EVALUATION OF IMMOBILIZING HORSERADISH PEROXIDASE AND ALCOHOL OXIDASE IN PVA-AWP POLYMER

BY

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THESIS

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ABSTRACT

Biosensor technology is a powerful alternative to conventional analytical techniques, harnessing the specificity and sensitivity of biological systems in small, low cost devices. One of the factors that affect the performance of a biosensor is the immobilization of the biological sensing element, or bioreceptor, on the transducer surface. The objectives of this study were to determine the effects of polymer concentration, ultraviolet (UV) light exposure, and film thickness on the activity and stability of horseradish peroxidase (HRP) and alcohol oxidase (AOX) when immobilized in a photo-crosslinkable and water-soluble polymer, poly (vinyl alcohol) azide-unit water pendant (PVA-AWP). The immobilized enzyme films were to be used in biosensing applications and their stability and activity were determined colorimetrically and electrochemically. Compared to other gel immobilization techniques, PVA hydrogels offer several advantages, such as better elasticity, low-toxicity, biocompatibility with enzymes and yeast cells, mechanical and long-term stability, and biodegradability.

To determine the activity and stability of immobilized HRP, UV–Vis spectroscopy was used to analyze changes in HRP structure since the position of the Soret absorption band at 402-403 nm of the enzyme's heme prosthetic group can provide information on protein conformation. Position of Soret band peaks occurred between 402-403 nm for HRP immobilized in agarose and PVA-AWP (3.8 and 5% w/v). Peak absorbance of the Soret band in AWP 5% (w/v) was found to be 22%, 30%, 25% higher than those using AWP 3.8% (w/v) for UV exposure times 5, 10 and 15 minutes, respectively. From the above results, it can be concluded that UV exposure did not affect the conformation of HRP immobilized in PVA-AWP and the activity was higher in AWP (5% w/v). There was no difference ($p < 0.05$) observed in the enzyme activity of HRP immobilized in PVA-AWP, for UV exposure times of 5, 10 and 15 minutes, and PVA-AWP concentrations of 3.8% (w/v) and 5% (w/v). However, enzyme activity was lower ($p < 0.05$) when HRP was immobilized in glutaraldehyde. There was also no difference ($p < 0.05$) in enzyme leaching for UV 5, 10 and 15 minute exposure when immobilized in PVA-AWP. However, enzyme leaching was higher ($p < 0.05$) for HRP immobilized in glutaraldehyde. It can

be concluded that HRP immobilized in PVA-AWP had higher activity and mechanical stability when compared to HRP immobilized in glutaraldehyde.

Results from the preceding tests were applied to immobilizing AOX in PVA-AWP. An AOX-PVA-AWP mixture containing 0.5 units of AOX activity was drop-coated on a screen printed carbon electrode. The immobilized AOX was tested with varying concentrations of ethanol solution ranging from 17.1 μM to 1.71 mM. The electrical current produced during the oxidation of hydrogen peroxide by cobalt phthalocyanine was measured chronoamperometrically. The magnitude of the current output was dependent on thickness of the film on the electrode. There was no difference ($p < 0.05$) observed in the rate of current output with change in ethanol concentration between AOX immobilized in PVA-AWP and non-immobilized or free AOX, but it was higher ($p < 0.05$) for AOX immobilized in glutaraldehyde. Enzyme activity decreased by 40%, 36% and 7% when stored for 24 hours at 23°C, 3°C and -17°C respectively. When AOX was immobilized in approximately 0.09 mm thick PVA-AWP polymer film, results showed that the lower limit of ethanol detection was 171.3 μM.

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CHAPTER 1
INTRODUCTION

Biosensors are widely applied to many fields such as biocatalytic process, medical care, food, environment, industries, security and defense (Alcock and Turner, 1994; Dennison and Turner, 1995; Kress-Rogers, 1996; White and Turner, 1997). Enzyme-based biosensors are analytical devices that utilize immobilized enzymes as biological recognition components and a transducer to generate a measurable response. The immobilized enzyme acts as a biocatalyst in a heterogeneous phase reaction in which the products are captured at the interface of the immobilized enzyme and transducer.

Since the second half of the last century, numerous efforts have been devoted to the development of insoluble immobilized enzymes for a variety of applications (Silman and Katchalski, 1966). These applications can clearly benefit from use of the immobilized enzymes rather than the soluble counterparts as reusable heterogenous biocatalysts. For example, the use of immobilized enzymes can reduce production costs by efficient recycling and control of the process (Vandamme, 1983; Schulze and Wubbolts, 1999); form the basis for stable and reusable devices for analytical and medical applications (Stetter, 1951; Clark and Lyons, 1962; Campbell et al., 1951; Watanabe et al., 1988; Chang, 1977; Klein and Langer, 1986; Kircka and Thorpe, 1986); and serve as selective adsorbents for purification of proteins and enzymes (Dunlap, 1974), tools for solid-phase protein chemistry (Bickerstaff, 1984; Martinek and Mozhaev, 1985), and microdevices for controlled release of protein drugs (Cristallini et al., 1997). It is recognized that the availability of a robust immobilized enzyme will enable early insight into process development and save costs not only in process development but also in production. However, the lack of guidelines for performance to be expected of an immobilized enzyme seriously hampers application of a rational approach to the design of such robust immobilized enzymes (Van Roon et al., 2002).

Enzymes belong to the category of natural catalyst which includes DNA, RNA and catalytic antibodies. A unique function of enzymes is that all the reactions they catalyze can be

performed sequentially, selectively and precisely under mild physiological reaction conditions. There is, however, no doubt that many are not ideal catalysts for industrial applications. For example in the manufacture of fine chemicals (Liese and Filho, 1999; Schulze and Wubbolts, 1999) and pharmaceuticals and their intermediates (Bommarius et al., 1998), enzymes are usually exposed to unnatural conditions such as elevated substrate concentrations, high pH, and temperature, and the presence of deleterious organic solvents. They must be used in the immobilized forms to reduce production cost by facilitating downstream processing such as recycling and separation (Tischer and Kasche, 1999).

The problem of enzyme immobilization is not how to immobilize the enzyme but how to achieve the desired performance for a given application by selecting an appropriate means of immobilization. Although enzyme immobilization and improvement of enzyme performance by immobilization share the same principles, the emphasis is different. The former is mainly associated with efforts to find suitable immobilization methods for enzymes that must be immobilized for certain applications. The immobilization technique developed is mainly intended to retain the major catalytic functions of the native enzymes. In contrast, improvement-by-immobilization is focused mainly on utilization of available immobilization techniques to alter (or improve) enzyme performance, to suit the desired application. The native enzyme might be not suitable for a desired process, because of its poor performance such as lower activity, or stability or selectivity. The technique to be developed should improve the performance of the enzyme besides immobilizing it.

Stabilization by immobilization has been studied since the 1970s, when immobilized enzymes became increasingly used in industrial processes, in which the cost-contribution of the immobilized enzyme is often the indicator of process viability (Clark, 1994). The stability of a native enzyme (i.e. a non-immobilized or modified enzyme) is principally determined by its intrinsic structure whereas the stability of an immobilized enzyme is highly dependent on many factors, including:

- the properties of its interaction with the carrier;
- the binding position and the number of the bonds;
- the freedom of the conformation change in the matrix;
- the microenvironment in which the enzyme molecule is located;

- the chemical and physical structure of the carrier;
- the properties of the spacer (for example, charged or neutral, hydrophilic or hydrophobic, size, length) linking the enzyme molecules to the carrier; and
- the conditions under which the enzyme molecules were immobilized.

Activity by immobilization is often regarded as an extra benefit rather than the primary goal of enzyme immobilization. Activity retention by carrier-bound immobilized enzymes is usually approximately 50%. At high enzyme loading, especially, diffusion limitation might occur as a result of the unequal distribution of the enzyme within a porous carrier, leading to a reduction of apparent activity (Janssen et al., 2002). The conditions for high activity retention are often marginal, often requiring laborious screening of immobilization conditions such as enzyme loading, pH, carrier and binding chemistry (Taylor, 1985). Next to the microenvironment effect mentioned above, it has been demonstrated that immobilized enzymes can be more active than the native enzymes, when the inhibiting effect of the substrate was reduced (Taylor, 1985).

The criteria for assessing the robustness of the immobilized enzymes remain the same – industrial immobilized enzymes must be highly active (high activity in a unit of volume, U/ml), highly selective (to reduce side reactions), highly stable (to reduce cost by effective reuse), cost-effective (low cost contribution thus economically attractive), safe to use (to meet safety regulations) and innovative (for recognition as intellectual property). As with the volume activity (U enzyme per gram of carrier used), most enzymes bound to carriers with particle sizes above 100 μm (minimum size requirements for a carrier-bound immobilized enzyme have a loading (or payload) ranging from 0.001 to 0.1 (Tischer and Kasche, 1999). The volume ratio of catalyst to reactor is usually in the range 10-20%. The productivity of most immobilized enzymes is still lower than in chemical processes, mainly because of the small number of active sites per mass of biocatalyst (low volume activity) (Straathof et al., 2002).

As a result, it is to be expected that the focus should be the development of a new method of enzyme immobilization that combines the advantages of carrier-free and carrier-bound methods. The new method should be able to provide high enzyme loading (close to that of carrier-free enzymes), high retention of activity, and broad reactor configurations. There is no currently available method that meets these criteria. A vast number of methods of immobilization

are currently available but how to have control over the performance of the immobilized enzyme remains elusive.

In this study, photolinkable polyvinyl (alcohol) azide-unit pendant water-soluble photopolymer (PVA-AWP) was used for immobilizing enzymes. PVA hydrogels have been widely used because of their rubbery elasticity, high degree of swelling in aqueous solutions, inherent non-toxicity and biocompatibility with enzymes, mechanical and long-term stability, and biodegradability. The photocrosslinking or degree of immobilization can be spatially and temporally controlled by the irradiation of light (Stammen et al., 2001; Li et al., 1998; Hyon et al., 1994; Boyd and Yamazaki, 1994). Horseradish peroxidase (HRP) and alcohol oxidase (AOX) are widely used enzymes in biosensing for the detection of hydrogen peroxide and ethanol respectively.

The objectives of this study were to:

1. Determine the effects of polymer concentration and ultraviolet (UV) exposure on the conformation, activity and stability of HRP immobilized in PVA-AWP polymer;
2. Determine the effect of film thickness of PVA-AWP and UV exposure on sensitivity of detection; and the effect of immobilizing AOX in PVA-AWP on biosensor sensitivity.

CHAPTER 2

LITERATURE REVIEW

2.1 Biosensor

A biosensor is described as a compact analytical device, incorporating a biological or biomimetic sensing element, either closely connected to, or integrated within, a transducer system (Figure 2.1). It uses specific biochemical reactions mediated by isolated enzymes, antibodies, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals (Velasco-Garcia and Mottram, 2003). Biosensors are a powerful alternative to conventional analytical techniques, harnessing the specificity and sensitivity of biological systems in small, low cost devices. Its applications are varied, ranging from agriculture - detecting of food borne pathogens and identification of infectious diseases in crops and livestock, measuring food quality parameters during processing, monitoring animal fertility and screening therapeutic drugs in veterinary testing (Velasco-Garcia and Mottram, 2003; Amine et al., 2006); health care – measurement of gases, blood, ions and metabolites required in the human body; to environmental monitoring. One of the factors that affect the performance of a biosensor is the immobilization of the biological sensing element, or bioreceptor, on the transducer surface.

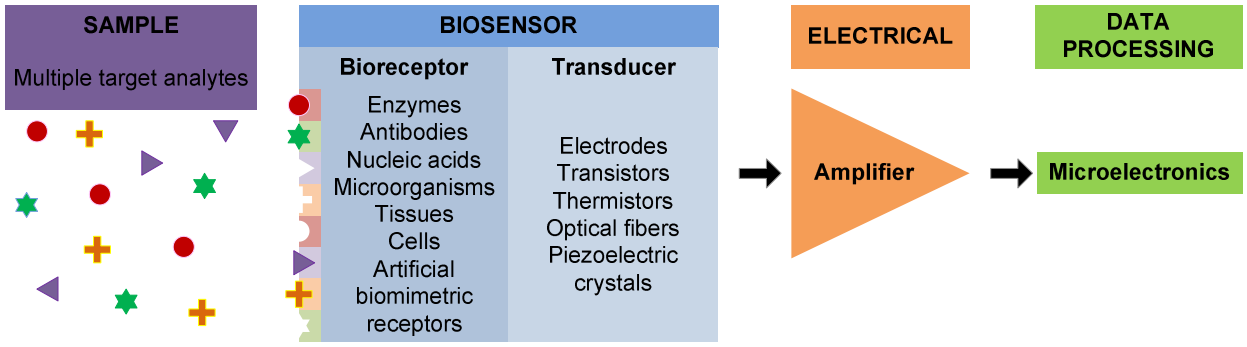

Figure 2.1. The sample comes into contact with a biosensor which recognizes the analyte and converts into sensible information.

