Nanofabrication and Characterization of an Enzyme-Less Electrochemical Biosensor for Creatinine Detection

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NANOFABRICATION AND CHARACTERIZATION OF AN
ENZYME-LESS ELECTROCHEMICAL BIOSENSOR
FOR CREATININE DETECTION

By

ANAS BELHARSA
B.S. Biomedical Sciences University of Central Florida, 2019

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
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in the College of Graduate Studies
at the University of Central Florida
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This study will reveal the fabrication and development of an enzyme-less biosensor for creatinine detection. The biosensor involves a periodically patterned nano-porous TiO2 deposited with Au nanoparticles via e-beam evaporation and a layer of Imprinted Polymer (IP) of acrylamide and bis-acrylamide to obtain a heterostructure of I-Au-TiO2. The detection methods of creatinine are based on electrochemical measurements using Cyclic Voltammetry (CV), Electrochemical Impedance Spectroscopy (EIS) and Differential Pulse Voltammetry (DPV). The IP-Au-TiO2 sensor shows a detection LOD of 0.0949ng/mL and 0.218ng/mL for EIS and DPV measurements, respectively. The nanofabricated biosensor was tested in the presents of urea, glucose, lactose, L-valine, and Glutamic acid and shows high specificity for creatinine due to the specific binding of the analyte to the imprinted polymer on the electrode. A comparison test was performed between the imprinted IP-Au-TiO2 versus Non-Imprinted (NI) NI-Au-TiO2 biosensors. the results show no specificity for the creatinine using NI-Au-TiO2 biosensor for the varied concentration from 0.1ng/ml to 1µg/ml compared to the I-Au-TiO2. However, The N-Au-TiO2 show enhanced specificity for creatinine in the presence of Localized Surface Plasmon Resonance (LSPR) at the interface of the Au nanoparticles and TiO2. The generated LSPR on the surface of the biosensor increased the sensitivity for creatinine due to charge separation and solution resistance between the sensor and mixture. This detection platform provided a promising result which can be easily expanded to detecting a variety of biomarkers linked to human diseases or pathogens such as bacteria or viruses for point of care detection.
ACKNOWLEDGMENTS

I would like to express my gratitude to the faculty members in the Nanoscience technology center at the University of Central Florida, who assisted me throughout my thesis project. Foremost, I would like to start by thanking Dr. Zhai and Dr. Kang for their huge support and guidance through every step of my research, and for providing me with the necessary chemicals needed to perform my experiments. I am also thankful to Dr. Swadeshmukul Santra and Dr. Laurene Tetard for their constant encouragement and useful suggestions all through my thesis project.
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CHAPTER ONE: INTRODUCTION

Creatinine is the byproduct of muscle metabolism from the precursor of creatine. In skeletal muscle, creatinine phosphate plus an Adenosine Diphosphate (ADP) is generated from a reversible reaction carried by creatinine kinase with the help of Adenosine Triphosphate (ATP) [1, 2]. The creatine phosphate act as energy storage to regenerate additional ATP during muscle stress through a non-enzymatic cyclization to form creatinine [3]. Since creatinine is the final product and no further degradation occurs or reabsorbed in humans, the molecule is able to diffuse out of muscle cells by the process of passive transport into the blood circulation and taking up by the renal system. In the kidney’s creatinine is considered as a waste product and up taking by the proximal tubules of the nephron through an anti-transporter known as SLC22A2 to finally be excreted in urine through the urinary tract [4]. The normal urine level of creatinine in a healthy individual is 40-300mg/dL for males and 37-250mg/dL for females [5, 6]. The discussed pathway above makes creatinine an extremely attractive molecule to understand the kidney’s functions by sensing its concentration from a urine sample. To accurately diagnose patients with chronic kidney disease or acute kidney Injury the Glomerular Filtration Rate (GFR) become crucial for assessing the kidneys function because of the level of GFR and creatinine are inversely proportional[7]. The electrochemical sensing is a great platform for detecting a small concentration of biomarkers in a serum sample with a simple potential voltage scan. Our fabricated sensor showed high specificity without the use of expansive biomolecules such as aptamers or antibodies for recognition. The high specificity was possible due to the imprinted polymer designed for creatinine, it showed no affinity for interfering molecules, unlike the response obtained by NI-Au-TiO₂ and Au-
TiO₂ with LSPR which lack specificity for creatinine using DPV and EIS. However, using the effect of LSPR on Au-TiO₂ generate a great enhancement in sensitivity but no specificity, it will be discussed in more details in the finding section.
CHAPTER TWO: LITERATURE REVIEW

