SPECIFIC AND NON-SPECIFIC BINDING OF PROTEINS AND NUCLEIC ACIDS ON CHEMICALLY MODIFIED RETICULATED VITREOUS CARBON ELECTRODES

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SPECIFIC AND NON-SPECIFIC BINDING OF PROTEINS 
AND NUCLEIC ACIDS ON CHEMICALLY MODIFIED 
RETICULATED VITREOUS CARBON ELECTRODES

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment of the
Requirements for the Degree
Master of Science
Chemistry

by
Hemangi Y. Shedge
May 2009

Accepted by:
Dr. Stephen E. Creager, Committee Chair 
Dr. Anthony Guiseppi-Elie 
Dr. George Chumanov
ABSTRACT

Proteins and nucleic acids play major roles in many important biological processes. Specific and rapid detection of these biomolecules is important for early and precise diagnosis of various genetic diseases and other medical conditions. However, accurate and inexpensive detection of proteins and nucleic acids has always been a challenge. One of the obstacles is their non-specific adsorption on the sensor surface which leads to the conformational changes in the proteins, sometimes causing their denaturation. The non-specific adsorption on the surface also leads to sensor fouling which induces changes in the availability of active sensor surface for detection of analytes and thus decreases the sensor response. Non-specific binding of biomolecules is therefore a major drawback of biosensor applications and its elimination or suppression needs to be carefully considered while developing biosensors.

The present work discusses various strategies to modify the surface of reticulated vitreous carbon (RVC) electrodes to suppress the non-specific binding of biomolecules. Both non-specific (NSB) and specific binding (SB) of neutravidin, oligonucleotides and enzyme labels were studied inside the pores of RVC electrodes that had been subjected to various modification schemes. The extent of NSB and SB of these biomolecules onto unmodified as well as modified RVC electrodes was compared by determining the initial rate of generation of an enzymatic product using linear scan voltammetry (LSV). A simple sandwich bioassay for detection of neutravidin (a deglycosylated version of avidin) was performed on the RVC electrode with the lowest NSB. The surface coverage
of these biomolecules inside the RVC pores was estimated by spectroscopic as well as by electrochemical method.
DEDICATION

I dedicate this work to my mother, Mrs. Kunda Yeshwant Shedge. I am indebted to her as it was not possible for me to reach at this position without her struggles and patience. Her love, support, and guidance nurtured and inspired me throughout my life. I am eternally grateful and wonderfully blessed to have her as my mother.
ACKNOWLEDGEMENTS

Firstly, I would like to thank my advisor, Prof. Stephen E. Creager for his guidance during these years. His patience and support helped me overcome many crisis situations and finish this dissertation.

I would especially like to thank my husband, Amit Palkar for his support which encouraged me to pursue and finish my Masters degree, especially during difficult times. His support and care helped me overcome setbacks and stay focused on my graduate study. His unwavering, unconditional love and quiet patience were undeniably the substratum upon which the past twelve years of my life have been built.

I am grateful to all my friends in Clemson and in India who have helped me stay sane through these difficult years and made my journey enjoyable. I greatly value their friendship and deeply appreciate their belief in me.

I am also thankful to my colleagues, Ashwin Rao, Nihar Ranjan, Jung min-oh, Rajgopal Rama and others who helped me during my Masters program.

I would sincerely like to thank my committee members, Prof. Anthony Guiseppi-Elie and Prof. George Chumanov for their time and help during the completion of this dissertation.
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CHAPTER ONE

1. INTRODUCTION

1.1 Electrochemical biosensors

Electrochemical biosensors consist of a biological entity that recognizes the target analyte and an electrochemical transducer that converts the bio-recognition event into a useful electrical signal. The fundamental principle of electrochemical biosensor operation is based on the production or consumption of ions or electrons which causes some change in the electrical properties of the solution which can be used as measuring parameter [1]. The electrochemical biosensor can be categorized based on either the immobilized bioelements such as enzymes, nucleic acids, antibodies, whole cells and microorganisms or on the basis of the measurement of electrical parameters like, amperometric, potentiometric and conductimetric signals (Figure 1.1).

![Figure 1.1 Schematic representation of the electrochemical biosensor.](image-url)
Electrochemical sensors have distinct advantages over other analytical systems in that they can be operated in turbid media while offering comparable instrument sensitivity. They are more amenable to miniaturization and are inexpensive compared to most other analytical techniques. Furthermore the equipment required for electrochemical analysis is simple and the continuous response of electrode system allows for an on-line control. Modern electro-analytical techniques offer very low detection limit (typically $< 10^{-9}$ M) using small volumes (1-20 µL) in a short period of time [2]. One example of an electrochemical biosensor is a handheld, home-use glucose monitoring device for diabetes treatment, which consists of a screen-printed enzyme electrode coupled to a pocket-sized current meter and easy-to-use self-testing glucose strips [3]. Such disposable enzyme electrodes generate the analytical information within 5–10 seconds using only 0.5–10 µL blood samples [4]. Similar to the glucose sensor there are many handheld devices in market, such as battery-operated clinical analyzers for rapid detection of multiple electrolytes and metabolites [5], blood gas analyzers [6], non-invasive hand-held biosensors for clinical diagnosis of periodontal disease [7], and automated handheld sensors for detection of carcinogens in agricultural products [8]. The development of implantable electrochemical sensors has also opened up completely new opportunities for more accurate, permanent and real-time measurements. Many implantable electrochemical sensors have been developed, examples of which are amperometric bio-chips for continuous in-vivo detection of glucose and lactate [9, 10], highly selective in-vivo detection of NO using natural biopolymers [11], simultaneous detection of catachol
and caffeine using implanted neuro-sensors [12], microfabricated implants in a variety of fields including drug delivery, tissue engineering and in-vivo biosensing [13].

A common element in most electrochemical biosensors is the presence of a suitable label that provides an electroactive substance for detection by the transducer. Enzyme based platforms have been popular for many years in the field of biosensors [14, 15]. Because of their high specificity, selectivity and catalytic (amplification) properties, enzymes have found widespread use as sensing elements in biosensors [16]. In an enzyme biosensor the enzyme is combined with a transducer to produce a signal that is proportional to target analyte concentration. This signal can result from release or uptake of gases, changes in proton concentration, or production or consumption of electro-active substance resulting from the enzyme catalyzed reaction. The transducer then converts this chemical signal into a measurable electrical response. The first enzyme-based sensor was developed by Clark and Lysons in 1962, who immobilized glucose oxidase onto an oxygen-sensing electrode to measure glucose concentrations [17]. Since then, there has been a remarkable growth in applications involving enzyme-based sensors with different substrates. A great number of enzymes belonging to class of oxido-reductase, hydrolase, lyases and ligase have been incorporated with transducers into various biosensors for applications in health care, medicines, food industry, environmental monitoring and monitoring of various metabolites in biological systems [18]. The first portable electronic device for sensing the blood sugar level utilized, glucose oxidase as an enzyme label. Enzyme based biosensors have also been widely used for detection of antibodies, nucleic acids, and microorganisms.
In electrochemical techniques one of the most important elements of a system is the working electrode on which the reactions of interest occurs. Therefore, the choice of working electrode is a vital choice for the experiment. In electrochemistry the electrode reactions occur over a wide range of potentials. It is necessary that these reactions must fall within the potential range of the working electrode without any interference from the electrode itself. Most common working electrode materials are carbon, gold, platinum, silver, and mercury drop and film electrodes [19]. Of these electrodes, carbon is often the material of a choice because of its inertness and low cost compared to other materials. It has good electrical and thermal conductivity, and high mechanical and dimensional stability. It is easy to handle and is available in a variety of physical structures and can easily be fabricated into composite structures [20, 21]. Various forms of carbon like glassy carbon, pyrolytic carbon, carbon black, carbon fiber, carbon nanotubes, fullerenes, HOPG and graphite have been used in many applications including in batteries, fuel cells, capacitors and sensors [21-23]. Glassy carbon is widely used as the electrode material in electrochemistry. Glassy carbon is an isotropic form of vitreous carbon which combines glassy and ceramic properties with those of graphite. It has very good electrical conductivity with a broad potential window compared to that of graphite [24]. It shows high resistance to corrosion and thermal shocks. Glassy carbon has high surface quality with excellent polishing characteristics [25]. It shows resistance in inert gas/vacuum up to 3000 °C and in air up to 600 °C. The surface of glassy carbon is biocompatible and can be modified with various functional groups [26, 27]. But unlike
other forms of carbon such as carbon black and active carbons, solid glassy carbon has comparatively low surface area and is not porous.

1.2 Reticulated Vitreous Carbon (RVC)

In the past few years a different form of glassy carbon has been widely used in many electrochemical applications. Reticulated vitreous carbon (RVC) is a porous glass-like carbon which combines some properties of glass with that of normal industrial carbon (Figure 1.2). The most common method used for synthesizing RVC is by impregnating a precursor with a thermosetting resin, followed by carbonization. The precursor foam is an open-cell polyether-based polyurethane foam consisting of a network of struts with continuous air passages. The resins used for impregnating polyurethane foam are generally epoxy/phenolic resins or furfuryl alcohol. The foam resin is typically dried and cured at 120 °C, followed by carbonization at 700-1000 °C [28, 29]. The RVC can easily be machined into electrodes of various geometrical shapes and sizes.
One advantage of using RVC in bioassays is that RVC provides high surface area per volume of analyte solution, which facilitates better analyte capture via surface-bound bioaffinity agents. Another advantage is that RVC provides a low resistance to fluid flow which facilitates surface washing steps which are nearly always required in bioassays. Use of RVC also prevents diffusive losses of redox-active analytes or redox-active molecules generated via labeling. This latter attribute can allow for accumulation of redox-active species in confined spaces, thereby decreasing the analysis time and improving detection limits for analyses that involve detection of redox-active molecules. RVC has high void volume up to 95-97% compared to solid glassy carbon (0%), which results in low pressure drop across in flowing streams [30]. The various comparative properties of RVC foam with solid glassy carbon are given in Table 1.
Table 1.1 Physical properties of RVC in comparison with solid glassy carbon [31]

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<td>Density (g cm(^{-3}))</td>
<td>0.048</td>
<td>1.65</td>
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<tr>
<td>Pore size (ppi)</td>
<td>5 - 100 ppi</td>
<td>-</td>
</tr>
<tr>
<td>Void volume (%)</td>
<td>95-97</td>
<td>0</td>
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<td>Specific resistivity (Ohm cm)</td>
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<td>0.001</td>
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<td>0.01-0.02</td>
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<td>Tensile strength (kg cm(^2))</td>
<td>3-12</td>
<td>2.3 x 10(^3)</td>
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<td>Hardness (Ω(^{-1}))</td>
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The electrochemical behavior of various redox molecules has been studied using RVC as an electrode. Sorrels’ et.al studied the electrochemical behavior of K\(_3\)Fe(CN)\(_6\) on RVC electrodes with different pore size distributions ranging from 30 ppi to 100 ppi. It was observed that on RVC electrode with 60, 80 and 100 ppi, the ferricyanide cyclic voltammetric redox current approaches zero after passing through an anodic or cathodic peak maximum when scanned at relatively slow rates (2mV s\(^{-1}\)) [32]. This behavior is a characteristic of thin-layer cells. In thin-cell systems the difference between cathodic and anodic peak potential is zero (\(\Delta E_p = 0\)) [33]. However, on the RVC electrode, \(\Delta E_p\) value was observed to fall in the range of 70 mV to 200 mV, depending on the scan rates. This
deviation from the thin cell system is attributed by Sorrels et. al to the non uniform current distribution and ohmic potential differences across the RVC.

The large surface area and high porosity of RVC material has attracted great interest, particularly for electrochemical applications. Wang et. al published an early review on RVC as a potential electrode material in electrochemistry, particularly in sensor development [31]. The RVC electrode has been used as a platform in many applications involving detection of glucose in blood plasma [34], NO$_2$ in air [35], and dopamine in the presence of ascorbic acid [36]. Along with sensor applications, RVC has also been used in various cell configurations for removal of metal ions from waste water and recovery of metals in mixed metal ion solutions [37, 38]. RVC has also been used in flow-through systems because of its porous nature [39, 40]. In the past few years, researchers have also reported on the use of RVC material in fuel cells and batteries [41, 42]. The high surface area, porosity, high current densities, high temperature resistance and inertness of RVC makes it an attractive material in many electrochemical applications.

1.3 **Factors affecting electrochemical biosensing**

Proteins and nucleic acids play major roles in many important biological processes. Specific and rapid detection of these biomolecules is important for early and precise diagnosis of various genetic diseases and other medical conditions. There are several techniques for studying and detecting these biomolecules such as western/southern blots, microscopy, spectrophotometry and enzyme assays [43, 44]. However accurate and inexpensive detection of protein and nucleic acid molecules has
always been a challenge. One of the obstacles while detecting these molecules is their adsorption onto solid surfaces such as storage containers, reactor walls, microwell plates, and sensor surface [45]. Adsorption of proteins on these surfaces often leads to conformational changes of protein molecules, sometimes causing denaturation and loss of activity. Also, the presence of irreversibly bound molecules on a sensor can make the measurement of other specific molecules difficult.