2.2 Immobilization of the Bioreceptor

Immobilization attaches the bioreceptors to an inert support material, thereby rendering them insoluble and enhancing their stability and activity. The flexibility and porosity of the immobilized bioreceptor layer must still allow for free diffusion of substrates and products into and out of the layer. Enzyme-based biosensors offer many advantages over conventional chemical methods, which mainly stem for their intrinsic specificity, sensitivity, and ability to operate under mild operational conditions (Vojinovic et al., 2006). The nature and specificity of enzymatic catalytic activity makes them excellent tools for chemical analysis and the reactions can be followed by simple, widely available spectroscopic or electrochemical methods (Azavedo et al., 2005). Since the development of the first enzymatic sensor comprising glucose oxidase, a multitude of enzyme-based sensors have been constructed. Nevertheless, their implementation in commercially successful instruments has been hampered, mainly because of the limited stability of the biological component (Gibson, 1999). The biological component should retain a high degree of stability not only during storage but also during operation (Gibson and Woodward, 1992). For enzyme-based biosensors, the enzymes can be immobilized by physical adsorption, covalent attachment, encapsulation, entrapment into various polymers and crosslinking (Kandimalla et al., 2006). Adsorption techniques are easy to perform, but the bonding of the enzymes to the transducer surface is often weak. Covalent linkage methods tend to be tedious and the compounds involved usually inactivate or reduce the activity of the enzymes. Entrapment in sol-gel systems allows for retention of water in the bioreceptor layer and promotes long-term stability of the enzyme, but the resulting beads or films are susceptible to cracking. There is a need, therefore, for an immobilization procedure that enhances the activity and stability of the enzymes without compromising the mass transfer of analytes through the bioreceptor layer. Additionally, the procedure would need to be practical and cost-effective to enable the mass production of portable enzyme-based biosensors for in situ environmental, bioprocess, food, and biomedical analysis.

The efficiency of an immobilization process can be measured by the following criteria:

- a high percentage of the enzymes must be initially retained in after immobilization;
- the enzymes must be mechanically stable and physically restrained from diffusing back into the substrate solution at a later time;

- the enzymes must be biocompatible with the immobilization medium; and
- the mass transfer of the analytes through the immobilized layer must not be limited.

Because enzymes are biological catalysts that promote the rate of reactions but are not consumed in the reactions in which they participate, they may be used repeatedly for as long as they remain active. However, in most of the industrial, analytical, and clinical processes, enzymes are mixed in a solution with substrates and cannot be economically recovered after the exhaustion of the substrates. This single use is obviously quite wasteful when the cost of enzymes is considered. Thus, there is an incentive to use enzymes in an immobilized or insolubilized form so that they may be retained in a biochemical reactor to catalyze further the subsequent feed. The use of an immobilized enzyme makes it economically feasible to operate an enzymatic process in a continuous mode.

The majority of enzyme immobilization methods can be classified into five main categories: adsorption, microencapsulation, matrix entrapment, crosslinking and covalent bonding (Figure 2.2).

2.2.1 Adsorption

Immobilization by adsorption is the simplest method and involves reversible surface interactions between the enzyme and the support material. The forces involved are mostly electrostatic, such as van der Waals forces, ionic and hydrogen bond interactions. These forces are very weak, but sufficiently large in number to enable reasonable binding. Existing surface chemistry between the enzyme and support is utilized so no chemical activation of modification is required and the enzyme structure is not altered (Messing, 1976; Woodward, 1985; Eggins 2002).

The procedure consists of mixing together the biological components and a support with adsorption properties for a period of incubation, followed by collecting the immobilized material and extensive washing to remove unbound biological components. This procedure offers several advantages such as little or no damage to enzymes; simple, economic and quick; no chemical changes to support the enzyme; and reversible to allow regeneration with fresh enzymes.

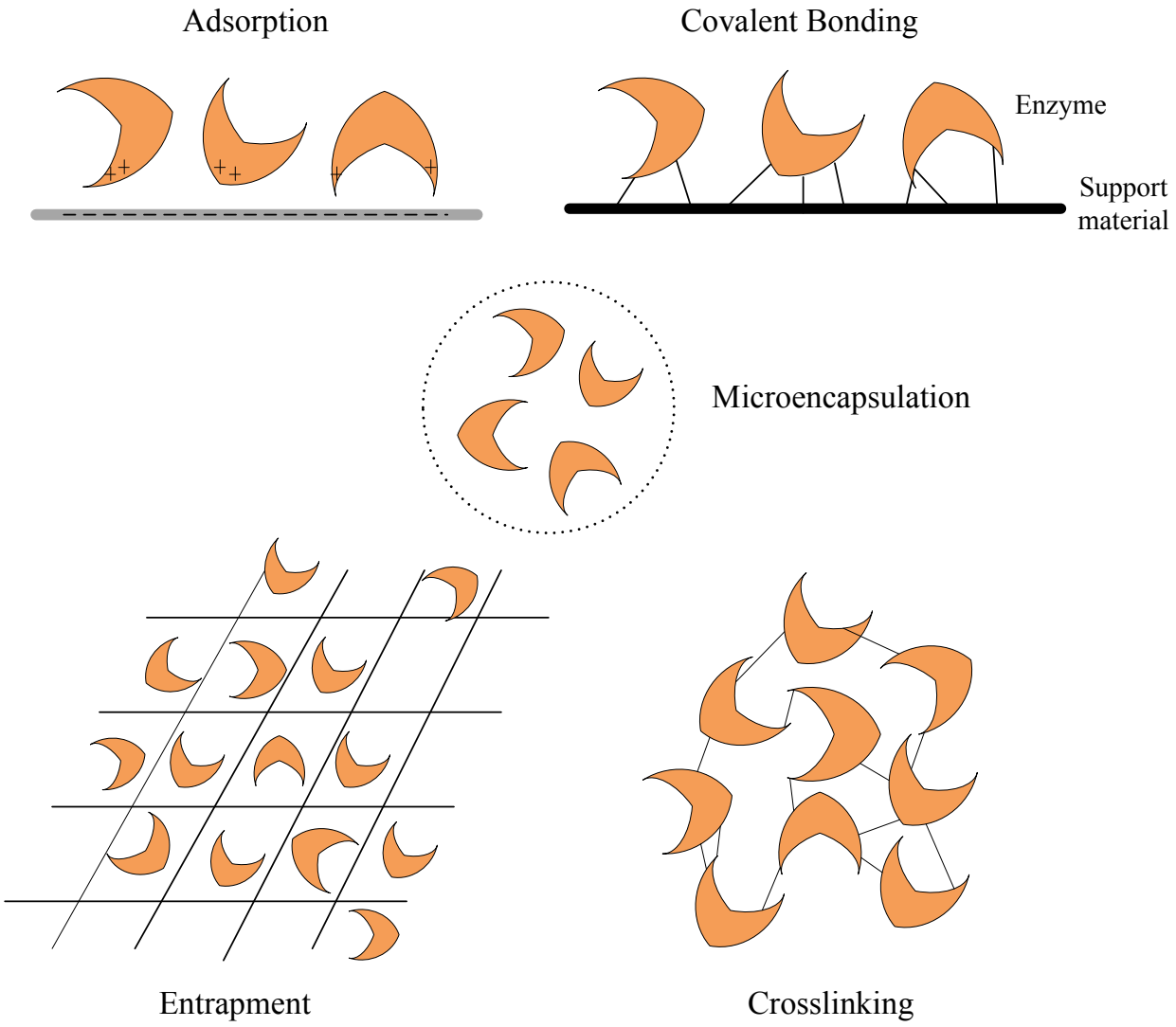

Figure 2.2. The majority of enzyme immobilization methods can be classified into five main categories: adsorption, covalent bonding, microencapsulation, entrapment and crosslinking.

However, the enzymes can leak from the immobilization medium; the binding is nonspecific; overloading on and steric hindrance by the matrix are commonly observed. Nonspecific binding occurs if substrate, product, residual contaminants are charged and interact with the support. This can lead to diffusion limitations and reaction kinetics problems (Goldstein, 1976; Rudge and Bickerstaff, 1984; Toher et al., 1990). Overloading the support can lead to low catalytic activity, and the absence of a suitable spacer between the enzyme molecule and the support can produce problems related to steric hindrance.

2.2.2 Covalent Bonding

Covalent bonding involves a carefully designed bond between a functional group in the biomaterial and the support matrix (Woodward, 1985; Porath and Axén, 1976; Cabral and Kennedy, 1991). The bond is normally formed between functional groups present on the surface of the support and functional groups belonging to amino acid residues on the surface of the enzyme. A number of amino acid functional groups are suitable for participation in covalent bond formation like the amino group (NH_2) of lysine or arginine, the carboxyl group (CO_2H) of aspartic acid or glutamic acid, the hydroxyl (OH) group of serine or threonine, and the sulfydryl group (SH) of cysteine (Srere and Uyeda, 1976).

For covalent bonding of enzymes to support materials, functional groups on the support material are activated by a specific reagent and the enzyme is added in a coupling reaction to form a covalent bond with the support material. The activation reaction is designed to make the functional groups on the support strongly electrophilic or electron deficient. In the coupling reaction, these groups will react with strong nucleophiles (electron donating), such as the amino functional group of certain amino acids on the surface of the enzyme, to form a covalent bond (Bickerstaff, 1995).

2.2.3 Microencapsulation

In microencapsulation, the biomaterial is held in place behind a membrane, giving close contact between the biomaterial and the transducer. This does not interfere with the reliability of the enzyme, and limits contamination and biodegradation. Many materials, such as alginate, nylon, and cellulose nitrate, have been used to construct microcapsules varying from 10-100 μm in diameter. The problems associated with diffusion of analytes are more acute and may result in rupture of the membrane if products from a reaction accumulate rapidly. The immobilized enzyme particle also may have a density similar to that of the bulk solution with consequent problems in reactor configuration and flow dynamics (Kierstan and Coughlan, 1991; Nilsson, 1987; Groboillot et al., 1994).

2.2.4 Entrapment

Enzymes immobilized by entrapment are free in solution, but restricted in movement by the lattice structure of a gel (Bickerstaff, 1995; O'Driscoll, 1976). Matrix entrapment has a fine wire-mesh structure and can more effectively hold smaller enzymes in its cages. The degree of crosslinking depends on the polymerization of the polymer and there is a degree of control over the matrix formation. Because there is a statistical variation in the mesh size, some of the enzyme molecules gradually diffuse toward the outer shell of the gel and eventually leak in to the surrounding medium. Thus, even in the absence of loss in the intrinsic enzyme activity, there is a need to replenish continually the lost enzymes to compensate for the loss of apparent activity. In addition, because an immobilized enzyme preparation is used for a prolonged period of operation, there is also a gradual, but noticeable, decline in the intrinsic enzyme activity even for the best method. Eventually, the entire immobilized enzyme packing must be replaced. Besides the leakage of enzymes, another problem associated with the entrapment method of immobilization is the mass transfer resistance to substrates, products, and inhibitors. Product inhibition may occur for some immobilized enzymes. Thus, ideally the network of crosslinking should be coarse enough so that the passage of substrate and product molecules in and out of a gel bead is as unhindered as possible. It can also have advantages since harmful cells, proteins, and enzymes are prevented from interaction with the immobilized biocatalyst (Brodelius, 1985; Bucke, 1983). Most polymerization reactions that cause crosslinking and gel formation in entrapment methods do not directly involve the formation of bonds between the support material and the enzyme molecules.

