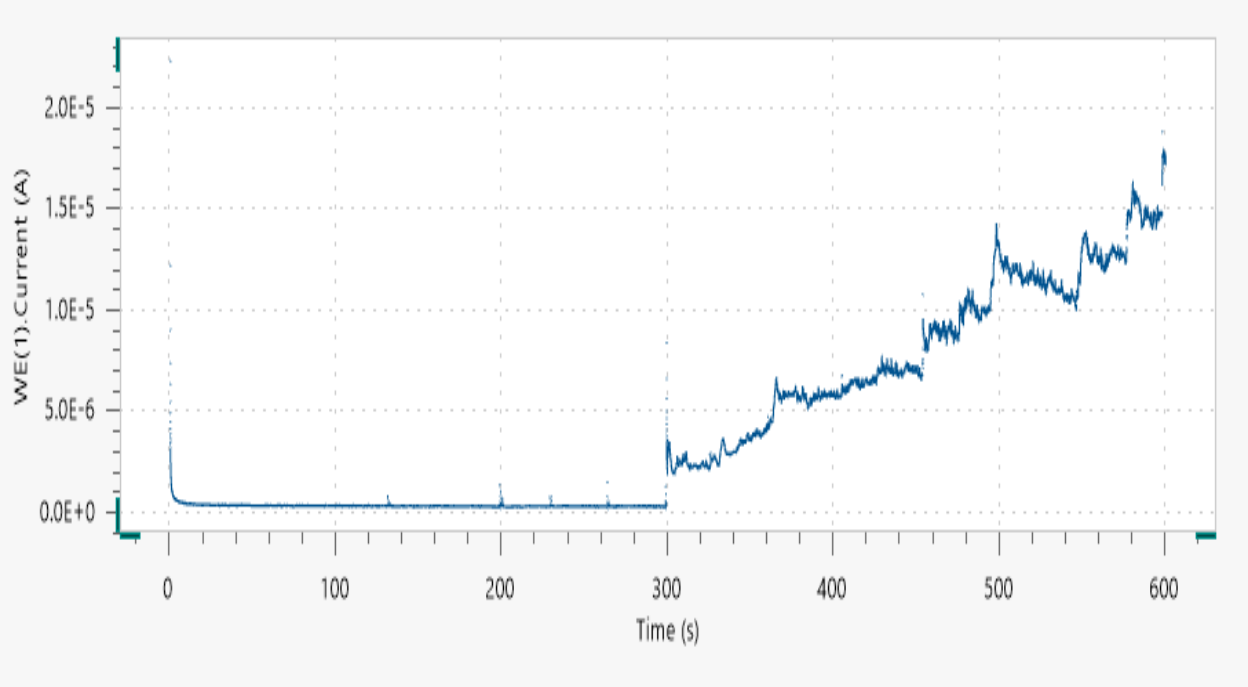


Figure 3.4: Chronoamperogram response of PGE in pH 7.0 PBS 5mM at 0.7V within 600secs, 100µL addition 100 µM H_2O_2 and ascorbic acid

Table 3.1: Properties of the working parameters

Parameters	**Optimum Condition**
Temperature	**25°C**
H_2O_2 concentration	**100mM**
H_2O_2 each addition	**100µL**
pH	**7.0**
Phosphate buffer concentration	**5mM**

CHAPTER FOUR
CONCLUSION

4.1 Conclusion

Over the years, enzyme biosensors have been developed for the detection of creatinine, some have been incorporated into biomedical sensor devices for clinical diagnosis. Incorporating an enzyme on the surface of the working electrode has an ability to increase the specificity of the biosensor. Silk fibroin used to modify the pencil graphite electrode is an excellent biomaterial for the construction of biosensors. And combining pure silk fibroin with other materials can prove to hold a huge chance in future biosensors applications. Although PGE is known for its fragility compared to other electrodes, it is advantageous and possesses properties which are attractive over other electrodes because of its low cost, it is quite easy to use, and readily available.

The use of the potentiostat/galvanostat is rapidly increasing as it is used lately for a lot of electrochemical applications. Chronoamperometry techniques can be used to study the chemical reaction that occurs and analyses the current time curve. Other methods or techniques are based on chronoamperometric technique. At the end of creatinine reaction, hydrogen peroxide is gotten which can be detected or investigated with chronoamperometric response observed as a staircase diagram.

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