Protein interactions at solid-water interfaces is a complex process that depends upon many factors such as type of surface, protein structure and concentration, temperature, time, pH, ionic concentration and buffer composition. Many theories have been proposed to understand the adsorption kinetics and thermodynamics of proteins on the surfaces [46]. Protein interaction with the surfaces can be specific and/or non-specific. The specific binding is described as the high affinity binding of molecules to the receptors of interests on the surface and it can be in the form of affinity, hydrogen or covalent binding. An example of affinity binding is the conjugation between avidin and biotin. Avidin is a tetrameric protein with four identical subunits each of which can bind to biotin (Vitamin B7) with a high degree of affinity and specificity. The dissociation constant ($K_D$) of the avidin-biotin complex is around $10^{-15}$ M which makes it one of the strongest known non-covalent bonds. Another type of specific binding is the hybridization between two single strands of DNA, which are complementary to each other via their matched base pairs. The DNA duplex is stabilized by the hydrogen bonding between bases present on each of the complementary strands of DNA [47]. [48].
However, specific binding is often accompanied by unwanted physiorption of proteins or nucleic acids under study. Non-specific binding (NSB) is the binding of molecules to the sites other than receptors of interests. The non-specific adsorption results from the various forms of interactive forces between proteins and surfaces such as hydrophobic/hydrophilic, Van der Waals forces, hydrogen bond and ionic interactions [49, 50]. The driving force behind protein adsorption is the energies associated with interactions of protein with surface, interactions of surface with water and interactions of protein with water [51]. These interaction energies could be either enthalpic or entropic or both. The enthalpic energies are dominated by the dispersive forces such as hydrophobic interactions, and the polar interactions such as dipoles, hydrogen bonding, and charge interactions. The entropic energies are associated with the water effects from both proteins and surfaces. The adsorption of proteins occurs on the surface when the energy of the adsorbed system is lower than the energy of the solution system. It has been observed that the protein adsorption on many surfaces occurs due to the interactions between the surface and the hydrophobic regions of the proteins. It has been well established that hydrophobic surfaces have a strong tendency to adsorb proteins irreversibly [52, 53]. The driving force for this action is unfolding of proteins on the surface which releases hydrophobically-bound water molecules from the solution-surface interfaces. This leads to a large entropy gain for the system. The presence of hydrophilic groups on the surface however results in binding of water molecules to the surface and thus reduces the free energy for adsorption of proteins. As the surface energy increases, the energy available from hydrophobic interactions decreases and hence adsorption of
proteins on hydrophilic surface is negligible compared with that on hydrophobic surface [54, 55]. Protein adsorption also depends on the orientation of protein available for adsorption. It is possible that the molecule arriving in one orientation may not bind to a surface, but it can bind very strongly to the surface in another orientation [56]. The adsorption of biomolecules on solid surfaces also depends on the charge present on both the biomolecules and the surface. Since proteins have both negative and positive charges, it can bind to the surface with opposite charge.

The non-specific adsorption of proteins and nucleic acids is the main cause of denaturation and loss of molecular functions. The non-specific adsorption of proteins on the surface leads to sensor fouling which induces changes in the availability of the sensor surface to analyte [57]. This alters the sensor response, making precise and specific detection of other biomolecules difficult (Figure 1.3). For example in test like ELISA, non-specific adsorption of antibodies-enzyme conjugates to the microwell plates instead of immobilized antigens gives rise to a false signal [58].

Figure 1.3 Non-specific binding of enzyme-conjugated antibodies and enzyme-conjugated nucleic acid probes onto the sensor platform.
Also in the case of analyte measurements in whole blood, blood plasma, or serum, the sensor response is often affected by non-specific binding of serum proteins onto the sensor surface [59, 60]. Adsorption of cells, proteins and other biological components onto the sensor membrane leads to membrane fouling and is one of the major causes of failing of implantable biosensors. Studies involving in-vitro protein and blood adsorption and in-vivo microdialysis studies have clearly shown detrimental effects of membrane biofouling on analyte transport and decreased sensor response [61, 62]. It has been observed that the presence of non-specific binding of immunoglobulins onto the sensor surface greatly influences the specific antibody-antigen binding kinetics [63-65]. In nucleic acid bioassays suppression of NSB of DNA onto sensor surfaces is essential for achieving sequence-specific DNA detection [66]. Non-specific binding of biomolecules is therefore a major drawback of biosensor application and its elimination or suppression needs to be considered seriously while developing biosensors.

1.4 Suppressing non-specific binding

In most biosensor applications, to improve the sensor response it is important for biomolecules to bind to a specific spot and in specific orientations. Minimizing NSB of molecules to maintain high signal-to-noise ratio is therefore one of the most important goals in sensor technology. Protein adsorption onto solid surfaces is influenced by the physical and chemical characteristics of the surface as well as the sample under test.

The rate and extent of protein adsorption onto a sensor surface can be altered by changing the surface chemistry of the sensor, modifying protein concentration and also varying ionic strength, pH and temperature of buffer solutions. Many attempts have been
made to eliminate the non-specific adsorption of biomolecules. One common way of suppressing NSB is through the use of commercially available blocking buffer such as Superblock RTM, sea block buffer, blockerit, blocker casin, fish skin gelatin, and bovine serum albumin (BSA) [67, 68]. Each of these blocking buffers imparts certain characteristics to the assay system and improves assay sensitivity and reduces background interference. Blockers are usually various additives that prevent nonspecific binding, but have no active part in the actual assay. Buffers containing 1-5% of these blockers are typically used as general purpose blocking agents. BSA-based blocking buffers are generally used in ELISA and western blot for suppressing NSB of immunoglobulins onto the sensor platform. Along with suppressing NSB, blocking buffers also help to stabilize the specifically bound proteins on the sensor surface by maintaining the optimal level of hydration. However, care must be taken while selecting the type and concentration of blocking buffer in bioassays. While developing any new bioassay, it is important to test several blockers as no single blocking agent is ideal for every case. Controlling the quantity of blockers is also essential as inadequate amount of blockers results in high background noise, while excessive concentration may mask the specific interaction of analyte with the receptor or inhibit the enzyme labels used in assay [69].

Another common approach for reducing NSB is to modify the surface in such a way that it resists the protein adsorption while enhancing the specific binding [70]. As discussed previously, the protein adsorption at biomaterial interfaces is related to the water of hydration present due to interfacial groups. Therefore the most obvious way of
suppressing the non-specific binding of proteins on the surfaces, is to make the surface more hydrophilic. Modification of surface with various neutral functional groups such as –OH, C=O etc has shown to suppress the NSB. Functionalization of the sensor surface can be achieved in various ways. Oxidation of carbon in gas phase increases the concentration of hydroxyl and carbonyl groups, while oxidation in liquid phase increases the number of carboxylic groups on the surface [71]. The oxidation of carbon can be achieved by treating the carbon with strong acids or by plasma or ozone treatment. Electrochemically assisted covalent attachment of molecules onto the surface is another way to modify the surface. Alcohols such as 1-butanol, ethylene glycol, triethylene glycol, 1, 2-propane diol etc. have been grafted electrochemically on carbon surfaces [27]. The neutral hydrophilic nature of these groups resists the unwanted protein adsorption on the surface.

Surface with charged functional groups are also often used to prevent NSB of proteins [72, 73]. The chemical or electrochemical reduction of aryl diazonium salt leads to grafting of aryl groups onto the surface. A wide variety of diazonium salt with end groups like –COOH, -NO₂, and NH₂ have been employed to functionalize the surface [74-79]. These groups impart either positive or negative charges to the solid surface. Proteins and nucleic acids experience electrostatic forces at these charged surfaces and can become adsorbed onto or repelled from the solid surface [80].

Polymers with different functionalities have also been used to alter the sensor surfaces [81]. Water soluble polymers like polyethylene glycol (PEG) and PEG-like materials are the most common and effective compounds used against NSB [82-89]. PEG
and similar polymers can be attached onto a variety of surfaces using various methods like adsorptive chemistry [90-92], self-assembled monolayer (SAM) [93-95] and PEG grafting [96-99]. The resistance to protein adsorption on certain PEG surfaces has been related to water structuring around the PEG which makes the protein binding energetically unfavorable [70, 100, 101]. Many studies have been carried out on a surface coated with PEG and it was observed that the molecular weight and density of PEG are important factors controlling protein adsorption [102-104].

Charged polymers like Nafion®, poly(acrylic acid), poly(ethyleneimine), carboxylate dendrimers and other polyelectrolytes also show an ability to block protein adsorption. The suppression of NSB at these charged surfaces occurs due to various interactions between charged polymers and biomolecules [57, 105-108]. Layer-by-layer adsorption of polyelectrolytes has also been used to prepare anti-fouling coatings on various surfaces against protein adsorption [109-113].

Novel hydrogel networks have also been of interest as biomaterials or biomaterial surfaces. Because of their high water content (> 50%) relative to many polymers, the hydrogel matrix shows low non-specific absorption of biomolecules [114]. Various hydrogel networks like poly(ethylene glycol) methacrylate [115, 116], poly(2-hydroxyethyl methacrylate) [117], thermally responsive polymer hydrogel brushes like poly(N-isopropyl acrylamide) (polyNIPAM) [118], and polysaccharide cross-linked hydrogels like cellulose and chitosan [119], and carboxymethylated-dextran [120] have been used as platforms in biosensor applications. Although many hydrogel matrices show relatively low NSB of proteins, they also allow for penetration of some macromolecules
as the average interchain distances in a hydrogel matrix can be several nanometers. Therefore these hydrogel networks are often used for cell encapsulation and to provide large number of reactive sites for molecular attachment.

An advantage of modifying the sensor surface with various functional groups instead of using blocking buffers is that doing so, it not only helps to suppress NSB but can also provide various groups for anchoring biomolecules specifically onto the surface. The combination of low NSB and specific binding is ideal for most biosensor applications.

1.5 Objective and summary of work

The primary objective of the work described in this thesis was to develop a new platform for detecting biomolecules. Reticulated vitreous carbon (RVC) was as an electrode selected because its 3D porous network provides a high surface area for better analyte capture, a low resistance to fluid flow and it prevents diffusive losses of redox-active analyte. RVC is easy to fabricate in any shape and size for the electrode material and is inexpensive and disposable. The RVC was fabricated into various electrode designs and characterized and tested for their use in electrochemical biosensor application. The secondary objective was to eliminate the non-specific binding of proteins which is a major cause of reduced performance of biosensor devices. The RVC electrode was fabricated with various functional groups mainly for two purposes. First, to suppress the non-specific binding of proteins and nucleic acids onto the RVC electrode and second, was to attach biomolecules specifically onto its surface.
The next chapter discusses non-specific (NSB) and specific binding (SB) studies of two enzyme conjugates namely, neutravidin-conjugated alkaline phosphatase (NA-ALP) and biotin-conjugated alkaline phosphatase (B-ALP) onto unmodified as well as modified RVC electrodes. The non-specific binding of these two enzymes was studies on 1) bare (unmodified) RVC, 2) bovine serum albumin (BSA) coated RVC, 3) phenylacetic acid covered RVC, 4) polyelectrolyte modified RVC, and 5) poly(ethylene glycol) (PEG) functionalized RVC. For the specific binding of NA-ALP enzyme conjugate, biotin molecules were introduced onto RVC electrodes that are functionalized with 1) BSA, 2) phenylacetic acid, 3) polyelectrolytes, and 4) PEG. For the specific binding of B-ALP enzyme, first biotin molecules were introduced onto the functionalized RVC, followed by immobilization of neutravidin molecules. The enzyme B-ALP was then immobilized onto the surface at the available neutravidin binding sites. Both non-specific and specific binding of enzymes were studied by determining the initial rate of conversion of an electro-inactive substrate (hydroquinone diphosphate, HQDP) into electro-active product (hydroquinone, HQ) using cyclic voltammetry (CV) and linear scan voltammetry (LSV). The RVC electrode with lowest NSB and comparatively high specific binding was used as a platform to carry out a neutravidin sandwich bioassay. The electrode was modified with biotin-PEO-amine and was incubated in varying concentrations of neutravidin analyte. The B-ALP conjugate was used as an enzyme label and HQDP as an enzyme substrate. The rate of conversion of HQDP into HQ was detected by LSV.

The following chapter covers the non-specific and specific binding studies of oligonucleotides and the NA-ALP enzyme conjugate onto the RVC electrodes. The
electrode with relatively high specific binding and lowest NSB from the previous work was used a platform in this work. The phenylacetic acid functionalized RVC was used to co-immobilize an amine-terminated capture oligonucleotide and an amine-terminated PEG. The hybridization of biotinylated complementary oligonucleotide from solution with capture probe at the RVC surface was accomplished. The hybridization event was detected using NA-ALP enzyme label and HQDP substrate in a manner similar to that used for the neutravidin assay. The DNA assay was carried out with various concentrations of complementary oligonucleotides on PEG modified RVC electrodes. The control experiments in the absence as well as in the presence of oligonucleotide probes were carried out. The number of oligonucleotides inside the pores of RVC electrode was estimated by fluorescence spectroscopy as well as by an electrochemical method. For the fluorescence experiment, the complementary oligonucleotide probe used was fluorescein labeled. The surface coverage of DNA on the RVC electrode was compared with theoretical value and experimental values published in the literature.

1.6 References


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CHAPTER TWO

2. EVALUATION OF NON-SPECIFIC BINDING SUPPRESSION SCHEMES FOR NEUTRAVIDIN AND ALKALINE PHOSPHATASE AT THE SURFACE OF RETICULATED VITREOUS CARBON (RVC) ELECTRODES.

2.1 Introduction

In biosensors the non-specific binding (NSB) of biomolecules onto the sensor platform often results in high background signal which decreases the sensor performance. Before developing a sensor, therefore it is very necessary to eliminate or minimize the non-specific binding of biomolecules to improve the signal-to-noise ratio.