Entrapment can be achieved by temperature-induced gelation (e.g., agarose or gelatin), organic polymerization by chemical or photochemical reaction (e.g., polyacrylamide), or precipitation from an immiscible solvent (e.g., polystyrene). The pore size of the gel and its mechanical properties are determined by the relative amounts of monomer and the lattice structure can be influenced. The formed polymer may be broken up into particles of a desired size, or polymerization can be arranged to form beads of defined size (Bickerstaff, 1997).

2.2.5 Crosslinking

In crosslinking, the biomaterials are joined to each other to form a large, three-dimensional complex structure. Covalent bond formation occurs between the biomaterial by means of a crosslinking reagent, such as glutaraldehyde (Broun, 1976). However, the toxicity of such reagents is a limiting factor in applying this method to living cells and many enzymes which might be damaged. In addition, the mechanical strength of the system is poor (Eggins, 2002).

Table 2.1. A comparison of different types of immobilization media used for immobilization.

Technique	Advantages	Limitations	Reference
Polyacrylamide (Entrapment)	1. Non-ionic 2. Properties of enzymes are only minimally modified 3. Diffusion of charged substrate and products not affected	1. Dimethylaminopropionitrile, the polymerization initiator, is highly toxic and must be handled with great care. 2. Monomer solution should be purged with nitrogen	Trevan and Grover, 1979
Calcium Alginate (microencapsulation)	1. Higher enzyme activity yields 2. Calcium ions can be easily displaced by other ions and hence, easy recovery of enzyme	Calcium ions can be easily displaced by other ions.	Ohlson et al., 1979; Vaija et al., 1982; Lee and Woodward, 1983
Gelatin (microencapsulation)	1. Requires only simple equipment and the reagents are relatively inexpensive and nontoxic 2. Retention rates of 25-50% of free enzyme 3. Mass transfer resistance relatively low	Rate of enzyme loss due to leakage is high	de Alteriis et al., 1985

Table 2.1 (cont.)

Avidin-Biotin (covalent bonding)	1. Strong noncovalent interactions which minimize leaching of enzyme 2. Specific binding 3. Greater operational stability	1. Lengthy process 2. Expensive	Goodno et al., 1981; Janolino and Swaisgood, 1982; Walsh and Swaisgood, 1994; Huang, et al., 1995; Janolino et al., 1996
Gluteraldehyde (crosslinking)	Thin layer crosslinking	1. Toxic and needs to be handled with care 2. Poor mechanical stability	Massafera et al., 2009
Polyvinyl (alcohol) having stilbazolium groups (PVA-Sbq) (matrix entrapment)	1. Rapid diffusion of substrate into the active membrane to maximize the catalytic rate 2. Biocompatible polymer 3. Better storage stability 4. Good mechanical resistance 5. Good response time	Operational stability of the immobilized enzyme is not very high from a commercial point of view.	Ichimura, 1980; Marty et al., 1992; Jeanty et al., 1998; Wan et al., 1999; Campas et al., 2005; Chang et al., 2007; Kudo et al., 2008

2.3 Polyvinyl (alcohol) Azide-Unit Pendant Water - Soluble Photopolymer (PVA-AWP)

PVA hydrogels have been widely used as an immobilization medium by entrapment because of their rubbery elasticity, high degree of swelling in aqueous solutions, inherent non-toxicity and biocompatibility with enzymes and yeast cells, mechanical and long-term stability, and biodegradability (Stammen et al., 2001; Li et al., 1998; Hyon et al., 1994; Boyd and Yamazaki, 1994). The photocrosslinking process is spatially and temporally controllable by the irradiation of initiation light. Azide derivatives are typically used as photolabeling reagents for biomolecules in aqueous solutions (Bayley and Knowles, 1977; Staros, 1980; Fink et al., 1980). Pyridine moiety (pK_a = 5.25) in the side chain of AWP (Figure 2.3) provided both water-solubility and adhesiveness to anionic surface, e.g., a glass substrate (Ishizuka et al., 2006). The thickness of the film layer depended on the concentration of AWP (Ito et al., 2005).

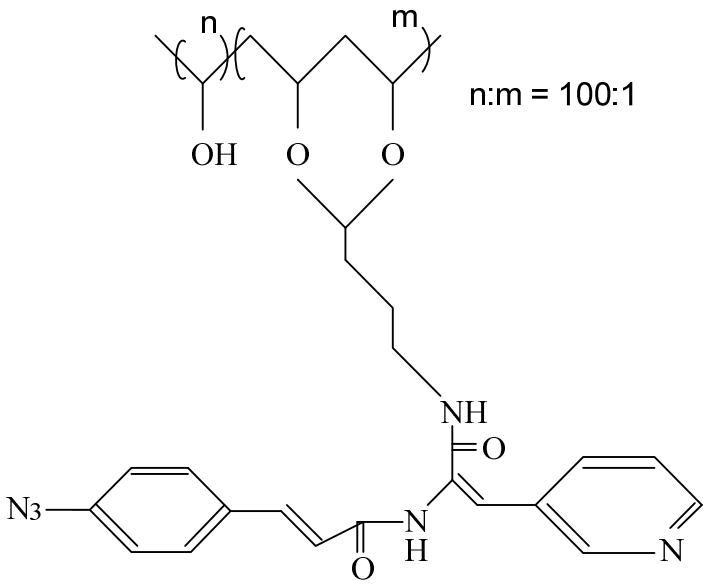

Figure 2.3. Chemical structure of AWP.

Oxidase enzymes and DNA immobilized in PVA-AWP polymer have been found to have better diagnostic sensitivity compared to sol-gel matrix or glutaraldehyde entrapment and the degree of immobilization in PVA-AWP can be controlled by spatially and temporally controlling the irradiation of ultraviolet light (Ishizuka et al., 2006; Iguchi et al., 2007; Gurban et al., 2008). PVA-AWP immobilizes enzymes, horseradish peroxidase and alcohol oxidase, by entrapment. Horseradish peroxidase and alcohol oxidase are widely used in biosensing.

2.4 Horseradish Peroxidase

Horseradish peroxidase (HRP) is an important heme-containing enzyme and is widely used in biosensing. It functions as an indicator in oxidase-based coupled enzyme assays, in enzyme immunoassay (Tijssen, 1985), cytochemistry (Oliver et al., 1984) and DNA probes (Renz and Kurz, 1984). It has great potential for use in biosensor configurations (Sanchez et al., 1990; Cowell et al., 1992). It has good stability at 37°C, high activity at a neutral pH, is nontoxic, and is used in conjugates to determine the presence of a target analyte in coupled enzyme assays, chemiluminescent assays, and immunoassays (Veitch, 2004). Its activity in organic solvents enhances its usefulness in these applications (Dordick, 1992; Kazandjian et al., 1986; Takahashi et al., 1984). Although it acts on a narrow range of peroxides, HRP can be used in a wide range of hydrogen donors, including a range of chromogenic and luminescent compounds (Childs and Bardsley, 1975; Bos et al., 1981; Whitehead et al., 1983; Thorpe et al., 1985; Tijssen, 1985). Its ability to form easily detectable compounds like these has contributed to its widespread use as a detection or probe system. HRP is used for the electrocatalytic reduction of hydrogen peroxide (H_2O_2).

HRP is a moderately stable protein. This is likely due to its tightly bound calcium ions, extensive glycosylation at up to eight different sites, and its four disulfide bridges (Welinder, 1979). Thermal inactivation of the commercial preparation deviates from a first-order decay at 75°C. HRP has good stability characteristics (Dunford, 1991), contributing to its widespread use. Stability of HRP can be improved by modifying the enzyme (Ryan et al., 1994). In oxidase-based sensing, the product hydrogen peroxide can be detected colorimetrically or electrochemically using HRP.

2.5 Alcohol Oxidase

Alcohol oxidase (AOX) is an oligomeric enzyme containing a strongly bound cofactor, flavin adenine dinucleotide (FAD), molecule per sub-unit (Vonck and Bruggen, 1990). Its quarternary structure ranges from four to eight identical subunits arranged in a quasi-cubic spatial distribution. It is produced by methylotropic yeasts (e.g., *Hansenula, Pichia, Candida)* located and assembled in peroxisomes. AOX has been explored in the development of biosensors for the detection of alcohols (Yildiz and Toppare, 2006). It uses molecular oxygen (O_2) as the

electron acceptor and oxidizes alcohols to their corresponding aldehydes. Enzyme activity may be followed by the decrease in the O_2 concentration or the production of hydrogen peroxide.

Applications of alcohol oxidase-based biosensors range from the quantification and detection of ethanol in liquid fermentation samples and to monitoring trace levels of ethanol and methanol in mammalian breath as a result of high gut flora (Patel et al., 2001; Mitsubayashi et al., 2005).

Chapter 3

EVALUATION OF IMMOBILIZING HORSERADISH PEROXIDASE IN PVA-AWP POLYMER

3.1 Introduction

One of the factors that affect the performance of a biosensor is the immobilization of the biological sensing element, or bioreceptor, on the transducer surface. Immobilization attaches the bioreceptors to an inert support material, thereby rendering them insoluble and enhancing their stability and activity. The flexibility and porosity of the immobilized bioreceptor layer must still allow for free diffusion of substrates and products into and out of the layer. For enzyme-based biosensors, the enzymes can be immobilized by physical adsorption, covalent attachment, entrapment into various polymers, microencapsulation or crosslinking (Kandimalla et al., 2006). There is a need for an immobilization procedure that enhances the activity and stability of the enzymes without compromising the mass transfer of analytes through the bioreceptor layer. Polyvinyl (alcohol) hydrogels have been widely used as an immobilization medium by entrapment because of their rubbery elasticity, high degree of swelling in aqueous solutions, inherent nontoxicity and biocompatibility with enzymes and yeast cells, mechanical and long-term stability, and biodegradability (Stammen et al., 2001; Li et al., 1998; Schemdlen et al., 2002; Hyon et al., 1994; Boyd and Yamazaki, 1994). One example of polyvinyl (alcohols) is polyvinyl (alcohol) azide unit water pendant polymer (PVA-AWP), which polymerizes upon exposure to ultraviolet (UV) light. Enzymes, such as horseradish peroxidase, can be entrapped and immobilized within the polymerized matrix and be used multiple times in biosensing and bioprocessing. Horseradish peroxidase (HRP), a heme-containing enzyme produced from horseradish roots, is an important and widely used enzyme in biosensing. It is used in conjugates to determine the presence of a target analyte in coupled enzyme assays, chemiluminescent assays, and immunoassays (Veitch, 2004). Optical absorption spectra of heme proteins and heme complexes exhibit an intense absorption band called Soret band at approximately 400 nm, attributed to a $\pi \rightarrow \pi^*$ electronic transition (Eaton et al., 1978; Eaton and Hofrichter, 1981; Makinen and Churg, 1983). The maximum Soret absorption band is at 403 nm for the heme

group of native HRP (Veitch and Williams, 1990; Kamiya et al., 2000). The position of the Soret absorption band of heme prosthetic group for heme proteins and intensity changes can provide information on protein conformation (George and Hanania, 1953; Herskovits and Jaillet, 1969; Uno et al., 1984). When HRP is denatured, the Soret band will shift or disappear. A shift of 1 to 2 nm in the position of the peak absorbance of the Soret band compared to the native state of the protein does not affect the biological activity of the heme protein but a shift of 5 nm or greater indicates a change in the structure of the enzyme (Liu and Hu, 2003; Xu et al., 2005).

In this study, horseradish peroxidase was immobilized in three types of immobilization media – agarose (HRP-agarose), glutaraldehyde (HRP-glutaraldehyde) and PVA-AWP (HRP-AWP). Agarose is a polysaccharide, whose monomer unit is a disaccharide of D-galactose and 3,6-anhydro-L-galactopyranose. In aqueous solutions below 35°C, the polymer strands of agarose are held together in a porous gel structure by non-covalent interactions like hydrogen bonds and electrostatic interactions which are broken when heated and are re-established on cooling. Agarose gels are formed by gelation though hydrogen bonding and electrostatic interactions thereby entrapping and immobilizing HRP. Since agarose neither absorbs nor polymerizes when exposed to UV light, the effects of UV exposure time on the conformation and activity of free HRP can be determined by measuring the position and magnitude of the Soret band of HRP immobilized in agarose (Wang et al., 2004).