The most common method for detecting creatinine is Jaffé procedure, however this method is based on a chemical reaction that can provide bias results due to the interfering side reactions [8, 9]. Studies show Picric acid of Jaffé procedure can react with a variety of molecules present in sample mixture such as molecules with ketone functional group, Vitamin C, bilirubin, antibiotics, and other drugs [5, 9-11]. Therefore, Jaffé reaction is an insufficient method for obtaining an accurate estimation of creatinine to diagnose patients with certain kidney diseases that require a narrow range of estimation levels. Other detection methods are based on liquid chromatography, High Performance Liquid Chromatography (HPLC), and enzymatic detection or a combination of methods [12-14]. However, those methods come with limitations such as time consumption for sample preparation, quantification, short lifetime, temperature controls and denaturation of the enzymes, and high cost or require trained lab technicians to obtained data and cannot be done at the point of care. Therefore, a more sophisticated methods needed to be developed. Recently the field of sensing by electrochemical detection become attractive due to the ability of detecting biomolecules with enzyme-less devices with high specificity and cost-efficient [15, 16]. In this study, we developed a novel biosensor for creatinine composed of a periodically patterned porous titanium dioxide encapsulated with gold nanoparticles to enhance the electrochemical sensing by hot electron transfer (figure 1-2). To further increase the sensitivity of the fabricated biosensor an imprinted polymer was coated around the target molecule to create a specific binding site similar to a lock and key model hypothesis for enzymes substrate interaction. The encapsulated gold nanoparticles enable greater sensitivity of the sensor compare to the bulk counterpart of a traditional gold electrode due to the substantially
larger surface area for the analyte binding and better reactivity at the interphase. These properties are generated due to the exposed gold atoms on the surface of the nanoparticles providing a superior tendency for the sensor to react with creatinine compare to the energetically stable bulk gold electrode showing unique chemical and physical properties. The immobilized nanoparticles inside the cavities increase the stability and prevent the issue of aggregation by acting as a template for hosting a network of the gold nanoparticles.
CHAPTER THREE: METHODOLOGY

The Ti foil used for fabricating the biosensor was 0.05mm thick. The foil was ultrasonically washed in ethanol and deionized water to remove any debris then rinsed two times with deionized water and allowed to air dry. A 3M solution of H₃PO₄ and HF was prepared for the electro-etching process. A 200mL of the 3M solution was heated at 95°C to evaporate the water molecules for four hours. The solution contains strong acid and must be stored in polytetrafluoroethylene material during anodization. The periodically patterned pours structures were initiated using a controlled anodization process for 4.5 hours at a 10V with a plated Pt as the counter electrode. A distance of 2.5cm between the counter electrode and the Ti foil is held constant throughout the anodization under a constant temperature of 75 °C. Uncontrolled temperature will oxidize the foil at a much higher rate it is important to keep the temperature constant during the entire etching process. After anodization, the Ti foil metallic color change into a blue color upon rinsing and air drying. The color change indicating the success of the anodization experiment with the etching solution to create TiO₂ and the formation of the nanocavities. An annealing process post anodization was done in open air furnace by rising the temperature to 500 °C at 5 °C/min for 3 hours and allowed to cool down to room temperature. After etching and annealing of the foil the TiO₂ is ready for the Au deposition.