This work presents and discusses various strategies for modifying reticulated vitreous carbon (RVC) electrode surface to suppress non-specific binding while also promoting specific binding of proteins, particularly neutravidin. RVC shows various advantages over commercial glassy carbon electrode such as high surface area per volume of analyte solution, low resistance to fluid flow, low diffusive losses of analytes trapped inside the pores of RVC [1]. RVC can be easily fabricated into various geometries and size and is inexpensive compared to glassy carbon electrode. But, like glassy carbon, the surface of RVC is hydrophobic which tends to promote protein adsorption. Therefore to accomplish bioassays with RVC, it is necessary to modify the surface of RVC in such a way that it will reduce or prevent non-specific binding but at the same time facilitate specific binding of analyte molecules onto the surface.
Nine separate RVC electrodes were fabricated and each electrode was modified using a different method to suppress NSB. Some of the modification schemes also included surface grafting of biotin groups to allow for specific binding of avidin-containing molecules. These electrodes were then subjected to adsorption of neutravidin (NA), neutravidin-conjugated alkaline phosphatase (NA-ALP) and biotin-conjugated alkaline phosphatase (B-ALP) to test the extent of non-specific and specific binding. For cases involving ALP, the amount of ALP bound onto the RVC surface was compared for each modified RVC electrode by obtaining the initial rate of conversion of hydroquinone diphosphate (HQDP, an ALP enzyme substrate) into hydroquinone (HQ) by surface-bound enzyme. The initial rate was determined from analysis of multiple linear scan voltammograms obtained following exposure of the electrode to a solution of HQDP. Since the initial rate of HQ generated is directly proportional to the amount of enzyme present, it is possible to compare non-specific and specific binding of ALP-containing molecules onto RVC surfaces by measuring the initial rate of HQ production. The modified RVC electrode with lowest non-specific binding was then used as a platform for a neutravidin sandwich bioassay, using biotinylated ALP as an enzyme label to detect captured neutravidin. A schematic representation of this neutravidin sandwich bioassay is illustrated in Scheme 2.1 below.
2.2 Experimental

2.2.1 Materials, chemicals & instruments

Reticulated vitreous carbon (RVC, 100 ppi) foam used for making electrodes was purchased from ERG Materials and Aerospace Corp. Oakland, CA. All chemicals were used as received from the suppliers, unless otherwise mentioned. Bovine serum albumin (BSA), biotinylated-bovine serum albumin (B-BSA), neutravidin (NA), biotinylated alkaline phosphates (B-ALP), biotinyl-3, 6-dioxaoctanediame (biotin-PEO-amine, M.W. 374.50), neutravidin conjugated alkaline phosphatase (NA-ALP), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Pierce Biotechnology. Hydroquinone (HQ), potassium ferricyanide and ruthenium hexamine trichloride were obtained from VWR and poly(acrylic acid sodium salt) (PAA, M.W. ~50,000 Da) & poly(allylamine
hydrochloride) (PAH, M.W. ~70,000 Da) was obtained from Aldrich. Amine-terminated polyethylene glycol (PEG-amine, M.W. ~5000 Da) was obtained from Nektar Therapeutics (previously Shearwater Corp). Water was purified using Millipore MilliQ Ultrapure Water Purification System. Reaction buffer (pH 8) consisted of 200 mM Tris, 150 mM NaCl and 10 mM MgCl₂. MES buffer (pH 6) was prepared by dissolving 50 mM of 2-(N-morpholino) ethanesulfonic acid and 500 mM NaCl. All electrochemical experiments were performed using a CH Instruments Inc. model CHI 660A electrochemical workstation with aqueous Ag/AgCl as a reference electrode and Pt wire mesh as a counter electrode unless otherwise mentioned. Scanning electron microscopic images of RVC were obtained using a Hitachi S3500 scanning electron microscope.

2.2.2 Characterization of reticulated vitreous carbon foam

The RVC foam (100 ppi) was characterized using various techniques. The scanning electron microscope image of RVC foam was obtained on Hitachi S3500 variable pressure scanning electron microscope. The pressure in the chamber was brought down to 30 Pa and the electron beam voltage was set to 150 KV to obtain images. The average pores size of RVC was obtained from SEM image. The surface area of RVC foam was calculated from BET measurement in-house. Surface area per volume of RVC, bulk density, ligament density and bulk resistivity values were obtained from the supplier. The electrodes were fabricated from RVC foam as specified in section 2.2.4 and the electrochemical response of RVC electrodes were tested using redox probes (K₃Fe(CN)₆, Ru(NH₃)₆Cl₃ and HQ). Double layer capacitance of RVC electrode was measured by applying a cyclic potential of 0.0 V – 0.01 V against Ag/AgCl reference.
electrode at a scan rate of 5 mV s$^{-1}$ in tris buffer (pH 8). The current and scan rate values were used to calculate double layer capacitance using the equation, $\Delta i = 2\nu C_{DL}$, where $\Delta i$ is the difference in anodic and cathodic currents obtained from a voltammogram, $\nu =$ scan rate of the applied potentials and $C_{DL}$ is the double layer capacitance of the electrode. The capacitance per unit area of the electrodes was obtained by normalizing the capacitance with respect to surface area. The internal volume of RVC electrode was determined by calculating the difference in the weight of dry electrode and the electrode filled with water followed by conversion of weight to volume using density of water (1 g cm$^{-3}$).

2.2.3  **Pretreatment of RVC foam**

The RVC foam was pretreated prior to fabrication, to remove any impurities. A large RVC cylinder of about 1 cm diameter and 2 cm height was cut and was soaked in 6 M HCl for 24 hrs to remove any metallic impurities present. The cylinder was thoroughly washed with water to remove the acid. The pH of the effluent was checked periodically until it become neutral. The cylinder was then soaked in methanol for 2 hrs to remove any organic impurities. The RVC cylinder was dried under a brief flow of nitrogen and kept in a vacuum oven at 110º C overnight. The pretreated RVC cylinders were used to make electrodes.

2.2.4  **Fabrication of RVC electrodes**

The pipette-type RVC electrodes were prepared from pretreated RVC foam using a syringe needle, shrink tube, gold wire and a micropipette tip. A cylinder (2 mm diameter x 4 mm height) of 100 ppi RVC foam was cut using 12 gauge syringe needle.
Electric contact was made by wrapping a gold wire (0.12 mm diameter) around the RVC cylinder. The RVC cylinder was then connected to a micropipette tip using polyolefinic heat shrink tube. The photograph of pipette-type electrode and schematic representation of RVC electrode is given in Figure 2.1 below. All RVC electrodes were tested electrochemically on CH instrument prior to modifications.

Figure 2.1 a) Photograph of RVC electrode with micropipette b) Schematic of RVC electrode.
2.2.5 **Synthesis of hydroquinone diphosphate (HQDP)**

The enzyme substrate used in this study was hydroquinone diphosphate (HQDP). HQDP is not commercially available therefore it was synthesized in the lab by combining two published procedures [2, 3]. The new procedure is less tedious as compared to the previously reported methods and is less time consuming. Hydroquinone (207 mg) was reacted with two equivalents of phosphorous oxychloride (0.948 mL) and two equivalents of anhydrous pyridine (0.306 mL) in anhydrous toluene (18.8 mL, for 0.1 M HQ) over 30 minutes. The mixture was refluxed for an additional 30 minutes and allowed to cool to ambient temperature. Pyridinium chloride which was formed in the course of reaction was removed by filtration through a pad of diatomaceous earth and rinsed with small a volume of dry toluene. The filtrate was concentrated in vacuo at 40 °C. The residue was then dissolved in water and the solution adjusted to pH 8.5 by addition of cyclohexylamine. Acetone was then added to initiate crystallization of hydroquinone diphosphate cyclohexylamine salt which was then converted to sodium salts by precipitation from methanol using sodium methoxide. Both cyclohexylamine and sodium salts are suitable for use in electrochemical bioassay. $^1$H NMR (D$_2$O) $\delta = 6.9$ (s, aromatic), $^{31}$P NMR (D$_2$O) $\delta = 3.4$ $\lambda_{max} = 274$ nm, Yield 670 mg.

2.2.6 **Synthesis of 4-aminophenylacetic acid diazonium tetrafluoroborate salt.**

4-aminophenylacetic acid diazonium tetrafluoroborate salt was synthesized according to the published procedure [4]. 4-aminophenylacetic acid (1.51 g, 0.01 mol) was solubilized by warming in 3 ml of 12 M HCl and minimum amount of water. Once the compound was solubilized, the solution was cooled in ice and the light brown
hydrochloride was precipitated. Then 0.76 g (0.11 mol) of NaNO₂, dissolved in minimum amount of water, was added with stirring under a stream of nitrogen. As soon as the precipitate was dissolved, solid NaBF₄, (1.47 g, 0.013 mol) was added and the mixture was stirred for 30 seconds and cooled rapidly below 0 °C. The pale yellow precipitate was filtered in a cooled glass frit under a stream of nitrogen, washed with a cooled 5% NaBF₄ solution to remove traces of acid followed by washing with ether and finally stored over phosphorus pentaoxide.

2.2.7 Fabrication of RVC electrodes for non-specific and specific binding studies.

Nine RVC electrode types were fabricated to study non-specific and specific binding of various enzyme-containing molecules. The RVC electrodes type I to type V were modified so as to prevent NSB, and electrodes type VI to type IX were modified so as to prevent NSB while also allowing for specific binding of targets via biotin-avidin chemistry. The structure of all modified electrodes is illustrated in Scheme 2.2. The fabrication and characterization of these electrodes is described below.

**Type I:** The type I electrode is a control that is not functionalized in any directed way. The electrode was pre-soaked in tris buffer for 5 minutes prior to exposure to B-ALP or NA-ALP enzyme solutions as described in Section 2.2.9.

**Type II:** The type II electrode was modified with BSA by simple adsorption. The electrode was incubated in 1 mg mL⁻¹ of BSA in tris buffer for 30 minutes followed by rinsing with tris buffer.

**Type III:** The type III electrode was prepared by functionalizing the RVC surface to produce exposed phenylacetic acid groups. The RVC electrode was first rinsed with
anhydrous acetonitrile and dried under nitrogen. The electrode was then modified by electrochemically reducing 5 mM of 4-amino phenylacetic acid tetrafluoroborate diazonium salt in anhydrous acetonitrile containing 0.1 M N(Bu)₄BF₄. The diazonium salt solution was purged with nitrogen for 15 minutes to remove dissolved oxygen prior to electrode modification. The reduction was accomplished by applying a 5-segment cyclic potential scan starting at 0.2 V followed by three forward scans up to -1.2 V and two backward scans to 0.6 V, at a scan rate of 50 mV s⁻¹. The electrode was then sonicated in acetonitrile to remove any adsorbed material and rinsed thoroughly with acetonitrile followed by rinsing with water.

**Type IV:** The type IV electrode was made using layer-by-layer (LBL) adsorption of polyelectrolytes onto the surface of a type III electrode. Poly(allylamine hydrochloride) (PAH) and poly(acrylic acid sodium salt) (PAA) were dissolved in pure water at a concentration of 1 mg mL⁻¹. The negatively-charged type III RVC electrodes were used for polyelectrolyte deposition beginning with a polycation by immersing the electrode in PAH solution for 15 minutes, followed by a wash in water for 5 minutes, and then immersion in the PAA solution for 15 minutes, followed by a wash for 5 minutes. Multilayer assemblies consisting of 4 layers each of PAA and PAH were prepared by consecutive adsorption of both polyelectrolytes.

**Type V:** The type V electrode was modified by covalent attachment of polyethylene glycol onto the RVC surface. The electrode was first functionalized with phenylacetic acid groups as described for type III electrode. The surface carboxylic acid groups were then activated by incubating the functionalized RVC electrode in 25 mM EDC and 25
mM NHS in MES buffer (pH 7) for 4 hrs. The covalent binding of amine-terminated PEG was carried out by treating the activated surface carboxylic acid groups with 1 mg mL\(^{-1}\) of PEG-amine in MES buffer for 2 hrs.

**Type VI:** The type VI electrode was first functionalized with activated phenylacetic acid groups in a manner similar to that described above for type V electrodes. The activated surface carboxylic acid groups were then allowed to react with a solution of 1 mg mL\(^{-1}\) of biotin-PEO-amine and 1 mg mL\(^{-1}\) PEG-amine in MES buffer for 2 hrs.

**Type VII:** The type VII electrode was prepared in a manner similar to type VI with biotin-PEO-amine but without the PEG-amine.

**Type VIII:** The type VIII electrode was first modified with polyelectrolyte multilayers as described for type IV electrode. The carboxylic acid groups of the outermost poly(acrylic acid) layer on the RVC electrode were then activated in 25 mM EDC and 25 mM NHS in MES buffer for 4 hrs. The activated surface carboxylic acid groups were then allowed to react with 1 mg mL\(^{-1}\) of biotin-PEO-amine in MES buffer for 2 hrs.

**Type IX:** The type IX electrode consists simply of an adsorbed layer of biotinylated BSA. An otherwise bare RVC electrode was incubated in a solution containing 1 mg mL\(^{-1}\) of biotin-BSA and 1 mg mL\(^{-1}\) of BSA in tris buffer for 30 minutes. The electrodes were rinsed with buffer and used for further experiments.
Scheme 2.2 Modification of RVC electrodes with different molecules (type I to type IX)

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<tr>
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<tr>
<td>II</td>
<td>Adsorption of BSA on RVC</td>
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<td>III</td>
<td>Modification with phenyl acetic acid</td>
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<td>IV</td>
<td>Layer by layer adsorption of polyelectrolytes on type III</td>
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<td>V</td>
<td>Immobilization of PEG-amine on type III</td>
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<td>VIII</td>
<td>Immobilization of B-PEO-amine on type IV</td>
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<td>IX</td>
<td>Adsorption of B-BSA on RVC</td>
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</table>
2.2.8 **Characterization of modified RVC electrodes**

Electrodes modified with charged groups were characterized by their ability to block cyclic voltammetric oxidation/reduction of two redox probes, Fe(CN)$_6$ and Ru(NH$_3$)$_6$. A cyclic potential scan between 0.7 V and -0.3 V was applied to RVC electrodes in 1 mM solution of K$_3$Fe(CN)$_6$ and -0.4 V and 0.2 V in 1 mM Ru(NH$_3$)$_6$Cl$_3$ solution in tris buffer (pH 8) at a scan rate of 10 mV/s, before and after modification with functional groups. The interaction between functionalized RVC surface and redox probe, Fe(CN)$_6^{3-}$ was also studied in different pH solutions using cyclic voltammetry. A cyclic potential of 0.7 V to -0.3 V was applied to each of the functionalized RVC electrodes in 1 mM solution of K$_3$Fe(CN)$_6$ in 0.1 M KCl (pH 1 to pH 10) at 10 mV/s. The formation of polyelectrolyte layers on type IV and VIII electrode surface was confirmed by studying the electrochemical response of Fe(CN)$_6^{3-}$ and Ru(NH$_3$)$_6^{4+}$ at alternate polyanion and polycation layers on RVC electrodes.