Glutaraldehyde is used for immobilizing enzymes by crosslinking where the enzymes are joined to each other to form a three-dimensional complex structure (Massafera et al., 2009). Immobilization of enzymes in glutaraldehyde by crosslinking is a standard and widely used method in biosensing. However, crosslinked enzymes exhibit low activity retention, poor reproducibility and low mechanical stability (Sheldon, 2007). In entrapment method of immobilization, the enzyme is entrapped while the material is being formed, producing low-leaching bioactive films (Turner et al., 2004). Leaching is the loss of enzyme from the immobilized films and is a measure of the mechanical stability of the films (Novick and Rozzell, 2005).

Colorimetric, chemiluminescent and fluorescent measurements are typically used to detect the activity of immobilized HRP. HRP is used to reduce hydrogen peroxide (H_2O_2) to water (H_2O) at the expense of hydrogen donor molecules (Figure 3.1). The most popular

substrate used is ABTS (2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) (Pappa and Cass, 1993). The oxidized form of ABTS has a bluish green color and is detected at 415 nm (Azavedo et al., 2005).

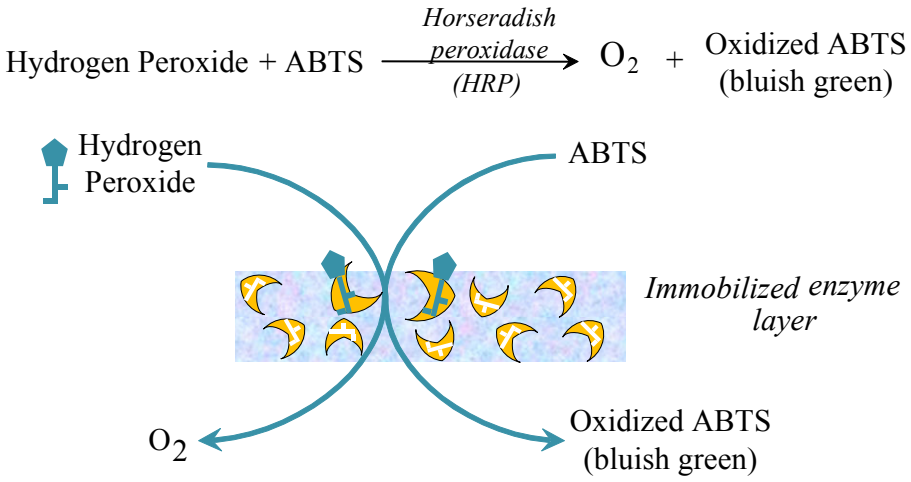

Figure 3.1. The oxidation of ABTS can be used to measure the activity of HRP during the reduction of hydrogen peroxide.

The objectives of this study were to determine

1. the effect of ultraviolet (UV) exposure on the conformation of HRP immobilized by entrapment method in PVA-AWP and agarose, as measured by the position and absorbance of its Soret band; and
2. the effect of immobilizing medium on the relative activity and leaching of HRP immobilized by entrapment method in PVA-AWP and crosslinking method in glutaraldehyde.

3.2 Materials and Methods

3.2.1 Chemicals and Other Reagents

Horseradish peroxidase (E.C. 232-668-6, 1550 units/mg solid) from *Pichia pastoris* was purchased from Sigma-Aldrich (St. Louis, MO). PVA-AWP was received from Toyo Gosei Co. (Chiba, Japan) in the form of 6% (w/v) aqueous solution. ABTS (2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), agarose, bovine serum albumin (BSA, 96%, w/v),

potassium phosphate (≥ 99.0%), dimethylformamide (DMF), hydrogen peroxide (30% w/w) were of analytical reagent grade and purchased from Sigma-Aldrich (St. Louis, MO). Hydrogen peroxide standard solution (0.3% w/w) was prepared immediately before use from a stock solution of 30% (w/w) by diluting in deionized water. Potassium phosphate buffer (100 mM, pH 7) solution was prepared with deionized water and stored at 4°C until use. HRP stock solution of 18 mg/ml was prepared in phosphate buffer to give the required activity per milliliter and was stored at -17°C.

3.2.2 Enzyme Immobilization in Agarose, Glutaraldehyde and PVA-AWP

HRP-Agarose Films

Agarose hydrogel was prepared by dissolving 0.15 g of agarose in 100 ml of boiling water and cooling the mixture at room temperature. A stock solution of agarose, DMF and HRP (18 mg/ml) was prepared in a 4:1:5 volumetric ratio and mixed using a vortex mixer. A 50 µl aliquot of the above mixture was spread onto a hanging drop slide and exposed to ultraviolet light (UVG-11, UVP, Upland, CA, 4 W and 7.1 x 4.5 cm^2) for 0, 5, 10 and 15 min. The HRP-agarose films were left to dry overnight at 4°C to facilitate proper binding of the enzyme to agarose and later rehydrated with 10 µl potassium phosphate buffer prior to testing (Figure 3.2).

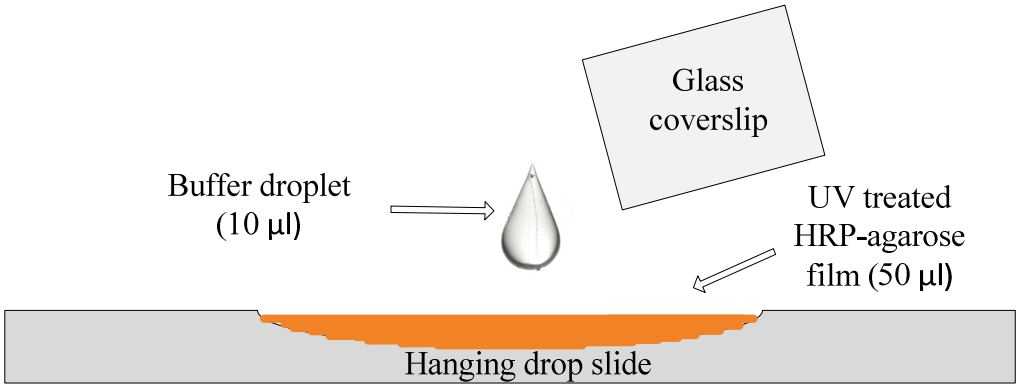

Figure 3.2. Immobilizing HRP in agarose required exposing the film to UV light for 0, 5, 10 or 15 min, drying the film for 12 hours at 4°C and rehydrating the films with 10 µl potassium phosphate buffer prior to testing.

HRP-Glutaraldehyde Films

A stock solution of glutaraldehyde (1% w/v), BSA (40 mg/ml) and HRP (0.1 units/µl) was prepared in a 1:3:6 volumetric ratio and mixed using a vortex mixer. A 25 µl aliquot of the

mixture was spread on the hanging drop slide and left to dry for 3 h at room temperature to allow for crosslinking between the enzyme and glutaraldehyde. The films were washed with 100 µl of buffer to remove any unbound enzyme prior to testing.

HRP-AWP Films

HRP-AWP films were prepared by mixing PVA-AWP at 5.0% or 3.8% (w/v) in potassium phosphate buffer with HRP (18 mg/ml) in a 1:1 volumetric ratio. The HRP-AWP mixture was kept warm at 40°C to reduce the viscosity of the solution. A 50 µl aliquot of the mixture was spread onto a hanging drop slide and exposed to UV light for 5, 10 or 15 min to promote photo-polymerization of the matrix (Figure 3.3). After polymerization, the films were rehydrated with 10 µl potassium phosphate buffer prior to testing.

For determining the activity of HRP, HRP-AWP films were prepared in the same way but using HRP (0.1 units/ µl) and 25 µl aliquot of the HRP-AWP mixture. Activity of free HRP was maintained at 1.25 units in each film. Instead of rehydrating the films with buffer, they were washed with 100 µl buffer solution to remove any unbound enzyme prior to testing.

BSA Films

For control experiments, BSA (18 mg/ml), a non-catalytic protein was used in place of HRP and BSA-agarose, BSA-glutaradehyde and BSA-AWP films were prepared using the same procedures above. Absorbance of the substrate on the films in the absence of enzyme demonstrates the ability of the porous membrane to facilitate substrate transport necessary for enzymatic activity and indicates that the immobilized enzyme is solely responsible for subsequent oxidation (Turner et al., 2004).

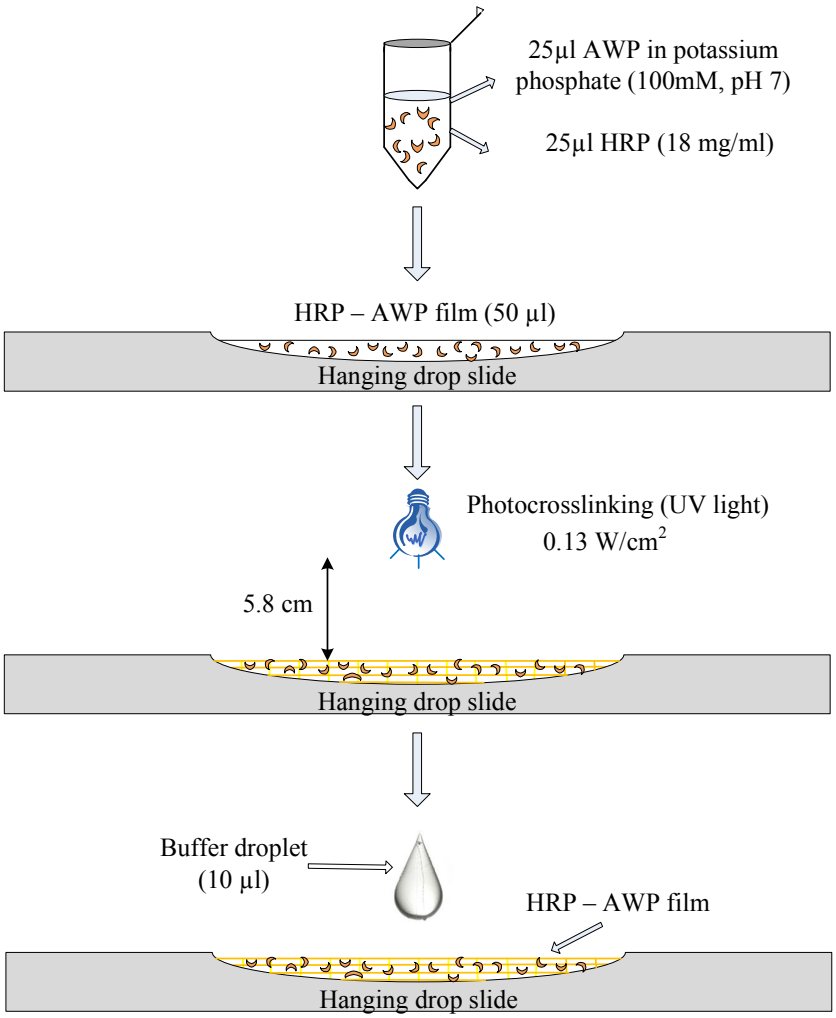

Figure 3.3. HRP-AWP mixtures were exposed to UV light for 5, 10, and 15 min and rehydrated with 10 µl potassium phosphate buffer prior to testing.

3.2.3 Experimental Design

Effect of UV Light on Conformation of Immobilized Enzyme

UV–Vis spectroscopy was used to analyze changes in HRP structure since the position of the maximum absorbance in the Soret absorption band occurs at 403 nm for native HRP. HRP immobilized in agarose was used as a conventional and equivalent method for comparison. The absorbances of the immobilized HRP and BSA films in agarose and PVA-AWP, between 300-500 nm, were measured using a Cary 5G spectrophotometer (Varian Inc., Palo Alto, CA) and recorded using Cary WinUV software.

Effect of Immobilization Medium on HRP Activity

A 20 µl aliquot of ABTS and 5 µl of hydrogen peroxide were added to the washed HRP-glutaraldehyde, HRP-AWP, BSA-glutaraldehyde and BSA-AWP films to determine the activity of HRP. The diffusion of ABTS and hydrogen peroxide through the films were monitored over a period of two min and the absorbance at 415 was measured using a Cary 5G spectrophotometer.

Effect of Immobilization Medium on Leaching of HRP

Leaching of HRP from the films was tested by washing the HRP films with 100 µl of buffer and measuring the activity, via ABTS assays, of the leached HRP in the buffer solution (Figure 3.4).