Au Deposition

The deposition of the gold layer was carried by Thermionics E-Beam Evaporator. A 20nm of Au film was deposited on the surface of TiO₂ with a deposition rate of 0.1Å/s. The deposited Au layer formed a dimerization around the cavities (figure 2d). Once the layer of Au is
placed on the electrode, the film was pressed at 90°C for 5min, it is believed that the pressing treatment prior to thermal CVD initiate the encapsulation of the gold nanoparticles into the nanocavities. The thermal CVD process was carried using CVD to secure the gold particles inside the TiO₂ cavities. The applied heat inside the opened system of CVD mobilizes the deposited gold with the help of the flowing inert gas to be inserted inside the 50nm pores on the surface of TiO₂. The CVD temperature parameters were set to 5°C/min up to 500°C and held constant for 4hours then naturally cool down to room temperature. N₂ gas was used as the inert gas for the experiment at a flow rate of 80sccm. The end product after Au deposition and annealing followed by CVD is an encapsulated heterostructure of Au-TiO₂.

Imprinting Polymerization Reaction
The surface chemistry modification of the gold nanoparticles in the TiO₂ is a significant step for conjugating the creatinine molecule prior to the imprinting polymerization reaction. The first step of the surface chemistry modification was the installation of a carboxylic acid functional group on the surface of the immobilized gold nanoparticles[17]. The carboxylation was carried using 45mg of carboxylic-polyvinyl-chloride in 20mL of dioxane. The mixture was applied to the surface of 4 electrodes with an area of 4cm². The carboxylic-polyvinyl-chloride provided a carboxylic acid functional group on the surface of the gold nanoparticles for the coupling reaction with the amine functional group of the creatinine analyte[17]. The coupling reaction was carried using 30mg of 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide (EDC) water-soluble reaction and 10mg of N-Hydroxy-succinimide in 30mL of DI water. The introduced EDC to the surface of the gold nanoparticles reacts with the carboxyl group forming O-Acylisourea as an intermediate[17-20]. The unstable intermediate then reacts with the N-Hydroxy-succinimide
forming a great leaving group that can be replaced by a nucleophilic substitution reaction using the primary amine of the creatinine molecule[18]. Creatinine structure can undergo tautomerization however, the nucleophilic substitution reaction with the formed leaving group will only favor the primary amine structure of creatinine to attack the electrophilic carbocation [21, 22]. To conjugate the creatinine to the carbonyl group, a 40mg of creatinine was dissolved in 100ml of PBS at physiological pH then filtered before immersing in solution. Once the electrodes are in the creatinine solution the nucleophilic substitution reaction takes place and nourishment of creatinine to the gold nanoparticles begins. The solution was allowed to react overnight at 15°C then washed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid to remove the byproducts and neutralize any reactive functional groups on surface of the electrodes by potentiation. The polymerization reaction around the creatinine was initiated using monomers of 30mg of Acrylamide, and 100mg N’,N’-methylenebisacrylamid[22], then 40mg of ammonium persulfate was added for the purpose of generating the radical initiators for the polymerization reaction to take place around the creatinine molecule[23, 24]. Once the acrylamide monomers are initiated they form a straight linear chains of polyacrylamide on surfaces of the electrode that contain the previously conjugated creatinine, while the N’,N’-methylenebisacrylamid act as linker between the linear chains of polyacrylamide to allow a tight uniform polymerization reaction around our target analyte[17]. The polymerization reaction take place in 50mL PBS solution with 1mL of TetraMethylEthylenediamine to speed up the polymerization reaction and free radical initiation [25, 26]. The polymerization reaction was stored at 15°C for 6 hours to allow the crosslinking between the monomers and the imprinting of the structure to take place. To obtain the final heterostructure of IP-Au-TiO₂, the bounded creatinine was extracted from the polymer by suspending the electrode vertically by a tweezer in ethanol and water for 3 hours
while stirring with high speed at 35 °C. After the extraction step the 4cm² biosensor was cut into 4 equal pieces to keep the area constant throughout the electrochemical measurements using EIS and DPV.