2.2.9 **Non-specific and specific adsorption of NA-ALP and B-ALP on modified RVC electrodes.**

NA-ALP and B-ALP enzyme solutions were prepared at a concentration of 50 µg mL$^{-1}$ in tris buffer (pH 8). The modified RVC electrodes were always rinsed with tris buffer and dried under brief flow of nitrogen gas prior to exposure to enzyme solutions. Non-specific adsorption of enzyme onto RVC electrodes was studied by incubating one set of type I-V electrodes in NA-ALP solution and a second set of type I-V electrodes in B-ALP solution for 30 minutes each. The electrodes were rinsed thoroughly with tris buffer and water and used for further studies. The specific binding of NA-ALP was
carried out on type VI-IX RVC electrodes through direct biotin-neutravidin binding. The electrodes were allowed to react with 50 µg mL\(^{-1}\) solution of NA-ALP in tris buffer for 30 minutes, followed by rinsing with buffer and water. For specific binding of B-ALP, the type VI-IX electrodes were first allowed to react with 25 µg mL\(^{-1}\) of neutravidin in tris buffer for 30 minutes, followed by rinsing with buffer to remove any adsorbed molecules. The enzyme B-ALP was then immobilized onto these RVC electrodes by incubating the electrodes in 50 µg ml\(^{-1}\) solution of B-ALP in tris buffer for 30 minutes. The electrodes were then further tested with enzymatic substrate HQDP to test for catalytic activity of enzymes that were attached onto the RVC surface.

2.2.10 **Enzyme substrate reaction on modified RVC electrodes.**

After specific and non-specific binding of NA-ALP and B-ALP onto RVC electrodes was accomplished as described above, the electrodes were subjected to ALP-catalyzed hydrolysis using HQDP as a substrate for ALP. The modified electrodes were exposed to a 1 mM solution of HQDP in tris buffer and catalytically-generated HQ was detected using cyclic voltammetry (CV) and a series of linear scan voltammograms (LSV). For CV, a cyclic potential of -0.3 V to 0.7 V was applied to the RVC electrode versus Ag/AgCl reference electrode. For LSV, a potential range of -0.3 V to 0.7 V was used in LSV scans at a scan rate of 100 mV s\(^{-1}\) with quiet time of 10 seconds prior to the start of each individual LSV scan. A continuous method for 10 scans was set up in such a way that each scan (including the 10 second quiet time) was automatically initiated immediately after the previous scan was completed. The time for the HQ oxidative peak to appear was noted in real time. The time when the electrode was dipped into the
solution was also noted and was set as the reference zero time. The increasing peak current with time was recorded at HQ peak potential \((E_p)\) and plotted against time. The initial rate of change of HQ peak current was calculated from the initial slope of a linear region of LSV curve, and the value was compared for all modified electrodes.

2.2.11 Neutravidin sandwich bioassay

A series of type VI electrodes was used to perform sandwich bioassays with neutravidin as analyte. Neutravidin solutions were prepared in a concentration range from 1 µg mL\(^{-1}\) to 20 µg mL\(^{-1}\) in tris buffer. Each of a series of type VI electrodes was incubated in a different neutravidin solution for 30 minutes each. The electrodes were then rinsed thoroughly with tris buffer followed by incubation in 50 µg mL\(^{-1}\) of B-ALP solution for 30 minutes, followed by exposure to a 1 mM HQDP solution to test for the amount of bound ALP via the catalytic conversion of HQDP to HQ using LSV as described above.

2.3 Results & discussion

2.3.1 Characteristic of RVC foam and RVC electrode

The electrode used in this work was made up of reticulated vitreous carbon foam (100 ppi). RVC is a porous material with interpenetrable strands of glassy carbon. The most common method used for synthesizing reticulated vitreous carbon is by impregnating the polyurethane foam precursor with furfuryl alcohol or epoxy/phenolic resins, followed by carbonization at 700-1000 °C [5, 6]. The electroanalytical versatility
of this material has been reviewed in the past decade indicating potential use of RVC material in electrochemical sensors [7-9]. Before fabricating the electrodes, RVC foam was pretreated as described in Section 2.2.3 and the SEM image of RVC foam was obtained (Figure 2.2).

![Figure 2.2 Scanning electron microscopic image of 100 ppi reticulated vitreous carbon foam.](image)

The physical characteristics of 100 ppi RVC foam are given in Table 2.1. The average pore diameter of RVC foam obtained from SEM image was around 230 µm. The double layer capacitance of RVC electrode was calculated to be 7.07 ± 0.04 µF cm$^{-2}$. The value was corrected by subtracting the capacitance of the gold wire used to make electric contact. The surface area of RVC was obtained from BET measurements performed in our lab [10] and other parameters of the RVC were obtained from the manufacturer [11]. The surface area of the RVC electrodes used in this work was around 1.3 cm$^2$ mg$^{-1}$ of
RVC foam (1 ± 0.2 mg of RVC material was used to make the electrodes). The free internal volume of RVC electrode was obtained to be ~ 10 ± 1 µL.

Table 2.1 Physical characteristics of reticulated vitreous foam (100 ppi)

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>VALUES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average pore diameter</td>
<td>230 ± 10 µm</td>
</tr>
<tr>
<td>Capacitance area⁻¹</td>
<td>7.07 ± 0.04 µF cm⁻²</td>
</tr>
<tr>
<td>BET surface area per gram of RVC</td>
<td>1278 ± 11 cm² g⁻¹</td>
</tr>
<tr>
<td>Surface area per volume of RVC</td>
<td>65 ± 0.1 cm² cm⁻³</td>
</tr>
<tr>
<td>Percent free volume</td>
<td>95 -97 %</td>
</tr>
<tr>
<td>Bulk density</td>
<td>6.2 ± 0.2 x 10⁻² g cm⁻³</td>
</tr>
<tr>
<td>Ligament density</td>
<td>1.8 ± 0.05 g cm⁻³</td>
</tr>
<tr>
<td>Bulk resistivity</td>
<td>5 x10⁻² Ω cm⁻¹</td>
</tr>
</tbody>
</table>

2.3.2 Evaluation of non-specific and specific binding of enzymes on RVC surface

The surface of RVC electrode was modified with different functional groups to study the extent of non-specific and specific binding of enzymes, NA-ALP and B-ALP. The attachment of enzymes on RVC electrodes was confirmed by studying the conversion of 1 mM electro-inactive HQDP substrate into electro-active HQ by enzymatic hydrolysis (Scheme 2.3). HQDP was used as an enzyme substrate because of
its stability over a broad potential range and excellent hydrolytic stability in aqueous medium. The enzymatic product (HQ) of HQDP shows reversible electrochemical oxidation at low potentials. This is important because it allows detection of HQ at potentials where interference due to common biological components, such as ascorbate and urate, is minimized. Multiple cyclic voltammograms of HQ in neutral or alkaline solutions indicate that it does not significantly passivate electrodes unlike other ALP substrates (4-aminophenyl phosphate and 1-napthyl phosphate) [2, 12].

Scheme 2.3 Hydrolysis of hydroquinone diphosphate (HQDP) substrate by an enzyme alkaline phosphatase (ALP) into hydroquinone (HQ) product. HQ undergoes reversible oxidation via, a two-electroon transfer and deprotonation to produce benzoquinone (BQ).

The cyclic voltammogram of HQDP in the absence of enzyme shows that the substrate is inactive over the potential window in aqueous medium (Figure 2.3a). The cyclic voltammogram of catalytically generated HQ shows well defined oxidation and reduction peaks on the RVC working electrode (Figure 2.3b). The HQ peak current is proportional to the concentration of HQDP and thus directly proportional to the enzyme concentration at a given time. It was observed that the repeated cycling of the electrode
(10 cycles) produces no deterioration (passivation) of electrode sensitivity, even at high (>1) millimolar concentrations of HQDP.

Figure 2.3 Cyclic voltammogram of a) HQDP in tris buffer on RVC with no enzyme added, b) HQ generated by catalytic conversion of 1 mM HQDP by an enzyme ALP in tris buffer (pH 8). The potential was recorded against Ag/AgCl reference at a scan rate of 10 mV s⁻¹.

All enzyme assays measure either consumption of substrate or production of product over time. The time-course of product formation of the enzyme-catalyzed
reaction can be determined by obtaining a plot of product concentration versus time. At the start of reaction, the product concentration increases linearly with time but at later times the curve starts to level off due to depletion of substrate and eventually the concentration of product reaches a plateau. It has been observed that fitting the time-course data of product formation is difficult, as the process cannot be easily reduced to a simple equation that expresses product concentration as a function of time [13, 14]. The most widely used method to approximate the reaction progress curve is defined by differential equations or by an implicit equation. But this method is complicated and requires a special mathematical program [15, 16]. Recently another more approximate method has been used to fit the data of enzyme-catalyzed reaction progress curves using a non-linear least squares fit [17]. The equation used in this method is, \[ I = I_{\text{max}} \frac{t}{(t_{1/2} + t)} \] (Equation 1), where \( I \) is the LSV current in amperes, which is proportional to the concentration of product generated at a given time \( t \), \( I_{\text{max}} \) is the saturation value of \( I \) which is achieved at long times when substrate has been fully converted into product, and \( t_{1/2} \) is the time required to elicit half of the maximum current response. This equation describes the data in a way that is consistent with the enzyme rate law but it is not directly derived from the law. In the present work, same method was used to estimate the initial rate of product formation from the growth in peak current with time for a series of LSV’s acquired at electrodes at which the enzyme-catalyzed reaction produces a redox-activate product.

In this work the RVC electrodes were modified with different functional groups in order to study non-specific and specific binding of two enzymes conjugates, NA-ALP
and B-ALP, onto the electrodes. The amount of enzyme bound to each electrode surface was compared by measuring the initial rate of HQ generation. It was assumed that the initial rate of HQ production is proportional to the initial of increase of the HQ peak current detected at each RVC electrode. For each electrode HQ peak current was recorded at +0.32 V with time. The recorded data (I versus t) for each electrode was fitted using equation 1 to obtain the parameters $I_{\text{max}}$ and $t_{1/2}$. The initial rate of product generation was then obtained by calculating slope of the tangent to the early region of the curve. The tangent was approximated by fitting a straight line to the initial part of the curve. To fit a straight line we generated current values for a time interval close to the origin using the parameters derived from equation 1.

The initial rate values of HQ generation for all the electrode types from Scheme 2.2 are given in Table 2.2 for adsorption of both NA-ALP and B-ALP. In each case two independent measurements were made, and the results of both measurements and their average values are reported. On type I to type V electrodes, the enzymes were adsorbed non-specifically and hence the amount of enzyme present on these electrode surfaces was comparatively lower than the amount of specifically bound enzymes on type VI to type IX electrodes. These data provide an overview of all the experiments; the following sections discuss individual experiments in more detail.

Before modifying the RVC electrodes, the NSB of enzymes was studied on unmodified electrode (type I). Figure 2.4a shows a series of linear scan voltammograms obtained to study the growth of the HQ current with time on a type I electrode following exposure to a 1 mM HQDP solution. The peak current at +0.32 V increases with time,
indicating the catalytic conversion of HQDP into HQ by non-specifically adsorbed enzyme inside pores of the type I RVC electrode. The initial rate of HQ generation was obtained from the slope of the initial region of the line in Figure 2.4b. The initial rate values for NSB of NA-ALP and B-ALP are given in Table 2.2. The reason for non-specific binding of enzymes onto type I RVC is most likely due to hydrophobic interactions between carbon surface and enzymes [18].
Figure 2.4  a) Linear scan voltammetry of HQ generated by hydrolysis of 1mM HQDP in tris buffer (pH 8) by an enzyme, NA-ALP adsorbed non-specifically on a type I RVC electrode.  b) HQ peak current recorded at 0.32 V (Figure 2.4a) with time.

The type II electrode was modified with BSA and NSB of NA-ALP and B-ALP was studied in a similar manner as described for type I electrode. The HQ peak current and the initial rate of HQ generation on this electrode are given in Figures 2.10 and 2.11 respectively. The initial rate value on type II electrodes was higher than those obtained for type I electrodes. The reason for higher non-specific binding could possibly due to electrostatic interactions between surface-bound BSA and the enzymes [19].

The type III RVC electrode was functionalized with phenylacetic acid groups by electrochemical reduction of 4-aminophenylacetic acid diazonium salt in anhydrous solvent. The modification mechanism is given below in Scheme 2.4.

Scheme 2.4  Electrochemical reduction of 4-aminophenylacetic acid diazonium on RVC electrode.

Figure 2.5 shows a cyclic voltammogram of 4-aminophenylacetic acid diazonium, tetrafluoroborate salt in acetonitrile on the RVC electrode. The irreversible reduction wave at -0.5 V versus Ag was attributed to a formation of 4-phenyl acetic acid radical from the diazonium salt due to a one electron-transfer redox reaction [20]. The reduction
peak of phenylacetic acid diazonium salt disappeared completely after the first cycle, indicating the electrode surface has been occupied by phenylacetic acid groups and further reduction of diazonium molecules in solution is blocked.

![Graph](image)

**Figure 2.5** Electrochemical reduction of 5 mM of 4-aminophenylacetic acid tetrafluoroborate diazonium salt in acetonitrile and 0.1 M NBut4BF4 at 50 mV s⁻¹ on the RVC electrode.

The blocking property of phenylacetic acid modified RVC electrode was confirmed by testing the electrochemical behavior of two redox probes, Fe(CN)₆⁻³ and Ru(NH₃)₆⁺³ in aqueous buffer on type III RVC electrode. Figures 2.6a and 2.6b shows cyclic voltammograms of 1 mM K₃Fe(CN)₆ and 1 mM Ru(NH₃)₆Cl₃ in tris buffer (pH 8) respectively, before and after modification of the RVC electrode with phenylacetic acid groups.
Figure 2.6 Cyclic voltammogram of a) 1 mM Fe(CN)$_6^{3-}$ and b) 1 mM Ru(NH$_3$)$_6^{3+}$ in tris buffer (pH 8) at 10 mV s$^{-1}$ on unmodified and phenylacetic acid modified RVC electrode.