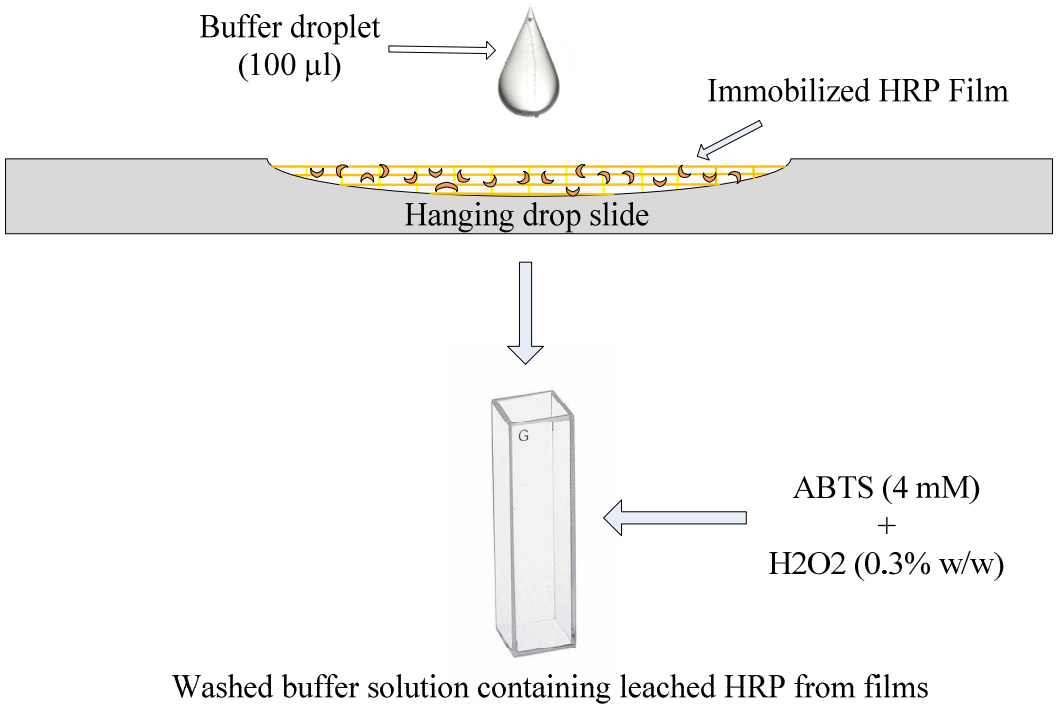

Figure 3.4. Immobilized HRP films were washed with buffer and the activity of leached HRP in the buffer was measured at 415 nm.

Data Analysis

Three replications were conducted for each type of film for determining the Soret band. Data were corrected by subtracting the values of absorbance (absorbance units) of BSA from that of HRP in the immobilized films, thereby removing the effect of the film on the enzyme (Figure 3.5). The first derivative of the absorbance values (AU) over wavelength was approximated

using a 10-point central difference numerical method. The position of the peak of the Soret band coincided with the wavelength at which the first derivate was zero or changed from a positive value to a negative value. This was repeated for the HRP-AWP films.

Regression analysis (R, Version 2.9.0, St. Louis, MO) was used to compare the linearity of the absorbance units (AU) over time for HRP-AWP, HRP-glutaraldehyde, BSA-AWP and BSA-glutaraldehyde films. The slope of the regression curve (AU/min) is the enzyme activity of the films. Data were corrected by subtracting the values of activity (AU/min) of immobilized BSA from that of immobilized HRP films. The experimental setup was arranged as a 2 x 3 factorial in a complete randomized design. Analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test (R, Version 2.9.0, St. Louis, MO) were used to compare the means of final enzyme activity values (AU/min). The level selected to show statistical significance was 5% ($p < 0.05$).

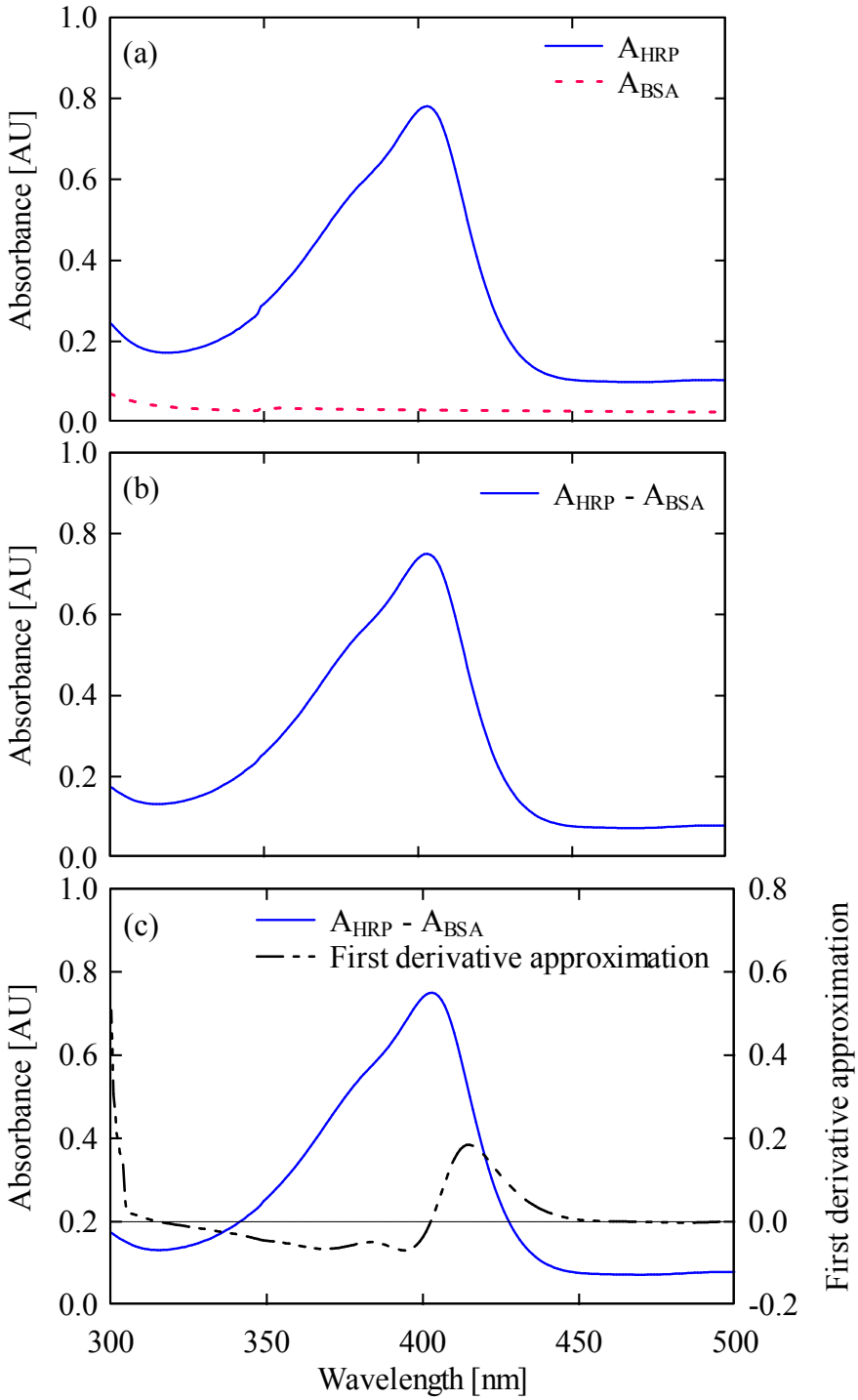

Figure 3.5. Data were corrected by taking (a) original immobilized HRP sample and immobilized BSA control and (b) subtracting the absorbance values of immobilized BSA from those of HRP. (c) The first derivative of the absorbance over wavelength was approximated using a 10-point central difference numerical method to determine the position of the peak of the Soret band which occurred at the point where the first derivative curve crossed the zero line.

3.3 Results and Discussion

3.3.1 Effect of UV Light on Conformation of Immobilized HRP

The position of Soret band peaks occurred approximately between 402-404 nm for HRP immobilized in agarose, PVA-AWP (3.8% and 5% w/v) (Figure 3.6). The position of the Soret band peak occurred at 404 nm for HPR immobilized in agarose and exposed to UV light for 15 min (Figure 3.6). This is due to longer exposure to UV light as compared to UV 5 and 10 min. The position of Soret band peak in native HRP occurs at 403 nm. A shift of 1 nm is not significant and the biological activity of the heme protein was not affected. Protein structure of the enzyme was not affected by entrapment with UV exposure times of 5, 10 and 15 min.

An increase in the Soret absorption indicates that some change at the active site might be responsible for the high enzyme activity. If there is no significant shift in the Soret band but enzyme activity is high, it signifies that the active site maintains a conformation very similar to that of the native enzyme although there might be a change in the bulk structure with a preserved tertiary structure but a few changes in its secondary structure (Zhou et al., 2002). Prolonged UV irradiation leads to protein denaturation (Neves-Petersen et al., 2007).The peak absorbance (absorbance units) of the Soret band decreased as the UV exposure time increased in HRP-agarose films. Peak absorbances of HRP-AWP films were higher than HRP-agarose films (Figure 3.7).

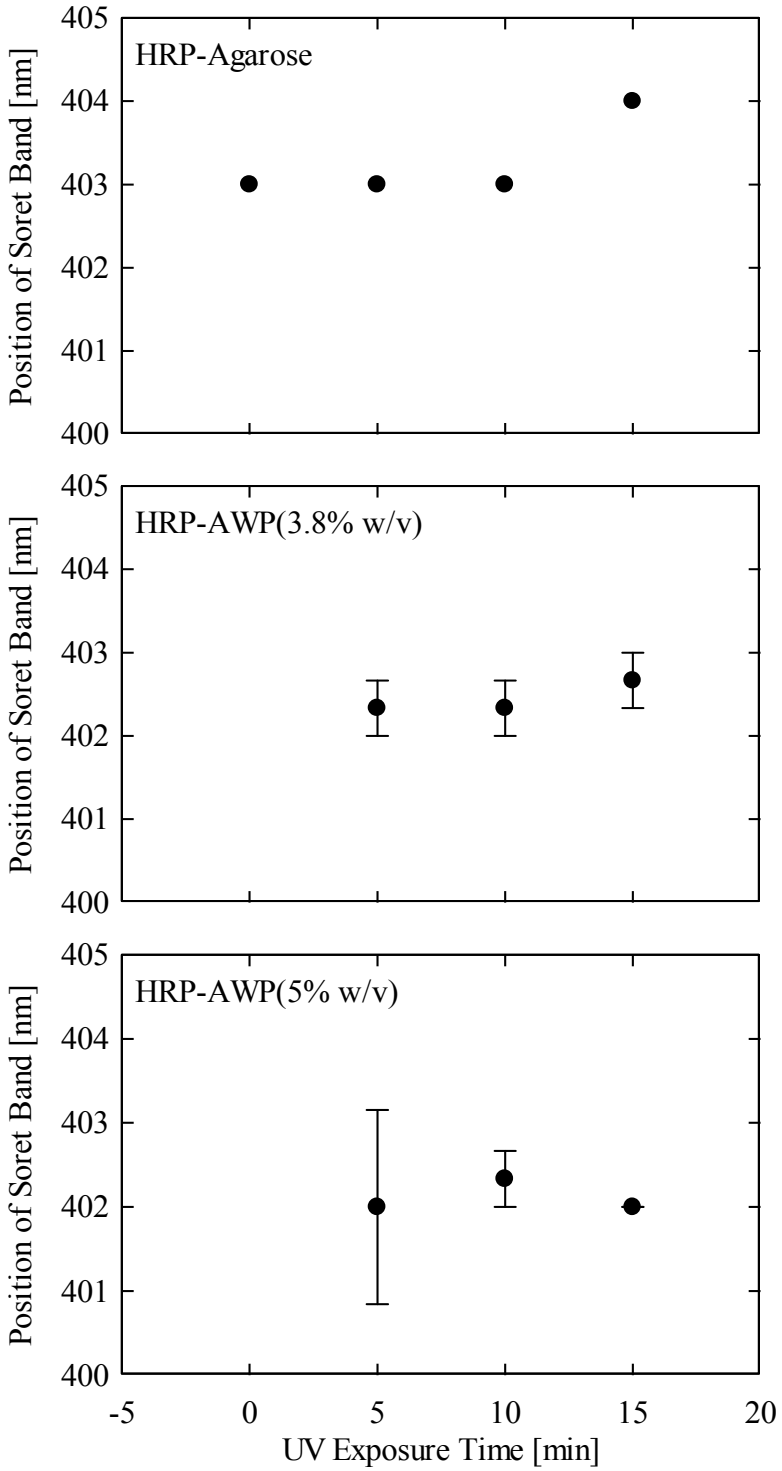

Figure 3.6. The average of three replications is reported for the position of the Soret band in HRP-agarose films at 0, 5, 10, 15 min, HRP-AWP (3.8 and 5% w/v) films at 5, 10, 15 min of UV exposure.