Non-Imprinted Biosensor

The non-imprinted sensor was fabricated following the same protocol of IP-Au-TiO₂. However, the NI-Au-TiO₂ lacking the creatinine conjugation step. After the 20nm gold deposition and thermal CVD, the Au-TiO₂ electrode was treated with carboxylic-polyvinyl-chloride followed by the polymerization reaction similar to IP-Au-TiO₂. The initiation and propagation of the polymerization reaction using ammonium persulfate with the monomer precursors in the absence of the creatinine molecule will not generate the imprinting structure above the gold nanoparticles. The purpose for fabricating the NI-Au-TiO₂ biosensor was to compare the sensitivity and selectivity of the IP-Au-TiO₂.
CHAPTER FOUR: FINDINGS

The anodization and gold deposition process were characterized using a scanning electron microscope (figure 2). The electro-etching of Ti foil using H₃PO₄ and HF oxidize the surface of the electrode forming TiO₂ with 50nm cavities (figure 2 a,b,c). The porous structure increases the surface area of the electrode and provides host cavities for the gold nanoparticles immobilization. A 20nm layer of Au was deposited on the TiO₂ (figure 2 d) the layer shows a connective network of gold around the cavities and inside the pores. However, after thermal CVD using CVD the gold layer is expected to be encapsulated inside the pores due to the constant gas flow and the applied heat to the sample [27]. Further characterization of the sensor was realized using UV-VIS during fabrication (figure 3). The absorbance spectra of TiO₂ show an exponential decay behavior response. Comparing the absorption spectrum of the oxide TiO₂ to Au-TiO₂, the spectrum shows a peak at 400nm due to the absorbance of the gold nanoparticles. After the polymerization reaction, the spectrum of IP-Au- TiO₂ showed two peaks the first at 275nm and the gold nanoparticles peak at 400nm. The difference in the absorbance spectrum response during fabrication promotes the achievement of the surface chemistry manipulation. The XPS spectrum (figure 4) shows the response of Au-TiO₂ as a way of characterizing the composition of the biosensor after anodization and Au deposition. The XPS hv interact with the surface atoms of the electrode and measure the photo ejected electrons on the surface of Au-TiO₂ to provide the survey peaks. The binding energy shows a narrow two peaks of O1s representing metal oxide of TiO₂. The Ti binding energy was identified by the peaks between 475eV and 460eV while the Au with valance state 0 of the nanoparticles are shown by two peaks at 90eV and 80eV. Based on the UV-VIS and XPS spectrum characterization we can
Cyclic voltammetry (CV) was used as the main characterization tool for the electrode during fabrication (figure 5). Based on the CV measurements, the voltammogram shows no significant peak after anodization but following the Au deposition and thermal CVD two massive peaks were obtained around -0.25V, and -0.5V. The recorded peaks are corresponding to the heterostructure of Au-TiO₂ compare to the oxidized Ti foil. The change in the magnitude of the peaks indicates the success of the experiments after each step of the fabrication because the surface chemistry of the sensor becomes distinct from the previous structures. From the voltammogram, it is important to point out the peaks of the creatinine conjugation, polymerization, and the extraction step. The peak at -0.5V becomes silent after the polymerization reaction, the observed disappearance of the peak is due to the confinement of the electrons in the gold nanoparticles trapped between the polymer and the creatinine molecule causing the loss of the peak in the voltammogram. The peaks were reobserved after the extraction steps indicating the availability of open channels created by the creatinine. The opened channels in the polymer allow the electrons to become mobile and free to transfer between the interface of the Au-TiO₂ and the solution mixture.