The redox peak of Fe(CN)$_6^{3-}$ observed on the unmodified RVC electrode disappeared completely after functionalization with phenylacetic acid groups. However, the redox peak of Ru(NH$_3$)$_6^{3+}$ was only slightly affected by the modification. The difference in the blocking property of phenylacetic acid layer on the RVC electrode for
different redox probes is due to the electrostatic interaction of phenylacetic acid groups on the RVC electrode surface and redox probes in solution. At pH 8, the surface phenylacetic acid groups are deprotonated (pKa = 4.28) and the RVC surface is negatively charged. The redox behavior of negatively charged Fe(CN)$_6^{3-}$ was therefore suppressed due to the negative Donnan potential established at the electrode surface. In order to confirm this hypothesis, the redox behavior of Fe(CN)$_6^{3-}$ was studied at unmodified and functionalized RVC electrode in a solution of different pH values. Figures 2.7a and 2.7b shows the cyclic voltammogram of ferricyanide on unmodified and phenylacetic acid modified electrode respectively at pH 1. At low pH values the surface carboxylic acid groups remain in the neutral form and negatively charged ferricyanide molecules can penetrate easily through neutral phenylacetic acid layer on the electrode surface.
As the pH increases, the surface carboxylic acid group deprotonates, giving rise to a negatively charged surface and thereby repulsing negatively charged ferricyanide molecules (Figures 2.8a and 2.8b). It was observed that at pH 10, the access of ferricyanide molecules to phenylacetic acid functionalized RVC had been completely blocked confirming the formation of phenylacetic acid layer onto the RVC electrode surface. The redox signal of ferricyanide on the unmodified electrode was also affected to some extent but it was not completely blocked. The peak splitting of ferricyanide however was larger on the unmodified RVC electrode at higher pH compared to that at lower pH. This probably was due to the deprotonation of functional groups that are already present on the bare RVC surface.

Figure 2.7 Redox behavior of 1 mM K$_3$Fe(CN)$_6$ in 0.5 M KCl (pH 1) on a) unmodified RVC and b) phenylacetic acid functionalized RVC electrode. All other conditions are same as used above.
The non-specific binding of NA-ALP and B-ALP was studied on the phenylacetic acid covered type III RVC surface and the initial rate of change of HQ peak current was determined to be $2.12 \times 10^{-7}$ A min$^{-1}$ for NA-ALP and $2.73 \times 10^{-7}$ A min$^{-1}$ for B-ALP. The initial rate values on this electrode were lower compared to those obtained on type I
and type II electrodes (Figures 2.10b and 2.11b). This finding indicates that comparatively less enzyme was adsorbed onto this surface. As discussed above, at pH 8, the surface phenylacetic acid groups are deprotonated and the RVC surface is negatively charged. Also the net charges on neutravidin (pI ~ 6.3) and alkaline phosphatase (pI~5.7) are negative at this pH. Adsorption of neutravidin or alkaline phosphates onto this electrode is comparatively low to due to repulsion between negatively charged surface carboxylic acid groups and negatively charged enzyme molecules. Also the carboxylate groups on RVC electrode make the surface relatively hydrophilic, thus adsorption of proteins on this surface is diminished compared to that on bare and BSA coated electrode [21]. However, the non-specific adsorption on the type III electrode was not entirely suppressed, and one possible reason for this is that the electrode surface might not completely be covered with the phenylacetic acid layer and some part of RVC electrode surface might be exposed to enzymes.

To achieve a more complete coverage with a negatively charged layer, another approach was used to modify RVC electrode. Polyelectrolytes with negatively charged functional groups are known to suppress the adsorption of negatively charged oligonucleotides [22] and other proteins [23]. The RVC electrode was modified by layer-by-layer adsorption of poly(allylamine) (PAH) and poly(acrylic acid) (PAA) as shown in Scheme 2.2 for the type IV electrode. PAH/PAA was chosen for the multilayer build-up because it has been shown to be bio-inert towards cell attachment [24]. The polyelectrolyte multilayers on phenylacetic acid functionalized RVC electrode were confirmed by testing the blocking property of each layer with K₃Fe(CN)₆ and
Ru(NH$_3)_6$Cl$_3$ redox probes in a similar way as described for type III electrode. Since polyelectrolytes are charged polymers, they can block or allow the penetration of redox molecules to the underlying electrode surface depending on the charge on redox probes. When the surface of the electrode is covered with positively charged polyelectrolyte layer, it should block the access of positively charged Ru(NH$_3)_6^{+3}$ and allow access to negatively charged Fe(CN)$_6^{-3}$. Similar behavior of redox probes was observed on the RVC electrode modified with polyelectrolytes (Figure 2.9, type IV).
Figure 2.9 Redox behavior of 1 mM K$_3$Fe(CN)$_6$ on RVC covered with a) polyallyamine and b) poly(acrylic acid) polyelectrolytes and 1 mM Ru(NH$_3$)$_3$Cl$_3$ on RVC covered with c) polyallylamine and d) poly(acrylic acid) polyelectrolytes. The measurement was done in phosphate buffer (pH 7) at a scan rate of 10 mV s$^{-1}$.

Figure 2.9a shows the electrochemical response of 1 mM ferricyanide in phosphate buffer (pH 7) on the first layer of positively charged PAH on the RVC electrode. At physiological pH, most of the amine groups of PAH remain protonated
(pKa ~8.5) and the negatively charged ferricyanide molecules could easily reach the electrode surface due to electrostatic attraction. However when the next layer of PAA was formed over the PAH layer, the charge polarity of the RVC surface was reversed. The surface of the RVC electrode was negatively charged due to dissociation of –COOH groups of poly(acrylic acid) (pKa ~ 4.28) and hence it repelled the negatively charged Fe(CN)$_6^{3-}$ away from the electrode surface (Figure 2.9b). The electrochemical response of positively charged redox probe, Ru(NH$_3$)$_6^{4+}$ was also studied on the RVC electrode covered with PAH and PAA. The suppression of redox peak of ruthenium hexamine on PAH surface was observed (Figure 2.9c) which was due to electrostatic repulsion between surface NH$_3^+$ groups and Ru(NH$_3$)$_6^{4+}$ molecules in solution. Similarly, opposite response was observed when the next layer of PAA was formed onto the RVC surface (Figure 2.9d).

The non-specific binding of NA-ALP and B-ALP on the outer PAA layer was studied by obtaining the initial rate of change of HQ peak current from LSV. It was observed that the initial rate of HQ generation was higher on the type IV electrode as compared to type I and III electrodes (Figures 2.10 and 2.11). Two mechanisms are possible for the non-specific binding of negatively charged enzymes onto a negatively charged PAA layer. The first is that patches of positive charges on proteins could behave similarly to multivalent counterions of the polyelectrolyte layer and thus the enzyme becomes adsorbed onto the negatively charged surface [25, 26]. The protein molecules can interact with polyelectrolytes via their positive charges even if the net charge on the protein molecule is negative. A second possibility is that there could be adsorptive
interactions between proteins and the inner positively-charged PAH layer. Even if the outer layer of the polyelectrolyte multilayer is terminating with a negatively-charged PAA, some positively charged groups of the inner polyelectrolyte layer can still emerge and interact with the negatively-charged proteins [27]. Also, although the experimental data showed the formation of multilayers on the RVC electrode (tested using redox probes); it is also possible that the entire electrode surface was not covered with the polyelectrolytes due to the porous nature of RVC (compared to a flat planar carbon). The exposed RVC surface may lead to adsorption of proteins.

The type V electrode was obtained by covalent attachment of polyethylene glycol (PEG) oligomers onto the RVC electrode. PEG is known for its ability to suppress protein adsorption due to its neutral hydrophilic groups [28-34]. Chain length and grafting densities are important parameters governing the inertness of PEG-derivatized surfaces to protein adsorption [35-39]. It is believed that PEG uses a “steric repulsion” and a hydration layer via hydrogen bonding around the PEG molecule to block the NSB of proteins [40, 41]. The initial rates of production of HQ on this electrode are $9 \times 10^{-8}$ A min$^{-1}$ and $7.69 \times 10^{-8}$ A min$^{-1}$ for NA-ALP and B-ALP respectively. These values are much lower than those observed for type I, II, III and IV electrodes (Figures 2.10a and 2.11b) indicating that much less enzyme was bound to the PEG-coated RVC surface. From these findings it was concluded that the type V electrode was most effective in suppressing the non-specific binding of NA-ALP and B-ALP and was further used in our subsequent studies of neutravidin and DNA bioassays.
Figure 2.10 a) Comparison of peak currents of catalytically generated HQ with time on the RVC electrodes modified with non-specific (type I-V) and specific (VI-IX) binding of enzyme NA-ALP b) The initial rate of change of HQ peak current obtained from the initial slope of curves in Figure 2.10a).
Figure 2.11 a) Comparison of peak currents of catalytically generated HQ with time on the RVC electrodes modified with non-specific (type I-V and VI-a*) and specific (VI-IX) binding of enzyme B-ALP b) The initial rate of change of HQ peak current resulted on type I-IX electrodes. The type VI-a* electrode represents the non-specific adsorption of B-ALP onto the type VI electrode in the absence of neutravidin analyte.

Along with non-specific adsorption, specific binding of NA-ALP and B-ALP enzymes was also studied on the RVC electrodes modified with biotinylated-BSA,
phenylacetic acid with biotin-PEO-amine, polyelectrolytes with biotin-PEO-amine and PEG with biotin-PEO-amine (Scheme 2.2, type VI – type IX). The specific binding of enzymes on these surfaces was studied in similar way using LSV. The initial rates of change of HQ peak current for all 4 electrodes are given in Table 2.2. The initial rate values for these electrodes were much higher than those obtained for type I to type V electrodes. The biotin groups present on the surface of RVC allowed the specific binding of NA-ALP and B-ALP conjugates and hence HQ production at a given time was much higher due to the large amount of ALP enzyme present. Figure 2.12a shows a plot of HQ peak current versus time on a type IX electrode where enzyme NA-ALP was specifically bound onto the B-BSA-coated RVC electrode. The HQ peak current increases with time linearly in the early time region, but becomes non-linear at later times. The saturation of current at later times was attributed to depletion of HQDP via hydrolysis by the surface-bound enzyme. As the concentration of enzyme on this electrode was high, due to specific binding, more substrate gets converted at a given time compared to that on type I to type V electrodes. Similar behavior was observed on type VI to type VIII electrodes indicating that specific binding of enzyme on these electrodes has occurred (Figures 2.10a and 2.11a). The initial rates of HQ generation on type VI to type IX electrodes are given in Figures 2.10b and 2.11b for NA-ALP and B-ALP respectively. The type IX electrode showed the highest signal which was attributed to the relatively large number of biotin groups (9 moles biotin / mole of BSA) available on the RVC surface for binding. The type VIII electrode with polyelectrolyte multilayers however showed relatively low initial rate values indicating that the specific binding of enzymes on this surface did not
occur to a very great extent. One possible cause for this behavior is that after activating or modifying the outer PAA layer, it is no longer negatively charged, so it could just rinse away. The initial rate value for type VI and VII electrodes differs marginally indicating the modification with PEG groups did not affect the specific binding on enzymes on the type VI electrode. After comparing all the electrodes, the type VI electrode was selected as a best platform for further studies of RVC-based sandwich bioassays because it has the lowest non-specific binding and relatively high specific binding.
Figure 2.12 a) Linear scan voltammetry of HQ generated by hydrolysis of 1 mM HQDP in tris buffer (pH 8) by an enzyme, NA-ALP specifically coupled to biotinylated BSA on the type IX RVC electrode. b) Increasing peak current of generated HQ recorded at +0.32 V (Figure 2.12a) with time.

Table 2.2 The initial rate of change of peak current of HQ generated by catalytic conversion of 1 mM HQDP. The values were obtained by calculating the initial slope of increasing peak current of HQ with time.