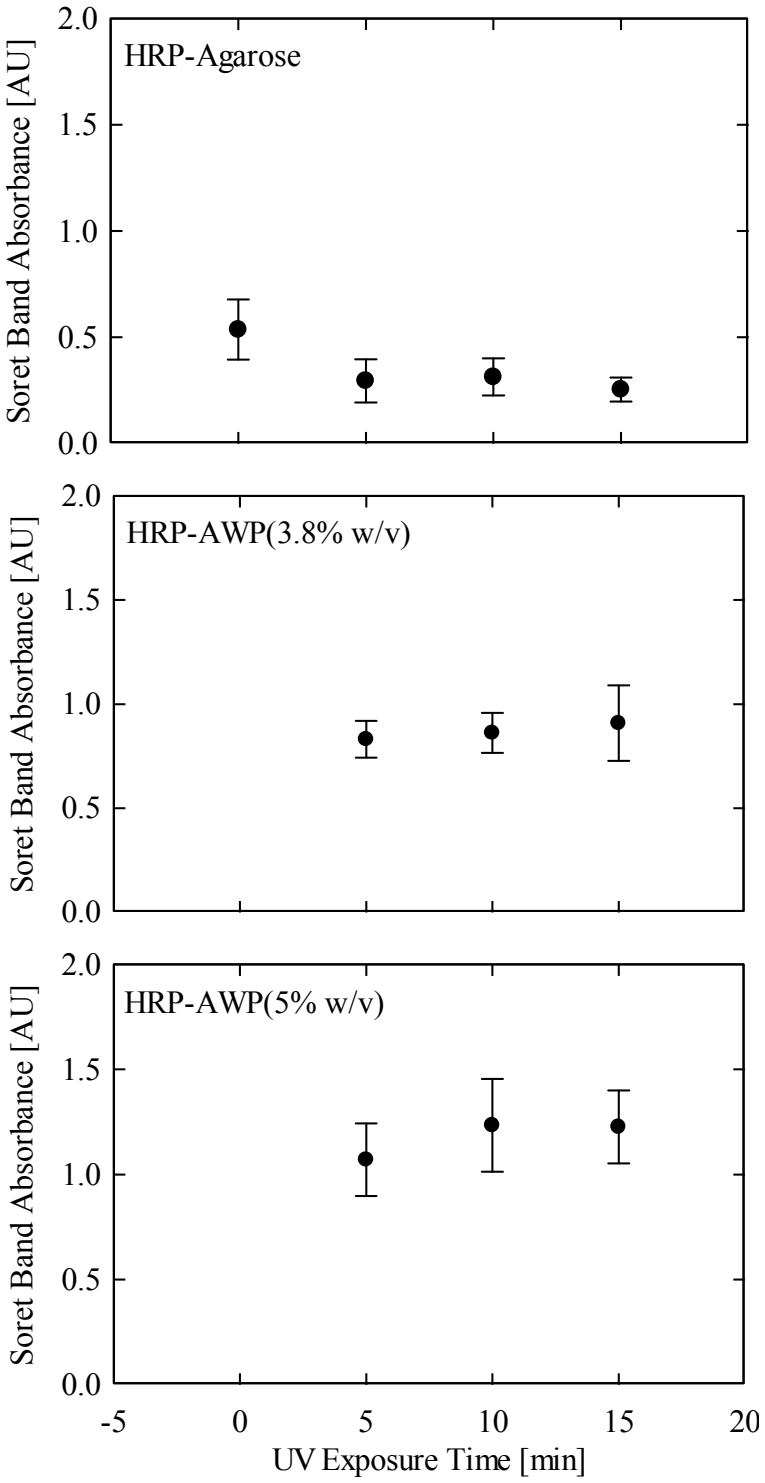

Figure 3.7. The average of three replications is reported for Soret Band absorbance in HRP- agarose films at 0, 5, 10, 15 min, HRP-AWP (3.8 and 5% w/v) films at 5, 10, 15 min of UV exposure.

Peak absorbances of Soret band in HRP-AWP films were higher than those in HRP-agarose films because there was no absorption of UV light by agarose during the exposure time (Figures 3.8 and 3.9).

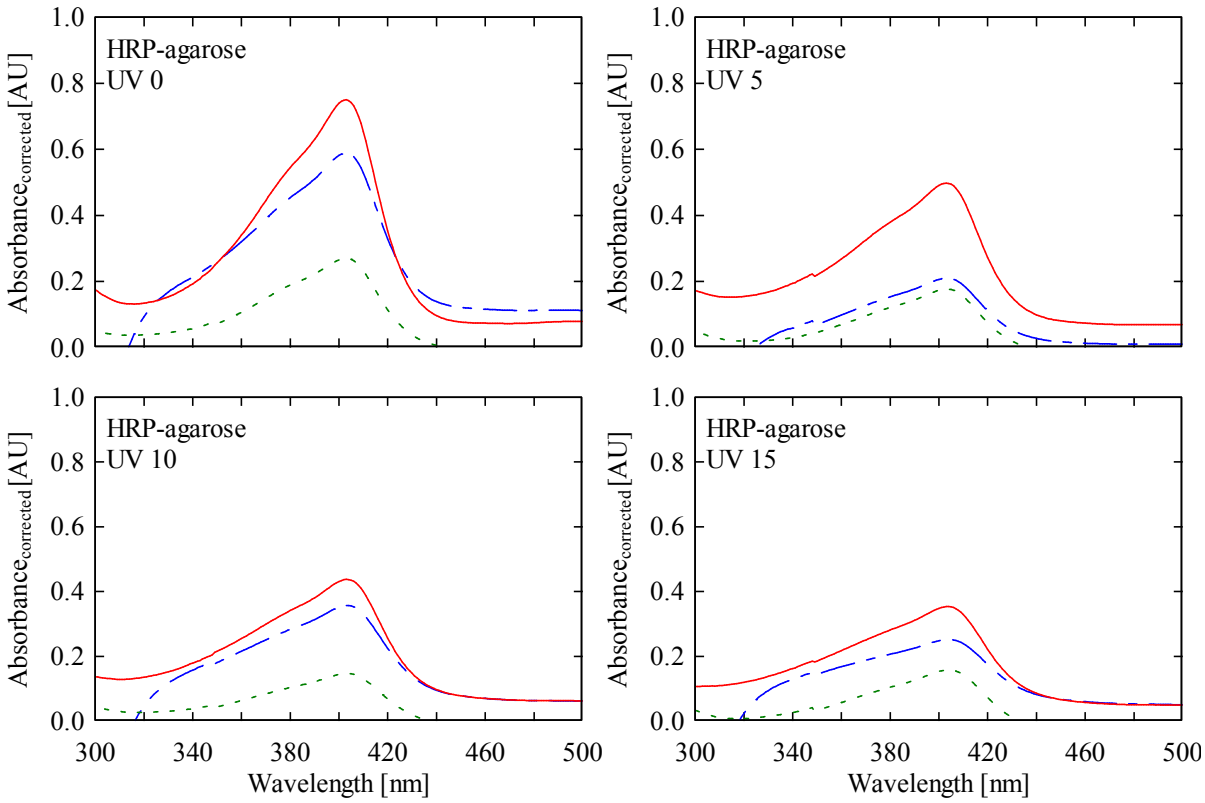

Figure 3.8. Three replications of corrected Soret absorption band are reported for HRP-agarose films exposed to UV light for 0, 5, 10 and 15 min.

Peak absorbance of the Soret band in AWP 5% (w/v) was found to be 22%, 30%, 25% higher than those using AWP (3.8% w/v) for UV exposure times 5, 10 and 15 min, respectively, because AWP (5% w/v) absorbed more UV irradiation than AWP (3.8% w/v), which shielded HRP from UV light (Figure 3.9).

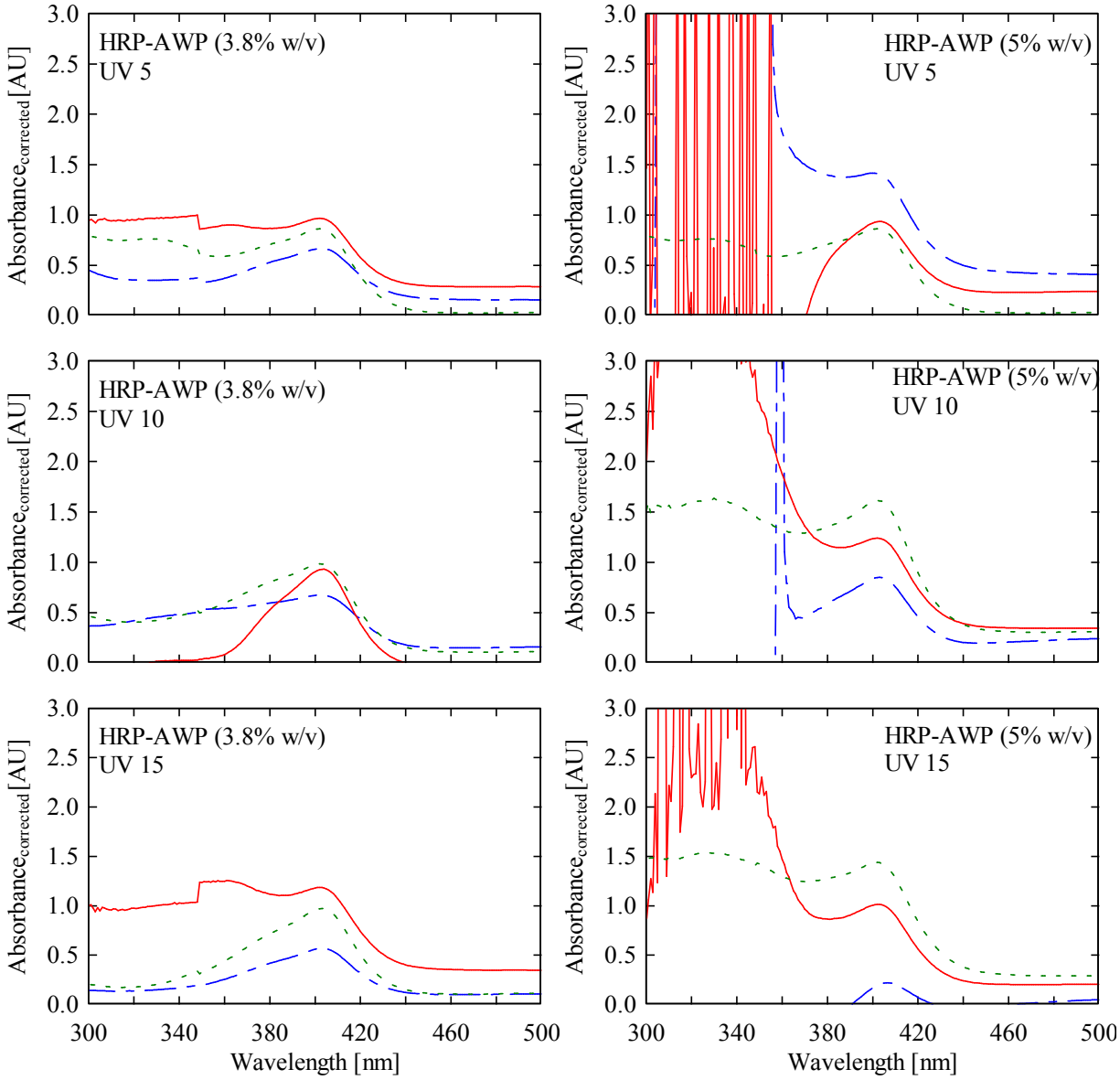

Figure 3.9. Three replications of corrected Soret absorption band are reported for HRP-AWP (3.8 and 5% w/v) films exposed to UV light for 5, 10 and 15 min. HRP-AWP (5% w/v) absorbed more UV light as compared to HRP-AWP (3.8% w/v) films.