**Measurements**

The detection strategy for creatinine was done using CH instrument electrochemical workstation using three cell electrodes. The electrolyzer contain 1cm² of the fabricated sensor as a working electrode, Pt foil as a counter electrode, and silver reference electrode. For testing the sensors, the analyte was devolved in PBS buffer containing 0.01M of potassium ferrocyanide at physiological pH using five different concentrations of 0.1ng/mL, 1ng/mL, 10ng/mL, 100ng/mL,
and 1µg/mL. The PBS solution with the desire concentrations provided an indirect oxidation of
the analyte using DPV and EIS [28, 29]. The modes of DPV and EIS was the preferred
measurement of choice due to the easier correlation between the concentrations and the fast
scanning of the samples. For the DPV measurements (figure 6) a potential voltage was applied
from -0.3V to 0.8V with a scanning rate of 1×10⁻³ V/s. The reported data of IP-Au-TiO₂ shows
strong sensitivity for creatinine with a trend of decreasing current as the concentration increases
from 0.1ng/mL to 1µg/mL (figure 6a). A calibration curve was derived from the DPV data of IP-
Au-TiO₂ (figure 6b). To obtain a positive slope of the calibration curve each measured
concentration was correlated to the blank sample at 0ng/mL of creatinine. The correlation of
DPVs was reported by subtracting the current of the blank sample from the measured
concentration and divided the difference in current by the blank current. The current data was
plotted in the y-axis while the x-axis represent the log concentrations in ng/mL. The calibration
curve showed a great linearity with an R²=0.9991 with a limit of detection (LOD) of
0.2176ng/mL. The sensor performance was also measured using EIS by applying a small
alternating current of 5mV then record the Nyquist plots (figure 7a). Based on the measured
concentration of the Nyquist plot, the charge transfer resistance increased as the concentration
increase. The increasing trend of the semicircle diameter of Nyquist plot is due to the capacitance
and resistance behavior of the fabricated IP-Au-TiO₂, as the concentration become greater the
resistance between the interface of the electrode and solution increases as more analyte bind to
the electrode. The higher concentration blocks the surface of the electrode causing the charge
transfer to increase. An equivalent circuit was realized for the IP-Au-TiO₂ (figure 8). The
creatinine concentration behaves as resistors, while the biosensor act as a capacitor in parallel to
resistor as the equivalent circuit representation based on the Nyquist plot fitting. The EIS charge
transfer resistance was plotted versus the log concentration of the analyte in ng/mL to derive a calibration curve (figure 7b). The calibration curve showed an $R^2$ value of 0.9998 with a detection limit of 0.09493 ng/mL. Based on the calibration curve and the linear relationship between the charge transfer resistance and the log concentration of the analyte, other concentrations can be directly quantified.

The IP-Au-TiO$_2$ response was also measured in the presence of interfering molecules at similar concentrations using DPV and EIS (figure 9a,b). Both calibration curve curves show a good linearity with an $R^2$ of 0.9968 and 0.9998 for DPV and EIS, respectively. It is important to note that both calibration curves changed in magnitude in the presence of interfering molecules but sustained good linearity. The change in the slope and magnitude of the curve is due to the higher resistance nature of the solution with interfering molecules compared to the resistance of creatinine solution alone.

The NI-Au-TiO$_2$ was tested as a control experiment for IP-Au-TiO$_2$ (figure 10). The DPV data for the ranging concentration of 0.1 ng/mL to 1 µg/mL showed a superimposable measurement between the varied concentrations. This observation illustrated the lack of sensitivity using NI-Au-TiO$_2$ due to the absence of the imprinting step of the creatinine. The lack of the specific binding site is the reason behind the overlapping measurements and inhibition of the electron transfer at the interface. Instead, the sensor reacted similarly to all measured concentrations due to the polymer acting as a barrier for the interface of the electrode.

The EIS data also supported the DPV observation for NI-Au-TiO$_2$ (figure 11). The Nyquist plot shows a semi-linear correlation between the measured concentrations indicating only diffusion is accruing at the interface of the electrode. The electrode acting as a Warburg impedance for EIS rather than the equivalent circuit presented for IP-Au-TiO$_2$ [30]. Based on this
observation we can conclude that the non-imprinted polymer on the Au-TiO\textsubscript{2} inhibit the sensing properties obtained by imprinted polymer.