<table>
<thead>
<tr>
<th>RVC Electrode Type</th>
<th>NA-ALP Initial rate of change of peak current of HQ in A min⁻¹</th>
<th>B-ALP Initial rate of change of peak current of HQ in A min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>a RVC1 RVC2 Average</td>
<td>RVC1 RVC2 Average</td>
<td></td>
</tr>
<tr>
<td>I 3.75 x 10⁻⁷ 3.30 x 10⁻⁷ 3.53 x 10⁻⁷</td>
<td>3.96 x 10⁻⁷ 4.31 x 10⁻⁷ 4.14 x 10⁻⁷</td>
<td></td>
</tr>
<tr>
<td>II 9.25 x 10⁻⁷ 1.25 x 10⁻⁶ 1.09 x 10⁻⁶</td>
<td>1.04 x 10⁻⁶ 1.28 x 10⁻⁶ 1.16 x 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>III 2.18 x 10⁻⁷ 2.06 x 10⁻⁷ 2.12 x 10⁻⁷</td>
<td>2.91 x 10⁻⁷ 2.54 x 10⁻⁷ 2.73 x 10⁻⁷</td>
<td></td>
</tr>
<tr>
<td>IV 5.03 x 10⁻⁷ 4.87 x 10⁻⁷ 4.95 x 10⁻⁷</td>
<td>- 7.75 x 10⁻⁷ 7.75 x 10⁻⁷</td>
<td></td>
</tr>
<tr>
<td>V 9.25 x 10⁻⁸ 8.95 x 10⁻⁸ 9.10 x 10⁻⁸</td>
<td>8.63 x 10⁻⁸ 7.25 x 10⁻⁸ 7.94 x 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>VI 1.82 x 10⁻⁵ 2.01 x 10⁻⁵ 1.92 x 10⁻⁵</td>
<td>1.85 x 10⁻⁵ 2.08 x 10⁻⁵ 1.97 x 10⁻⁵</td>
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</tr>
<tr>
<td>VII 2.35 x 10⁻⁵ 2.18 x 10⁻⁵ 2.27 x 10⁻⁵</td>
<td>2.16 x 10⁻⁵ 2.83 x 10⁻⁵ 2.50 x 10⁻⁵</td>
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</tr>
<tr>
<td>VIII 3.17 x 10⁻⁶ - 3.17 x 10⁻⁶</td>
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</tr>
<tr>
<td>IX 4.06 x 10⁻⁵ 4.83 x 10⁻⁵ 4.45 x 10⁻⁵</td>
<td>5.10 x 10⁻⁵ 4.86 x 10⁻⁵ 4.98 x 10⁻⁵</td>
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</table>

2.3.3 Neutravidin sandwich bioassay

Type VI electrode with lowest non-specific and comparatively high specific binding was used as a platform for a neutravidin sandwich bioassay. Neutravidin, a deglycosylated form of avidin was used as an analyte and B-ALP was used as an enzyme label to allow for detection of captured analyte. A set of five type VI RVC electrodes was
treated with five different solutions containing different concentrations of neutravidin and the initial rate of conversion of HQDP to HQ by surface-immobilized B-ALP was studied at each electrodes. Figure 2.13a shows a series of plots of peak current of generated HQ with time for electrode exposed to solutions containing different concentration of neutravidin. The initial rate of change of HQ peak current was calculated from the initial slope for all five concentrations and a calibration curve was obtained by plotting the initial slope against neutravidin concentration (Figure 2.13b). The initial rate of HQ production was found to increase with increasing concentration of neutravidin. With increasing neutravidin concentration, the number of B-ALP molecules captured on the RVC surfaces increases, and thus the amount of HQ generated at a given time varies with the amount of B-ALP on the electrode. The calibration curve for neutravidin concentration was found to be linear with regression line having the equation $y = 2.13 \times 10^{-7} x \pm 0.1 + 6.51 \times 10^{-8}$ ($R^2 = 0.993$).
Figure 2.13 a) Plot of HQ peak current obtained from LSV plotted against time for concentrations of neutravidin analyte (0-20 µg mL⁻¹) in tris buffer at a scan rate of 100 mV s⁻¹ b) The initial rate of HQ generation plotted against various concentrations of neutravidin.

A control experiments in the absence of neutravidin shows a very low initial rate value (8 \times 10^8 \text{A min}^{-1}) indicating that substantial binding of B-ALP occurs only in the presence of neutravidin analyte (Figure 2.11b, type VI-a*). The lower detection limit was estimated using the equation $DL = \left(3\sigma_{\text{blank}}\right) / m$, where $\sigma$ is the standard deviation for the blank $3.67 \times 10^{-9} \text{A min}^{-1}$, and $m$ is the slope of calibration curve of Figure 2.13b which is equal to $2.13 \pm 0.1 \times 10^{-7} \text{A min}^{-1} \mu\text{g}^{-1} \text{mL}^{-1}$. The limit of detection for neutravidin on type VI RVC electrodes was estimated to be $52 \pm 2 \text{ng mL}^{-1}$ and the absolute detection was $5.2 \pm 0.2 \text{ng}$ for the sample size of 100 µL used for this analysis.

It is instructive to compare the analytical figures of merits for the present assay with those of previously described assays. The detection limit of type VI RVC-based sensor for neutravidin is close to that of recently reported voltammetric detection method
for avidin [42]. The detection limit of type VI RVC-based sensor for neutravidin is lower than that obtained with the colorimetric quantitation of avidin in a competitive inhibition method [43] and in a sandwich assay [44, 45].

The sensitivity of the RVC-based electrodes studied in this work could be affected by several factors. One important factor is the ability to reproduce the RVC electrodes for each experiment. Since RVC is porous in nature, an inhomogeneous pore distribution can results in mechanical instability of the electrode. Another important factor is possible variation in contact resistance between the gold contact wire and the RVC foam that could result in an uneven potential distribution across the electrode. Therefore, care was taken while fabricating the RVC electrodes to have consistent size and volume and only the electrodes with resistance less than 50 Ω were used for all the experiments.

2.4 Conclusion

For electrodes of type I to type V, the initial rate of HQ generation was much lower compared to that observed on type VI to type IX electrodes. The initial rate values were attributed to non-specific and specific binding of NA-ALP and B-ALP on RVC electrodes modified in different ways. Both phenylacetic acid and polyethylene glycol-based electrode modification schemes were successful in suppressing the non-specific binding of the NA-ALP and B-ALP enzymes conjugates on RVC electrodes. The electrode modified with PEG (type VI) showed the lowest non-specific binding and also comparatively high specific binding when some biotin was included in the electrode modification method to serve as a capture agent for neutravidin. The initial rate of HQ generation versus neutravidin concentration in a neutravidin sandwich assay was linear
over the neutravidin concentration range studied. The lowest concentration of neutravidin that can be detected on a type VI RVC electrode was 52 ± 2 ng mL\(^{-1}\) and the absolute detection of 5.2 ± 0.2 ng was achieved.

2.5 References


CHAPTER THREE

3. ELECTROCHEMICAL DETECTION OF OLIGONUCLEOTIDES ON RVC FUNCTIONALIZED WITH POLYETHYLENE GLYCOL

3.1 Introduction

In recent years nucleic acid and DNA hybridization sensor technology has gained much attention. The diagnosis of disease and analysis of pathogens in environmental, food or clinical samples often involves detection of nucleic acids and proteins [1, 2]. Various platforms have been developed to detect the DNA sequence and its hybridization [3, 4]. A key parameter for a successful DNA sensor is a stable hybridization between two single-stranded DNA chains which are complementary to each other [5]. To achieve good sensitivity, the signal arising from sequence-specific DNA hybridization must be high enough compared to the signal from the background or signal due non-specific binding.

This chapter describes the attempts made to detect 32-mer oligonucleotide inside the pores of reticulated vitreous carbon (RVC) electrode. The schematic representation of RVC based DNA sensor is given in Scheme 3.1. The RVC electrode was functionalized with phenylacetic acid prior to poly(ethylene glycol) (PEG) and DNA immobilization. Modification with phenylacetic acid and PEG groups helps to suppress the non-specific binding (NSB) of DNA and enzymes onto the RVC surface. The amine-terminated capture oligonucleotide probe and amine-terminated PEG was co-immobilized onto the phenylacetic acid modified RVC electrode. Hybridization with a complementary
biotinylated oligonucleotide probe was accomplished to demonstrate the oligonucleotide detection. The hybridization event was detected using neutravidin-conjugated alkaline phosphatase (NA-ALP) label onto the surface immobilized biotinylated complementary oligonucleotide. The hydrolysis of hydroquinone diphosphate (HQDP) substrate into hydroquinone (HQ) by surface-bound NA-ALP enzyme was detected using linear scan voltammetry (LSV). Control experiments were carried out in order to study the specific and the non-specific binding of oligonucleotides and NA-ALP on the RVC electrodes. A spectroscopic experiment was also carried out in order to determine the number of oligonucleotide probes immobilized inside the pores of the RVC electrode. The surface coverage of DNA inside the pores of the RVC electrode was compared with the theoretical as well as experimentally reported coverage of oligonucleotides.

Scheme 3.1 DNA hybridization on the RVC electrode modified with phenylacetic acid and PEG.
3.2 Experimental

3.2.1 Materials and methods

All chemicals were used as received unless otherwise mentioned. Neutravidin-conjugated alkaline phosphatase (NA-ALP), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC), N-hydroxsuccinimide (NHS) and fluorescein were purchased from Pierce Biotechnology. Hydroquinone (HQ), potassium ferricyanide and ruthenium hexamine trichloride were purchased from VWR. Hydroquinone diphosphate (HQDP) and 4-aminophenylacetic acid diazonium tetrafluoroborate salt were synthesized according to published procedures [6-8]. The detailed procedures for synthesis of HQDP and diazonium salt are described in Chapter 2. Hybridization buffer was 2x saline sodium citrate (SSC) buffer (pH 7) diluted from commercial 20 x SSC buffer (Sigma) and storage buffer for oligonucleotides was TE buffer (10mM tris (hydroxymethyl) aminomethane (tris) + 1mM EDTA)(pH 7.5). Tris and MES buffer were prepared as described in Chapter 2. All the electrochemical experiments were performed using a model CHI 660A electrochemical workstation from CH Instruments Inc (Austin, Texas) with aqueous Ag/AgCl as a reference electrode and Pt wire mesh as a counter electrode unless otherwise mentioned. Fluorescence experiments were performed on a custom built fluorescence spectrophotometer by Photon Technologies International (PTI). The melting temperature (Tm) of DNA duplex was determined on Cary 1 UV spectrophotometer. Oligonucleotides were obtained from Sigma Genosys. The oligonucleotides are 32-mer long DNA sequence purified by reversed-phase HPLC (Sigma Genosys). The
oligonucleotide sequence used was the same as previously reported in the literature (Table 3.1) [9, 10].

Table 3.1 Oligonucleotide sequence (from 5’ to 3’)

| Capture probe (32mer): NH₂C₆ACCACGACGTTGTTAAAACGACGGCCAGCTCTAT |
| Complementary target: Biotin-ATAGACTGGCCGTCGTTTTTACAACGTCGTGGT |
| probe 1(32mer) |
| Complementary target: Fluorescein-ATAGACTGGCCGTCGTTTTTACAACGTCGTGGT |
| probe 2(32mer) |
| Non-complementary : Biotin-CAAAACGTATTTTGTAACAAT |
| probe (21mer) |

3.2.2 Fabrication of the RVC electrodes

The RVC electrodes were functionalized to study specific and non-specific binding of oligonucleotides inside the pores of RVC electrodes. The modification protocol is given in Table 3.2 below.

Table 3.2 Experimental conditions used in DNA hybridization assay for specific and non-specific binding of oligonucleotides and enzyme on the RVC electrode surface.

<table>
<thead>
<tr>
<th>RVC</th>
<th>Phenylacetic Acid 5 mM</th>
<th>EDC/NHS 25 mM</th>
<th>PEG 1 mg mL⁻¹</th>
<th>Capture Probe 50 μg mL⁻¹</th>
<th>Target Probe 10 μg mL⁻¹</th>
<th>NA-ALP 25 μg mL⁻¹</th>
<th>HQDP 1 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>2</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>3</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>4</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>NC</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>5</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>6</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

* NC - Non-complementary oligonucleotide
All RVC electrodes were first functionalized with phenylacetic groups as described in Chapter 2. In brief, the electrodes were first rinsed with anhydrous acetonitrile and dried under nitrogen. The electrodes were then modified by electrochemically reducing 5 mM of 4-amino phenylacetic acid tetrafluoroborate diazonium salt in anhydrous acetonitrile containing 0.1 M $\text{N}($Bu$_4$)$_2\text{BF}_4$. The diazonium salt solution was purged with nitrogen for 15 minutes to remove dissolved oxygen prior to electrode modification. The reduction was accomplished by applying a 5 segment cyclic potential starting at 0.2 V followed by three forward scans up to -1.2 V and two backward scans to 0.6 V, at a scan rate of 50 mV s$^{-1}$. The electrodes were then sonicated in acetonitrile to remove any adsorbed material and rinsed thoroughly with acetonitrile followed by rinsing with high purity water.

3.2.3 **Immobilization of amine-terminated oligonucleotide probe and PEG onto RVC electrodes**

The amine-terminated oligonucleotide probe and amine-terminated PEG were co-immobilized on the RVC electrodes (Table 3.2, type 2 electrode) by covalent binding between the amine groups of the oligonucleotide and the PEG and the activated phenylacetic acid groups on the RVC surface. The phenylacetic acid groups were activated by incubating the acid-modified electrode in 25 mM EDC and 25 mM NHS in MES buffer (pH 7) for 4 hrs followed by rising with water. The electrodes were then incubated in a TE buffer (pH 7.5) containing 50 $\mu$g mL$^{-1}$ of amine-terminated capture probe and 1 mg mL$^{-1}$ of amine-terminated PEG for 4 hrs. Another set of RVC electrode
(type 1) was modified with only amine-terminated oligonucleotide in similar way as described for type 2 electrode, but without the amine-terminated PEG.

3.2.4 Hybridization protocol

The RVC electrodes modified with the capture oligonucleotide probes were subjected to hybridization with biotin-terminated complementary DNA strands. The electrodes were first washed thoroughly with TE buffer to remove any unreacted amine-capture oligo probe followed by rinsing with water. The electrodes were pre-saturated with 2x SSC buffer for 10 minutes prior to hybridization. Hybridization with the complementary probe was carried out by incubating the RVC electrode in 100 µL of a solution of 10 µg mL\(^{-1}\) biotinylated oligonucleotide (probe 1) in 2x SSC buffer for 10 hrs.