3.3.2 Effect of Immobilization Medium on HRP Activity

There was no difference ($p < 0.05$) observed in the activity of HRP immobilized in PVA-AWP, for UV exposure times of 5, 10 and 15 min, and PVA-AWP concentrations of 3.8% (w/v) and 5% (w/v) (Figure 3.10). Enzyme activity was lower ($p < 0.05$) when HRP was immobilized in glutaraldehyde (Table 3.1).

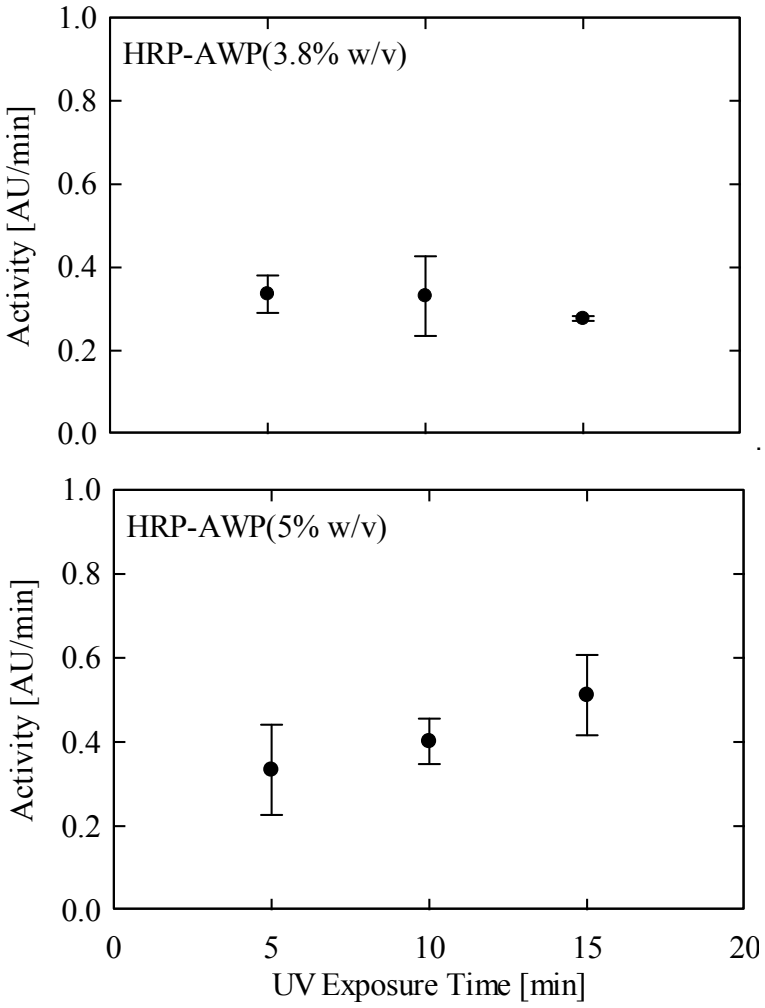

Figure 3.10. Enzyme activity was measured over a period of 2 min in HRP immobilized in PVA-AWP (3.8 and 5% w/v) for UV exposure times 5, 10 and 15 min.

Table 3.1. HRP activity values (AU/min) in HRP-AWP and HRP-glutaraldehyde films (means of triplicate runs ± 1 standard error).

Immobilization medium	UV exposure time[c] (min)	PVA-AWP Concentration (% w/v)[c]			Mean[b] (concentration and UV)
		3.8	5	Mean[a] (UV)	
PVA-AWP	5	0.34 ± 0.08	0.33 ± 0.11	0.34a	0.37 ± 0.13A
	10	0.33 ± 0.17	0.40 ± 0.05	0.37a	
	15	0.31 ± 0.01	0.52 ± 0.10	0.42a	
Mean[a] (Concentration)		0.32A	0.42A		
Glutaraldehyde (1% w/v)					0.11 ± 0.02B

[a] Mean enzyme activity values followed by the same letter in a column (abc) or row (AB) were not different ($p < 0.05$)

[b] Mean enzyme activity values followed by the same letter in the same column (AB) were not different ($p < 0.05$)

[c] LSD value across UV exposure times and 3.8, 5% (w/v) concentrations were 0.1657 and 0.1353, respectively.

Enzymatic activity is inversely proportional to the concentration of glutaraldehyde because extensive crosslinking may result in a distortion of the enzyme structure (i.e., the active site conformation) (Chui and Wan, 1997). With this distortion, the accessibility and accommodation of the substrate may be reduced, thus affecting the retention of biological activity. The relative concentration of enzyme to glutaraldehyde should also be considered (Okuda et al., 1991). Low concentrations of enzyme and glutaraldehyde tend to induce intramolecular crosslinking by enhancing the probability that glutaraldehyde functional groups will react with the same enzyme molecule (Zaborsky, 1973). Conditions should be chosen carefully to favor intermolecular crosslinking between enzyme molecules instead of unwanted intramolecular links, which could also be formed (Broun, 1976; Bano and Saleemuddin, 1980; Gupta, 1993).

From the above results, it can be concluded that glutaraldehyde crosslinking has changed the structure of immobilized HRP leading to lower activity when compared to immobilization in PVA-AWP.

3.3.3 Effect of Immobilization Medium on Leaching of HRP

There was no difference ($p < 0.05$) observed in the activity of leached HRP from HRP-AWP films, for UV exposure times of 5, 10 and 15 min, and PVA-AWP concentrations of 3.8% (w/v) and 5% (w/v) (Figure 3.11). The activity of leached HRP from HRP-glutaraldehyde film was higher ($p < 0.05$) when compared to HRP-AWP films (Table 3.2). For leaching, the films were washed with buffer. Enzymes prefer aqueous environments creating a distinct possibility for enzyme leaching during washing processes (Turner et al., 2004). The higher activity of the buffer containing the leached HRP from HRP-glutaraldehyde when compared to HRP-AWP films indicates higher mechanical stability of HRP-AWP films.

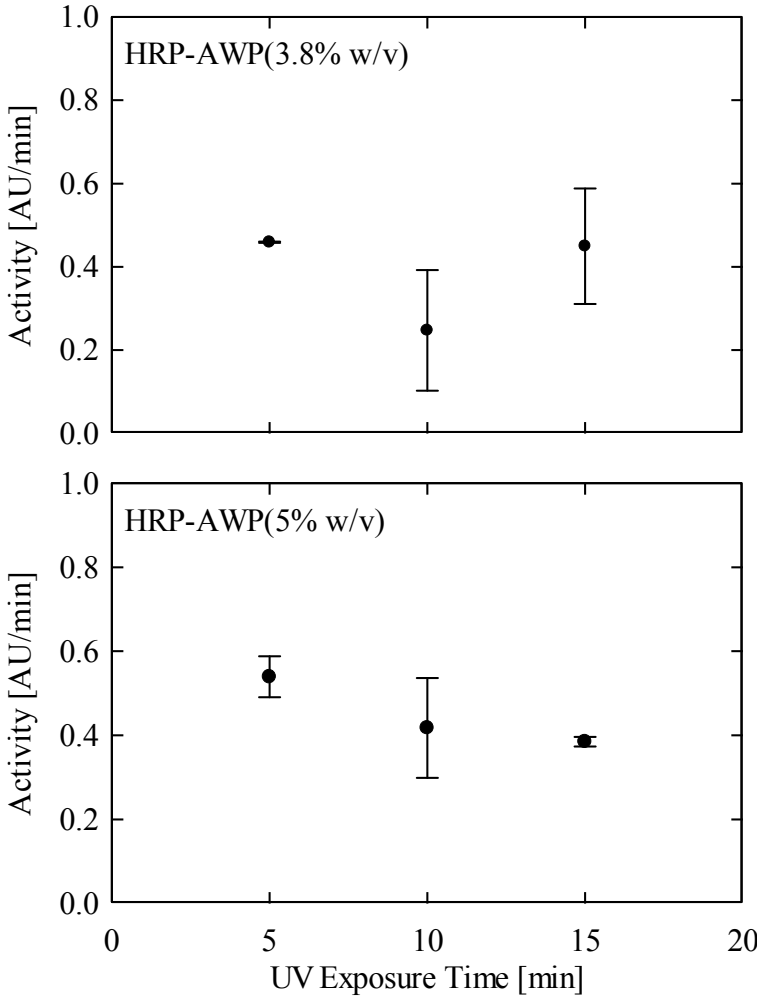

Figure 3.11. Leaching was measured in HRP immobilized in PVA-AWP (3.8 and 5% w/v) films for UV exposure times 5, 10 and 15 min.

Table 3.2. Leached HRP activity values (AU/min) in HRP-AWP and HRP-glutaraldehyde films (means of triplicate runs ± 1 standard error).

Immobilization medium	UV exposure time[c] (min)	PVA-AWP Concentration (% w/v)[c]			Mean[b] (concentration and UV)
		3.8	5	Mean[a] (UV)	
PVA-AWP	5	0.46 ± 0.00	0.54 ± 0.05	0.50a	0.42 ± 0.17A
	10	0.25 ± 0.15	0.42 ± 0.12	0.33a	
	15	0.45 ± 0.14	0.39 ± 0.01	0.42a	
Mean[a] (Concentration)		0.39A	0.45A		
Glutaraldehyde (1% w/v)					0.71 ± 0.11B

[a] Mean enzyme activity values followed by the same letter in a column (abc) or row (AB) were not different ($p < 0.05$)

[b] Mean enzyme activity values followed by the same letter in the same column (AB) were not different ($p < 0.05$)

[c] LSD value across UV exposure times and 3.8, 5% (w/v) concentrations were 0.2118 and 0.1729, respectively.

3.4 Conclusions

The positions of the Soret band absorbance peaks occurred between 402-404 nm for free and immobilized HRP in PVA-AWP. The peak absorbance of the Soret band decreased as UV exposure of HRP-agarose films increased. The peak absorbance of the Soret band in PVA-AWP (5% w/v) was found to be 22%, 30%, 25% higher than those using PVA-AWP (3.8% w/v) for UV exposure times 5, 10 and 15 min, respectively. From the above results, it can be concluded that UV exposure did not affect the conformation of HRP immobilized in PVA-AWP and the activity was higher in AWP (5% w/v).

There was no difference ($p < 0.05$) observed in the activity and leaching of HRP immobilized in PVA-AWP, for UV exposure times of 5, 10 and 15 min, and PVA-AWP concentrations of 3.8% (w/v) and 5% (w/v). Enzyme activity was lower ($p < 0.05$) and leaching was higher ($p < 0.05$) when HRP was immobilized in glutaraldehyde. It can be concluded that

HRP immobilized in PVA-AWP had higher activity and mechanical stability when compared to HRP immobilized in glutaraldehyde.

Chapter 4

EVALUATION OF IMMOBILIZING ALCOHOL-OXIDASE IN PVA-AWP POLYMER

4.1 Introduction

Immobilization is a double edged sword. On one hand immobilization attaches the bioreceptors to an inert support material in a manner rendering them insoluble and fixing their position in space, so they can be effectively utilized in continuous processes; on the other, immobilizing the bioreceptor can reduce the bioactivity because of a compromise in the mass transfer of analytes through the bioreceptor layer. The stability and activity of alcohol oxidase (AOX) immobilized in glutaraldehyde, which has been effectively demonstrated in the development of assays and devices for measuring ethanol content, was compared to AOX immobilized in a polyvinyl (alcohol) azide-unit pendant water-soluble photopolymer (PVA-AWP). PVA hydrogels offer several advantages, such as better elasticity, low toxicity, biocompatibility with enzymes and yeast cells, mechanical and long-term stability, and biodegradability (Stammen et al., 2001; Li et al., 1998; Hyon et al., 1994; Boyd and Yamazaki, 1994). PVA-AWP polymerizes upon exposure to ultraviolet (UV) light. Enzymes, such as AOX, can be entrapped and immobilized within the polymerized matrix and be used multiple times in biosensing and bioprocessing (Gurban et al., 2008). AOX catalyzes the oxidation of lower primary aliphatic alcohols to the respective aldehydes with oxygen as the electron acceptor and releases hydrogen peroxide (H_2O_2), which is electrochemically detectable using cobalt phthalocyanine (CoPC) modified screen printed carbon electrodes (Veenhuis et al., 1983; Azavedo et al., 2005; Wring and Hart, 1992) (Figure 4.1). Immobilization using horseradish peroxidase performed in the previous study was to determine whether PVA-AWP and UV exposure cause a change in the conformation of the enzyme. However optical measurement of enzyme activity was not practically feasible.