LSPR Response

The Au-TiO\textsubscript{2} sensor showed no specificity for creatinine with overlapping data for DPV measurements. However, when the sensor was used as a photoelectrochemical biosensor the LSPR phenomenon substantial enhanced the signals and increased the sensitivity under solar light. The DPV measurements for the varied concentrations provided a similar trend to IP-Au-TiO\textsubscript{2} in presence of LSPR (figure 12a). The increased sensitivity of the Au-TiO\textsubscript{2} in the presence of LSPR provided a faster acceleration of the charge separation at the interface of the gold-particles and increase in the electrons transfer rate. The incident solar light generates a resonant condition of a propagating wave through the interface of the periodically patterned Au-nanoparticles surface creating a natural oscillation frequency. The natural oscillation frequency was most effective under blue monochromatic light which enhanced the oscillation amplitude, the UV-vis shows and observation at 400nm. The generated dielectric evanescent field on the Au-TiO\textsubscript{2} serve as an advantage comparer to NP-Au-TiO\textsubscript{2} by showing a decrease in current as the concentration of creatinine increases for the purpose of generating a calibration curve(figure 12b). The $R^2$ value obtained from Au-TiO\textsubscript{2} using DPV was 0.9969 with a limit of detection of 0.4086ng/m. The LOD obtained using LSPR is slightly lower than the IP-Au-TiO\textsubscript{2} of 0.2176ng/mL. However, both values exceed the LOD of Jaffé procedure and enzymatic detection of 2500ng/mL and 700ng/mL respectively [31]. The biosensor response using EIS was also enhanced using LSPR (Figure 13a). A linear calibration curve was obtained with an $R^2$ value of 0.9995 and a LOD of 0.1603ng/mL (figure 13b). The issue with LSPR-Au-TiO\textsubscript{2} is selectivity,
the EIS and DPV data was not reproducible in the presence of interfering molecules (figure14). The biosensor suffered from the limitation of selectivity which can be a major issue regarding analytical specificity for creatinine. The interfering molecules calibration curve of LSPR-Au-TiO₂ was derived from an average of five consecutive EIS and DPV measurements. Overall, the IP-Au-TiO₂ biosensor showed a superior analytic detection with high specificity and selectivity compare to NI-Au-TiO₂, and LSPR-Au-TiO₂ because of imprinted polymer acting as specific binding site for creatinine.
Figure 1: Schematic representation for the fabricated heterostructure of MI-Au-TiO2: step 1, Chemical etching via anodization process; step 2, 20nm Au layer deposition via Thermionics E-Beam Evaporator; Step 3-4, Pressing treatment at 90 C followed by thermal CVD; Step 5, conjugation of a carboxylic acid to the Au particles; Step 6, water soluble EDC reaction for binding creatinine to carboxylic acid; Step 7-8, initiation of molecular imprinted polymerization reaction around the creatinine followed by extraction.
Figure 2: SEM images a-b) Post anodization of Ti forming periodically patterned TiO\textsubscript{2} nanocavities; c-d) Post Au deposition but Prior pressing treatment at 95 °C

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Figure 5: a) Cyclic voltammetry measurements during the fabrication steps b) Zoomed image for peaks analysis.
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Figure 7: a) Nyquist plot of Electrochemical Impedance Spectroscopy measurements using IP-Au-TiO₂ for concentration ranging from 0.1ng/mL-1 µg/mL. b) EIS Calibration curves of IP-Au-TiO₂.
Figure 8: Equivalent circuit representation model.

Figure 9: Calibration curves of IP-Au-TiO₂ in the presence and absence of interfering molecules  a) EIS calibration curve b) DPV calibration curve.
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Figure 14: Calibration curves of Localized Surface Plasmon on Au-TiO$_2$ in the presence and absence of interfering molecules a) EIS calibration curve b) DPV calibration curve.

Figure 15: Calibration curves of IP-Au-TiO$_2$ in the presence and absence of interfering molecules a) EIS calibration curve b) DPV calibration curve.
CHAPTER FIVE: CONCLUSION

In this study we presented the fabrication and characterization of a periodically patterned Au-TiO$_2$ with imprinted and non-imprinted polymer for creatinine sensing. The detection method involves electrochemical and photoelectrochemical sensing platforms using DPV and EIS measurements for quantifying the concentrations ranging from 0.1ng/mL to 1µg/mL. Both IP-Au-TiO$_2$ and Au-TiO$_2$ show good analytic detection for creatinine with good linearity of the calibration curve and reusability over 20 weeks. The Au-TiO$_2$ showed the greatest response under blue monochromatic light due to the natural oscillation frequency of LSPR providing higher sensitivity for quantifying creatinine. However, the LSPR-Au-TiO$_2$ was surfing from specificity in the presence of interfering molecules urea, glucose, lactose, L-valine, and Glutamic acid due to the lack of specific binding sites. The Detection limit of IP- Au-TiO$_2$ was calculated to be 0.0949ng/mL and 0.218ng/mL for EIS and DPV, respectively. The imprinted polymer provided a higher level of specificity similar to a lock and key model of enzymes substrate interaction. Only the targeted creatinine is able to bind the biosensor while other molecules are ignored.
LIST OF REFERENCES