3.2.5 Labeling protocol

The NA-ALP label was attached onto the surface of RVC electrodes that were modified with DNA hybrid (as described above) through formation of a neutravidin-biotin (5’ end of complementary DNA) complex. The electrodes modified with DNA duplex were first washed thoroughly with 2x SSC buffer to remove any unreacted oligonucleotides followed by rinsing with water. The electrodes were then rinsed with tris buffer (pH 8). The enzyme conjugate was immobilized on the electrodes by filling the electrodes with a 25 µg mL\(^{-1}\) solution of NA-ALP conjugate in tris buffer for 2 hrs. Finally the RVC electrodes were rinsed thoroughly with tris buffer and water and dried under a stream of nitrogen.
3.2.6 **Control experiments**

The non-specific binding of NA-ALP conjugate and non-complementary oligo probe onto the PEG modified RVC electrodes were studied. The control experiments were carried out in the absence of either capture or target oligonucleotide probes. The experimental steps are given in Table 3.2 and the experimental conditions are the same as given in above sections. The type 3 electrode was functionalized with the layer of phenylacetic acid, PEG and capture oligo probe. This electrode was used to study NSB of NA-ALP in the absence of the target complementary oligonucleotide. The type 4 electrode was used to study the binding of non-complementary oligonucleotide. The electrode was first fabricated with phenylacetic acid to attach amine-terminated capture probe and amine-terminated PEG followed by incubation in 10 µg mL$^{-1}$ of the biotinylated non-complementary strand (probe 2) in 2x SSC buffer for 10 hrs. The electrode was then exposed to a solution of NA-ALP conjugate to label the biotinylated non-complementary oligo probe on the electrode surface. The type 5 electrode represents the electrode functionalized with phenylacetic acid and PEG, but no capture oligo probe. The NSB of the complementary target (probe 1) and NA-ALP were studied on this electrode in the absence of the amine-terminated capture probe. Finally type 6 electrode represents the electrode fabricated with phenylacetic acid groups, PEG and capture probes but without activating the surface phenylacetic acid groups. The binding of target oligo probe and NA-ALP conjugate were carried out onto the type 6 electrode in similar way as described for the type 2 electrode.
3.2.7 DNA assay at various concentrations of complementary oligonucleotides

The RVC electrode functionalized with phenylacetic acid groups was used to study the effect of varying concentration of complementary probes in DNA assay. The phenylacetic acid functionalized RVC electrodes were modified with amine-terminated capture probe and amine-terminated PEG in a way similar as described in Section 3.2.3. A series of these electrodes was used to perform a hybridization assay with biotinylated complementary oligonucleotide. Complementary oligo solutions were prepared in a concentration range from 10 ng mL\(^{-1}\) to 10 µg mL\(^{-1}\) in 2x SSC buffer. Each of series of RVC electrodes was incubated in a different biotinylated complementary oligo solution for 10 hrs. The electrodes were then washed thoroughly with 2x SSC buffer followed by rinsing with tris buffer (pH 8). The NA-ALP conjugate label was then immobilized onto the modified RVC electrodes in a similar way as described in Section 3.2.5.

3.2.8 Detection

A comparative binding of oligonucleotides and NA-ALP enzyme conjugate on all electrodes was studied by measuring the initial rate of conversion of 1 mM HQDP substrate into HQ by surface-bound NA-ALP enzyme. This method was described in Chapter 2 in the context of detecting other biotinylated biomolecules. A brief description on the method is as follows. Electrodes were exposed to 1 mM solution of HQDP in tris buffer and catalytically generated HQ was detected using linear scan voltammetry (LSV). For LSV, a continuous method for 10 scans was set up in such a way that each scan was automatically initiated immediately after the previous scan was completed with quiet time of 10 seconds prior to the start of each individual scan. The time for the HQ oxidative
peak to appear was noted in real time. The time when the electrode was dipped into the solution was also noted and was set as the reference zero time. The increasing peak current with time was recorded at HQ peak potential \(E_p\) and plotted against time. The initial rate of change of HQ peak current was calculated from the initial slope of a linear region of LSV curve as described in Chapter 2 and the value was compared for all modified electrodes.

3.2.9 **Melting temperature (Tm) of 32-mer DNA duplex**

The number of complementary oligonucleotide molecules that can bind to immobilized capture probes inside the pores of RVC electrode was determined using a fluorescence spectroscopic method. To determine the number of oligonucleotide probes attached onto the RVC electrode, the hybridized DNA captured inside RVC pores were denatured at temperature above Tm of duplex. The melting temperature of DNA duplex was first determined by formation of DNA duplex in solution and then denaturing the duplex. The experimental procedure is as follows. A solution of 1 x 10\(^{-5}\) M of capture and complementary target oligo probe in water was mixed together and heated at 90 °C for 30 minutes. The solution was then cooled at room temperature and incubated at 4 °C for 24 hrs for hybridization. After hybridization, the solution was transferred into 1 mL quartz tube and placed inside the Cary 1 UV spectrophotometer. The melting of DNA hybrid was carried out by heating the solution from 20 °C to 98 °C at the rate of 3 °C min\(^{-1}\), followed by cooling down to 20 °C at the same rate. The absorbance of DNA was recorded at 260 nm during the experiment. The Tm of duplex was determined from the plot of absorbance versus temperature.
3.2.10 **Fluorescence spectroscopic detection**

The RVC electrodes were first modified with phenylacetic acid and amine-terminated capture probes as describe in Section 3.2.2 and 3.2.3. The electrodes were then incubated in 100 µL of a 10 µg mL\(^{-1}\) solution of fluorescein-conjugated complementary oligonucleotide (probe 2, Table 3.1) in 2x SSC buffer for 10 hrs. The electrodes were then rinsed thoroughly with buffer and water and dried under stream of nitrogen. The modified RVC foam was then carefully separated from the micropipette tip and placed into 1 mL of water. The DNA hybrid was denatured by heating the solution at 90 ºC for 15 minutes. The solution was then transferred into a fluorescence cuvette and the volume was made up to 2 mL with water. The fluorescein-tagged oligonucleotide solution was excited at 480 nm and its fluorescence intensity was measured at 518 nm. The concentration of fluorescein-tagged oligonucleotide that was released in the solution from RVC electrode surface was obtained using a standard addition method. Constant volumes of 2 µL of 1x 10\(^{-6}\) M fluorescein-conjugated oligonucleotide were added to the unknown sample solution and the fluorescence intensity was recorded at 518 nm following each addition. In a control experiment the phenylacetic acid functionalized RVC electrodes were incubated with fluorescein-conjugated oligonucleotide in the absence of the capture probe. The fluorescence intensity was recorded in a similar manner as described above.
3.3 Results and discussion

3.3.1 Specific and non-specific binding of oligonucleotides and NA-ALP on RVC electrodes

The RVC electrode modified with phenylacetic acid and PEG groups was used to study DNA hybridization inside the pores of RVC. The PEG-modified RVC electrode was selected because it showed lowest NSB of NA-ALP (Chapter 2). All the experiments were repeated on two sets of RVC electrodes (RVC 1 and RVC 2). Figure 3.1a shows the LSV current for HQ generated on type 1 and type 2 electrodes, also on type 3 to type 6 electrodes. The rate of HQ generation on all RVC electrode types is given in Table 3.2. The HQ peak current on type 1 and type 2 electrodes was observed to increase with time indicating the conversion of HQDP into HQ by surface-bound enzyme NA-ALP conjugate. This also proves that the immobilization of DNA duplex on the RVC electrode has occurred. The initial rate of increase of HQ peak current which is proportional to the rate of conversion of HQDP into HQ is given in Figure 3.1b). The initial rate values for type 1 and type 2 electrodes are $2.8 \times 10^{-5}$ A min$^{-1}$ and $1.9 \times 10^{-5}$ A min$^{-1}$ respectively. It was observed that the signal for the type 2 electrode onto which PEG was co-immobilized along with the capture probe, was smaller than that for type 1. One possible explanation for this finding could be that some of the PEG groups on the RVC surface could possibly be blocking the access of target oligo probes and thus hindering the hybridization of some of the target oligonucleotides with surface immobilized capture oligo probes.
Figure 3.1 a) Plot of HQ peak current generated with time on type 1 – type 6 electrodes. b) The initial rate of change of HQ peak current calculated from the initial slope of curves in Figure 3.1a).

Figure 3.1a) also shows a series of plots of HQ peak current versus time for control experiments described in Table 3.2. The non-specific binding of NA-ALP conjugate was studied on the type 3 electrode in the absence of complementary target strand. The rate of change of peak current was $6.2 \times 10^{-7}$ A min$^{-1}$ indicating that much
less enzyme was adsorbed onto this electrode surface. The low NSB onto type 3 electrode could possibly be due to electrostatic repulsion between the negatively-charged oligonucleotide and the negatively-charged NA-ALP at pH 8 (pI of neutravidin ~ 6.3 and ALP ~ 5.7) [11]. The negative charge on DNA is attributed to presence of phosphate ions in the backbone of DNA molecules. The neutral hydrophilic groups of PEG chain also contribute to low NSB of enzyme (Chapter 2). The type 4 electrode was used to study hybridization between a capture probe and a non-complementary biotinylated oligonucleotide probe. The initial rate value on this electrode was comparatively lower than that obtained for the type 2 electrode, indicating low non-specific binding of non-complementary oligo strand with the capture probe. The non-specific binding of the target probe was also studied on the PEG–modified RVC electrode in the absence of a capture probe (type 5 electrode). The initial rate value was relatively low indicating no binding has occurred in the absence of capture probe and NSB was suppressed due to the hydrophilic PEG groups. The type 6 electrode shows the non-specific binding of oligonucleotide and NA-ALP enzyme conjugate, in the absence of carboxylic acid activating agents EDC and NHS. The initial rate of change of HQ peak current on this electrode was $1.02 \times 10^{-6}$ A min$^{-1}$ which was much lower than the signal obtained for otherwise similarly treated electrodes with EDC-NHS activation (type 2 electrode). This finding proves that no covalent coupling of the target probe with surface carboxylic groups could occur in the absence of EDC and NHS. The weak signal observed was probably due to physisorption of oligonucleotides and/or NA-ALP onto the RVC surface.
Table 3.3 The initial rate of change of HQ peak current generated by catalytic conversion of 1mM HQDP. The values were obtained by calculating the initial slope of increasing peak current of HQ with time.

<table>
<thead>
<tr>
<th>RVC Electrode Types</th>
<th>Initial rate of change of HQ peak current</th>
<th>A min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RVC 1</td>
<td>RVC 2</td>
</tr>
<tr>
<td>1</td>
<td>2.81 x 10⁻⁵</td>
<td>3.12 x 10⁻⁵</td>
</tr>
<tr>
<td>2</td>
<td>2.07 x 10⁻⁵</td>
<td>2.68 x 10⁻⁵</td>
</tr>
<tr>
<td>3</td>
<td>6.22 x 10⁻⁷</td>
<td>5.89 x 10⁻⁷</td>
</tr>
<tr>
<td>4</td>
<td>1.20 x 10⁻⁶</td>
<td>9.78 x 10⁻⁷</td>
</tr>
<tr>
<td>5</td>
<td>4.13 x 10⁻⁷</td>
<td>4.46 x 10⁻⁷</td>
</tr>
<tr>
<td>6</td>
<td>1.0 x 10⁻⁶</td>
<td>1.17 x 10⁻⁶</td>
</tr>
</tbody>
</table>

3.3.2 Melting temperature of DNA duplex

The number of oligonucleotide probes immobilized inside the RVC pores was determined by denaturing a fluorescein-conjugated DNA duplex from the surface of RVC into the solution and subsequently measuring the fluorescence intensity of the released fluorescein-labeled oligonucleotides. For denaturation the melting temperature of 32-mer DNA duplex was first determined by recording the absorbance of DNA duplex in water with increasing temperature. Figure 3.2 shows the plot of absorbance of DNA duplex as it melts over the temperature range. From the data, the Tm of the DNA duplex with biotin-
conjugated complementary oligo was determined to be 61.98 °C (Figure 3.2a) and that
with fluorescein-conjugated complementary oligo was 60.96 °C (Figure 3.2b).

Figure 3.2 Melting temperature of 32-mer DNA duplex with a) biotin-conjugated complementary
oligo, b) fluorescein-conjugated complementary oligo. The absorbance was recorded at 260 nm
against temperature in 1 x 10⁻⁶ M solution of DNA duplex in water.
3.3.3 **Quantification of amount of capture oligonucleotides on the RVC surface**

The number of capture oligonucleotide inside the pores of RVC electrode was determined by measuring the fluorescence intensity of fluorescein-tagged hybridized complementary oligonucleotide. The solution containing unknown concentration of denatured fluorescein-tagged oligo (obtained from the RVC electrode as described in Section 3.2.10) was excited at 480 nm and the emission was recorded at 510 nm. Figure 3.3a shows the fluorescence spectra for standard addition of exactly 2 µL of 1 x 10^{-6} M solution of fluorescein-oligo to the cuvette containing unknown concentration of fluorescein-tagged oligonucleotides that were released from the RVC electrode surface. The increasing fluorescence signal was due to a series of 2 µL additions of a 1 x 10^{-6} M solution of fluorescein-conjugated oligo in water to the unknown solution. A control experiment in the absence of amine-terminated capture probe shows very weak signal for fluorescein fluorescence prior to standard addition, which may be attributed to physically (non-specifically) adsorbed fluorescein-tagged DNA onto the RVC surface.

The standard addition curve was obtained by plotting the fluorescence intensity against the concentration of fluorescein-tagged oligonucleotide (Figure 3.3b).
Figure 3.3 Fluorescence spectrum of fluorescein-conjugated oligonucleotide (unknown + 1 x 10^{-9} M in water) of standard fluorescein-oligo excited at \( \lambda = 480 \) nm. b) Fluorescence intensity recorded at \( \lambda = 518 \) nm with increasing concentration of fluorescein-oligo.

The fluorescence signal from the non-specific adsorption was subtracted from each signal value. The concentration of unknown (surface bound) fluorescein-oligo was obtained by extrapolating the line to get \( x \)-intercept (Figure 3.3b). From the graph, the
concentration of surface-bound fluorescein-conjugated oligonucleotide that was released in the solution was estimated to be $4.2 \pm 0.4 \times 10^{-9}$ M or $8 \times 10^{-12}$ moles for 2 mL of sample size. From this value the surface coverage of DNA inside the pores of the RVC electrodes was estimated to be $6 \pm 0.5 \times 10^{-12}$ moles cm$^{-2}$ (surface area of RVC electrode $\sim 1.3$ cm$^2$). The surface density of 32-mer DNA molecules inside the pores of the RVC electrode was estimated to be $4 \times 10^{12}$ probes cm$^{-2}$.

This value was compared with an estimate of the coverage of DNA obtained from the approximate size of DNA. The cross-sectional radius of a DNA chain is estimated to be 0.6 to 0.7 nm [14, 15]. The surface density of the close packed DNA molecules oriented normal to the surface with this radius would be $6 \times 10^{13}$ to $9 \times 10^{13}$ probes cm$^2$. But this calculation does not take into account the additional steric requirements of counter-ions and water of hydration and therefore would be likely to overestimate the surface coverage of DNA [16]. The surface density of DNA is also known to be inversely proportional to the number of nucleotides in the DNA strand. It was observed that as the DNA probe length increases above 24-mer, the DNA strands start to behave as flexible coil-like polymer chains and adsorb side by side onto the surface thus decreasing the surface density [17]. The possible reason for low surface coverage of DNA on RVC surface compared to the geometrically estimated value is probably the long oligo strand (32-mer) used in this study. However the surface density of $4 \times 10^{12}$ molecules cm$^{-2}$ of oligo inside RVC pores was comparable to the surface coverage values of 3 to $4 \times 10^{12}$ DNA molecules cm$^{-2}$ reported for 32-mer DNA on a glassy carbon electrode [10, 18, 19] and 2 to $3 \times 10^{12}$ molecules cm$^{-2}$ on gold [20, 21].
It is also possible to estimate the number of DNA molecules immobilized onto the RVC electrode by analysis of the rate of catalytically generated HQ. The initial rate of HQ generation on the type 1 RVC electrode was considered. The initial slope of the progress curve for type 1 electrode was found to be $2.8 \times 10^{-5}$ A min$^{-1}$ (Table 3.3, type 1). The units A min$^{-1}$ can be converted into moles min$^{-1}$ by using an equation for correlation of HQ redox peak current to the concentration of HQ used for detection. The redox peak current of HQ was obtained for various concentration of HQ in tris buffer (pH 8) and the values were plotted. Figure 3.4 shows the plot of HQ peak current versus HQ concentration obtained from LSV signal. The current behavior was observed to be linear with the concentration.

![Figure 3.4](image)

Figure 3.4 Plot of various concentration of HQ (0.005, 0.01, 0.5, 0.1, 0.5 mM) versus HQ peak current obtained from LSV.

The equation for linear fit was $y = 7.5 \times 10^{-5} x$ (Equation 1), where $x$ is the concentration of HQ in mM and $y$ is corresponding peak current in ampere. Using this
equation, the initial slope $2.8 \times 10^{-5} \text{ A min}^{-1}$ of curve in Figure 3.1 for RVC type 1 electrode was converted into $3.7 \times 10^{-9} \text{ moles min}^{-1}$ of HQ generated in 10 $\mu$L inside RVC. The specific activity of NA-ALP (obtained from supplier) is 208 units $\text{ mg}^{-1}$ of protein which is equal to $208 \mu\text{moles min}^{-1} \text{ mg}^{-1}$. It was assumed that the specific activity of enzyme will remain same at the electrode surface. From this value the amount of NA-ALP immobilized inside pores of RVC was calculated to be $1.8 \times 10^{-5} \text{ mg}$ which corresponds to $7.7 \times 10^{-14} \text{ moles of NA-ALP (M.W. of NA-ALP ~ 230 KDa)}$. By assuming 1:1 binding between NA-ALP and surface bound biotinylated oligonucleotide, the number of DNA immobilized onto RVC electrode was estimated to be $7.7 \times 10^{-14} \text{ moles area}^{-1}$ of RVC electrode which is equal to $6 \times 10^{-14} \text{ moles cm}^{-2}$ or $3 \times 10^{10} \text{ probes cm}^{-2}$. For a control experiment in the absence of complementary oligonucleotide probe (type 3), the moles of DNA inside the RVC pores was obtained to be $9 \times 10^{-16} \text{ moles cm}^{-2}$. From these findings the surface coverage value of oligonucleotides on the RVC electrode estimated from the electrochemical method was almost hundred times lower than the coverage value estimated from the fluorescence method. The difference in surface coverage values obtained from fluorescence and electrochemistry is possibly due to the assumptions made during the electrochemical experiments. In electrochemical determination of surface coverage, it was assumed that the specific activity of NA-ALP enzyme provided for p-nitrophenyl phosphate substrate will also be same for HQDP. The second assumption was that the specific activity of enzyme will remain same after immobilizing onto the RVC electrode surface. It was also assumed that the binding between NA-ALP and biotinylated oligonucleotide is in 1:1 ratio.
3.3.4 Concentration variation of complementary DNA

A set of four type 2 RVC electrodes was treated with four different solutions containing different concentrations of biotinylated complementary oligonucleotides and the initial rate of conversion of HQDP by surface immobilized NA-ALP was studied at each electrodes. Figure 3.5a) shows a series of plots of peak current of generated HQ with time for electrodes exposed to solutions containing different concentration of complementary DNA. The initial rate of change of HQ peak current was calculated from the initial slope for all four concentrations and plotted against oligonucleotide concentration (Figure 3.5b).
The DNA hybridization assay was carried out on the PEG-modified RVC electrode with various concentrations of complementary oligonucleotides. It was observed that the electrochemical response as indicated by the initial rate of HQ generation via enzyme-catalyzed hydrolysis of HQDP on RVC electrodes was not linear with the oligonucleotide concentration. A possible reason for this deviation from linearity is the saturation of capture DNA binding sites present on the RVC surface. However at low concentration of 10 ng mL$^{-1}$, the number of moles of complementary DNA present in 100 µL solution was calculated to be $1 \times 10^{13}$ moles. This value is almost 40 times lower than the estimated surface coverage ($4 \times 10^{12}$ moles cm$^{-2}$) of capture DNA obtained from fluorescence spectroscopy (Section 3.3.3). Therefore saturation of binding sites at this concentration could not be possible.
Another possible reason could involve the co-immobilization of PEG molecules along with the capture oligonucleotide probes. It is speculated that the long chains of PEG groups on the RVC electrode surface is possibly blocking the access of complementary probes for hybridization. The PEG used in this assay has molecular weight of 5000 Da with the 113 repeat units. If each PEG monomer unit is assumed to be 0.35 nm in length [12], then the length of PEG chain can be estimated to be 60 nm. The estimated length 32-mer DNA used in this study is determined to be 10 nm by assuming the length being determined by the distance between two base pairs (0.33 nm) [13]. As the length of PEG molecules is about six times larger than that of 32-mer DNA, it could be possible that the long PEG chain present on the RVC electrode could possibly be interfering in the DNA hybridization. It is also possible that the distribution of PEG molecules inside RVC pores is not uniform. In areas on the electrode where a single capture DNA strand is surrounded by PEG chains, it becomes inaccessible for hybridization with its complementary probe, whereas in cases where multiple DNA strands are present in a given region the hybridization becomes more pronounced (Figure 3.6). This could lead to a cumulative effect on the various electrodes resulting in the number of capture sites available for binding being much lower than expected. This in turn could make is more likely that all available capture sites could be easily saturated, as was apparently observed in the studies of variable complementary DNA concentration.
3.4 Conclusion

The DNA hybridization was successfully carried out inside the pores of the RVC electrode. The initial rate of HQ generation on type 1 and type 2 electrodes was determined to be $2.9 \times 10^{-5}$ A min$^{-1}$ and $2.3 \times 10^{-5}$ A min$^{-1}$ respectively. These values are higher than that obtained on type 3, 4, 5 and 6 electrodes indicating specific binding of DNA and NA-ALP conjugate on the RVC electrode. Control experiments on PEG modified RVC electrodes (types 3, 4, 5, and 6) showed low signal compared to that obtained with DNA assay (type 2). This finding indicated that the PEG has been effective in suppressing the NSB of oligonucleotides and NA-ALP enzyme conjugates. The DNA assay with various concentration of complementary oligonucleotide on PEG functionalized RVC electrode however showed non-linear behavior. The probable reason for this behavior was attributed to the interference of PEG groups on the RVC surface during hybridization. The surface coverage of DNA molecules inside the pores of RVC
electrode was estimated to be $4 \times 10^{12}$ probes cm$^{-2}$ from fluorescence spectroscopic method, which is close to the previously reported data in the literature. The surface coverage of DNA estimated from electrochemical method, however was hundred times lower ($3 \times 10^{10}$ molecules cm$^{-2}$) than that estimated from fluorescence method.

3.5 References


CHAPTER FOUR

4. CONCLUSION AND FUTURE DIRECTIONS

4.1 Findings and conclusion

The objective of this project was to develop a new platform with high sensitivity and low detection limit for the detection of proteins and oligonucleotides. Reticulated vitreous carbon (RVC) was selected as a working platform for electrochemical bioassay because of its 3D porous structure and various advantages over other electrodes. The RVC material was fabricated into electrodes of desired size and shape. The surface area of the electrode was determined to be 1.3 cm$^2$ mg$^{-1}$ and the analysis volume of RVC electrode was 10 ± 1 µL. The electrodes showed partial characteristics of a thin-layer cell which is consistent with the previously reported characteristics for RVC electrodes (Chapter 1).

To use the RVC electrode as a platform for electrochemical bioassay, the surface of the RVC electrode was functionalized with various functional groups such as bovine serum albumin (BSA), phenylacetic acid, polyelectrolytes and poly(ethylene glycol) (PEG). These functional groups were used to anchor biomolecules such as biotin, neutravidin, neutravidin-conjugated alkaline phosphatase (NA-ALP) and biotin-conjugated alkaline phosphatase (B-ALP) specifically onto the RVC electrode surface. The non-specific adsorption (NSB) of these biomolecules was also studied on the functionalized RVC electrodes. Electrodes modified with phenylacetic acid and PEG
were able to successfully suppress NSB as compared to the unmodified RVC electrode and RVC electrodes modified with BSA and polyelectrolytes.

The specific binding of NA-ALP and B-ALP conjugates was highest on the biotinylated BSA modified RVC electrode due to the presence of large number of surface-bound biotin molecules. BSA modified electrode however showed high NSB compared to that on phenylacetic acid and PEG modified RVC. The polyelectrolyte modified RVC was not successful with specific binding of NA-ALP and B-ALP conjugates, probably due to the crashing of an outer poly(acrylic acid) layer after its activation with EDC/NHS. From the specific and non-specific binding experiments, it was concluded that the RVC functionalized with PEG showed competitively better results and was used as a platform for performing neutravidin and DNA bioassay.

The neutravidin sandwich bioassay was carried out on RVC electrodes modified with PEG and biotin-PEO-amine. The neutravidin analyte was sandwiched between surface bound biotin-PEO-amine and B-ALP enzyme label. The calibration curve was obtained by treating the RVC electrodes with various concentrations of neutravidin. The calibration curve showed linear behavior with neutravidin concentration. The concentration detection limit of PEG-modified RVC electrode for neutravidin assay was determined to be 52 ± 2 ng mL$^{-1}$ and the absolute detection limit of 5.2 ± 0.2 ng of neutravidin was achieved. The control experiment with the absence of neutravidin showed negligible signal indicating suppression of non-specific binding of B-ALP due to PEG groups.
The detection of 32-mer oligonucleotide was carried out on the RVC electrode modified with phenylacetic acid and PEG and a capture DNA strand. The non-specific binding of amine-terminated capture oligo probe, biotin-terminated complementary oligo probe, biotinylated non-complementary oligo and NA-ALP was also studied. The low signal values obtained from control experiments indicated that the PEG groups were effective in blocking the NSB of oligonucleotides and NA-ALP.

The DNA hybridization assay was carried out on PEG functionalized RVC electrodes with various concentrations of complementary oligonucleotides. It was observed that the electrochemical response of RVC electrodes was not linear with the oligonucleotide concentration. The possible reason for this deviation from linearity could be the saturation of binding sites present of the RVC surface, even at a low concentration. Another reason could be the co-immobilization of PEG molecules along with the capture oligonucleotide probes. It was speculated that the high of density of long chain PEG molecules and their non uniform distribution inside the pores of RVC electrode could have possibly blocked the access of complementary probes for hybridization.

The surface coverage of DNA molecules on the RVC electrode was determined using fluorescence spectroscopy and an electrochemical method. The surface coverage of oligonucleotides obtained from fluorescence method was estimated to be $6 \times 10^{-12}$ moles cm$^{-2}$ which is comparable with the surface density value for 32-mer DNA on glassy carbon reported in the literature (Chapter 3). The coverage value estimated from the electrochemical method was $6 \times 10^{-14}$ moles cm$^{-2}$ which is hundred times lower than that obtained from fluorescence method. The difference in surface coverage values obtained
from fluorescence and electrochemistry is possibly due to the assumptions made during the electrochemical experiments. In electrochemical determination of surface coverage, it was assumed that the specific activity of NA-ALP enzyme provided for p-nitrophenyl phosphate substrate will also be same for HQDP. The second assumption was that the specific activity of enzyme will remain same after its immobilization onto the RVC electrode surface. It was also assumed that the binding between NA-ALP and biotinylated oligonucleotide is in 1:1 ratio.

4.2 Future directions

Reticulated vitreous carbon could potentially be use as a platform for biosensor with its various advantages over other electrode materials. However care should be taken while fabricating the RVC material into electrodes. The inhomogeneous pore distribution of RVC material results in mechanical instability of the electrode and makes the fabrication of electrodes with consistent size and shape difficult. This factor could also limit the use of RVC as an electrode material for quantitative analysis. Electric connection between RVC foam and gold wire should be made carefully to avoid uneven potential distribution across the electrode. It was observed that sometimes air bubbles can get trapped inside the pores of RVC. The presence of these air bubbles give rise to large oxygen background current which interferes mainly in reduction studies. Therefore care should be taken to remove these air bubbles prior to analysis.

The neutral PEG molecules were effective in suppressing the NSB of biomolecules inside the pores of RVC. However one must consider the concentration and chain length of PEG while immobilizing it inside the RVC pores for bioassay, especially
while studying oligonucleotide assay. While performing DNA assay with RVC electrodes, other factors such as capture oligonucleotide concentration, target oligonucleotide concentration, immobilization time, hybridization time and effect of enzyme label concentration should be considered as these parameters are important to achieve successful hybridization.