In this study, alcohol oxidase was immobilized in two immobilization media – glutaraldehyde (AOX-glutaraldehyde) and PVA-AWP (AOX-AWP). Glutaraldehyde is used for immobilizing enzymes by crosslinking where the enzymes are joined to each other to form a three-dimensional complex structure (Massafera et al., 2009). Immobilization of enzymes in glutaraldehyde by crosslinking is a standard and widely used method in biosensing. However, glutaraldehyde is toxic which limits its application to living cells and many enzymes as they might be damaged (Eggins, 2002).

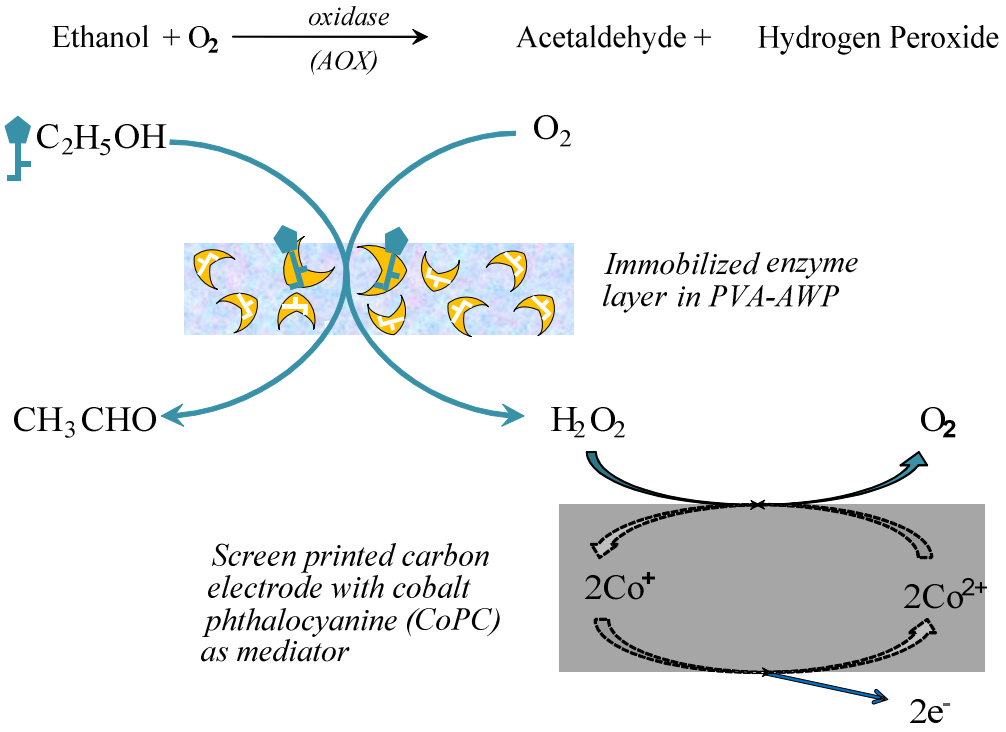

Figure 4.1. Alcohol Oxidase catalyzes the oxidation of ethanol to release hydrogen peroxide. The redox reactions between hydrogen peroxide and the CoPC mediator produce a flow of electrons that is proportional to the amount of ethanol in the sample.

The objectives of this study were to:

1. determine the effect of PVA-AWP film thickness and UV exposure time on the sensitivity of detection;
2. determine the effect of immobilizing AOX in PVA-AWP on biosensor sensitivity as compared to a conventional immobilization medium, glutaraldehyde; and
3. assess the stability of AOX immobilized in PVA-AWP after 24 h at different storage temperatures.

4.2 Materials and Methods

4.2.1 Chemicals and Other Reagents

Alcohol oxidase (AOX, EC. 232-971-3, 30 Units/mg protein) from *Pichia pastoris* was purchased from Sigma Aldrich (St. Louis, MO) as a phosphate-buffered 30% sucrose solution. PVA-AWP was received from Toyo Gosei Co. (Chiba, Japan) in the form of 6% (w/v) aqueous solution. Bovine serum albumin (BSA, 96% w/v), glutaraldehyde (25% w/v), potassium phosphate (≥ 99.0%), and hydrogen peroxide (30% w/w) were of analytical reagent grade and purchased from Sigma-Aldrich (St. Louis, MO). Potassium phosphate buffer solutions (100 mM, pH 7) were prepared with deionized water and stored at 4^{o}C until use. Ethanol standard solutions were prepared by dilution in deionized water immediately before use.

4.2.2 Methods

Biosensor Preparation

Electrochemical tests were carried out using screen printed carbon electrodes (SPCE, Figure 4.2). The SPCE cell consisted of a CoPC-mediated carbon working electrode and a 60/40 Ag/AgCl electrode which served as the reference and counter electrode. The electrode cell was arranged in a circular configuration, with the working electrode in the center (27.61 mm^2) and the reference/counter electrode around it (41.7 mm^2). The gap between the two electrodes was 1.12 mm.

Chronoamperometric measurements were performed using an electrochemical analyzer or potentiostat (WaveNow, WEB50-EDU, Pine Instrument Company, Grove City, PA), at a potential of 400 mV vs. Ag/AgCl. Electrocatalytic oxidation of hydrogen peroxide occurs readily at this potential (Boujtita et al., 2000). Chronoamperometry was performed on non-immobilized AOX, AOX immobilized in PVA-AWP (AOX-AWP) and AOX immobilized in glutaraldehyde (AOX-glutaraldehyde) films. A 20 µl volume of ethanol solution (0.02-1.7 mM) was deposited over the entire electrode cell ensuring that both the working and reference/counter electrodes were covered (Figure 4.2). Current response to ethanol sample at different concentrations of ethanol solutions were calculated from the resulting chronoamperograms (Figure 4.3).

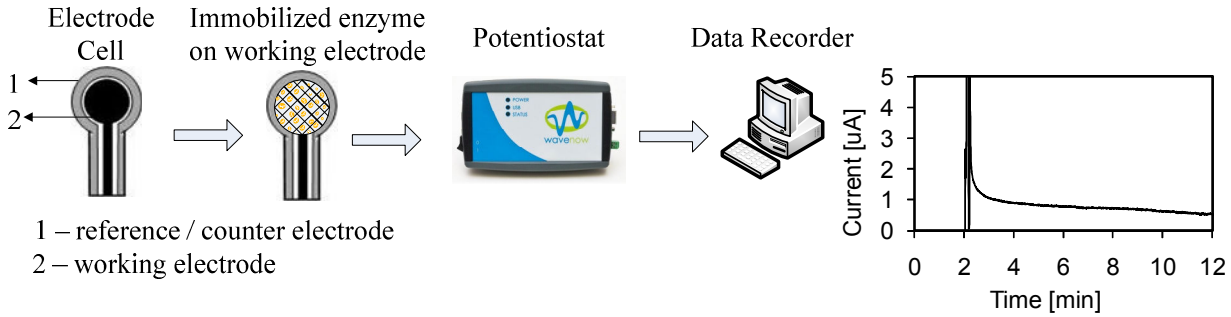

Figure 4.2. An AOX biosensor was prepared by dropcoating the enzyme-immobilizing medium on the working surface of the electrode. Ethanol solution was spread over the entire working and reference/counter electrodes and the current response was measured using a potentiostat.

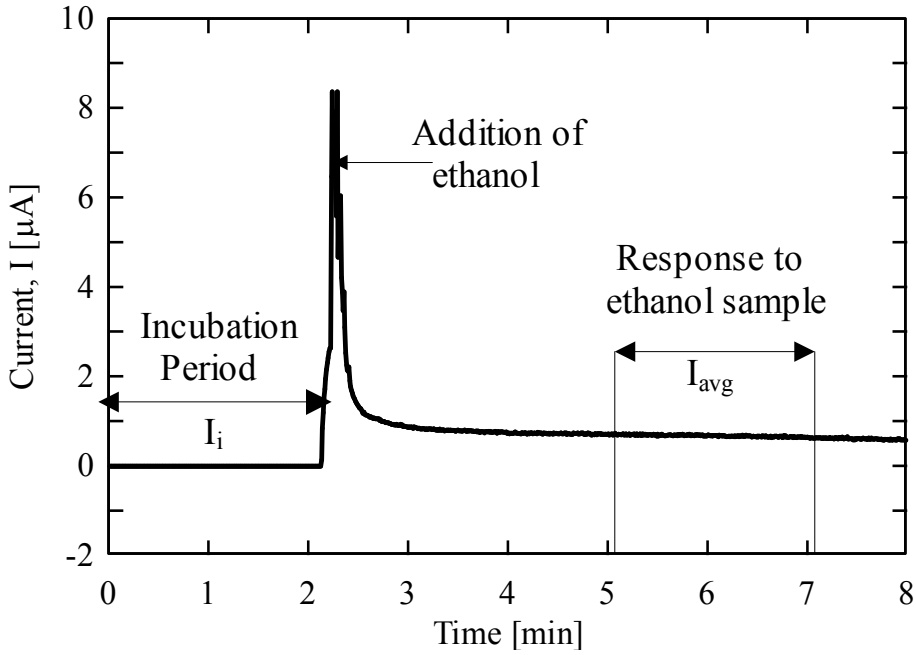

Figure 4.3. An incubation period of 2 min was used before adding ethanol to the electrode and the current response recorded between 5 to 7 min from the start of the test was averaged (I_{avg}) and later corrected by subtracting the average response during the incubation period (I_i).

Enzyme Immobilization in Glutaraldehyde and PVA-AWP

AOX stock solutions of 0.1 U/µl, 0.2 U/µl and 0.4 U/µl were prepared in potassium phosphate buffer containing 100 mM potassium chloride and 1% (v/v) Tween 20 to give the required enzyme activity per milliliter. The AOX stock solutions were stored at -17°C until use.

Tween 20 is a surfactant that is used to lower the surface tension of a liquid, enhancing the contact angle between liquid and the electrode surface. AOX-immobilizing medium solutions were dropcoated over the working surface of the electrode to yield an enzyme loading of 0.5 U (0.02 U/mm^2) per electrode.

AOX-Glutaraldehyde Films

A stock solution of glutaraldehyde (1% w/v), BSA (40 mg/ml) and AOX (0.2 U/µl) was prepared in 1:3:6 volumetric ratio and mixed using a vortex mixer. A 4 µl aliquot of the enzyme-glutaraldehyde mixture was dropcoated on the working electrode and left to dry for 3 h at room temperature to allow for crosslinking between the enzyme and glutaraldehyde.

AOX-AWP Films

PVA-AWP was diluted to 5% (w/v) in potassium phosphate buffer. The PVA-AWP solution was kept warm at 40°C to reduce its viscosity. PVA-AWP and AOX (0.4 U/µl) were mixed in 1:1 volumetric ratio. An aliquot of the AOX-AWP mixture on the electrode was exposed to ultraviolet light (UVG-11, UVP, Upland, CA, 4 W and 7.1 x 4.5 cm^2) to promote polymerization of the PVA-AWP medium and entrapment of AOX (Figure 4.4). The films were left to dry for 3 h at room temperature before testing.

To determine the volume of PVA-AWP to be used as immobilization medium above and UV exposure time, hydrogen peroxide (H_2O_2) step experiment was conducted. Three volumetric aliquots of PVA-AWP (5% w/v) and BSA (40 mg/ml) in 1:1 volumetric ratio were immobilized on the electrode using the procedure described for immobilizing AOX in PVA-AWP. BSA was used as a noncatalytic protein in place of AOX. The films were left to dry for 3 h at room temperature before testing.

Non-immobilized AOX Films

Non-immobilized AOX films were prepared by dropcoating 5 µl of AOX (0.1 U/µl) on the working surface to yield an enzyme loading of 0.5 U per electrode. The films were left to dry for 3 h at room temperature before testing.

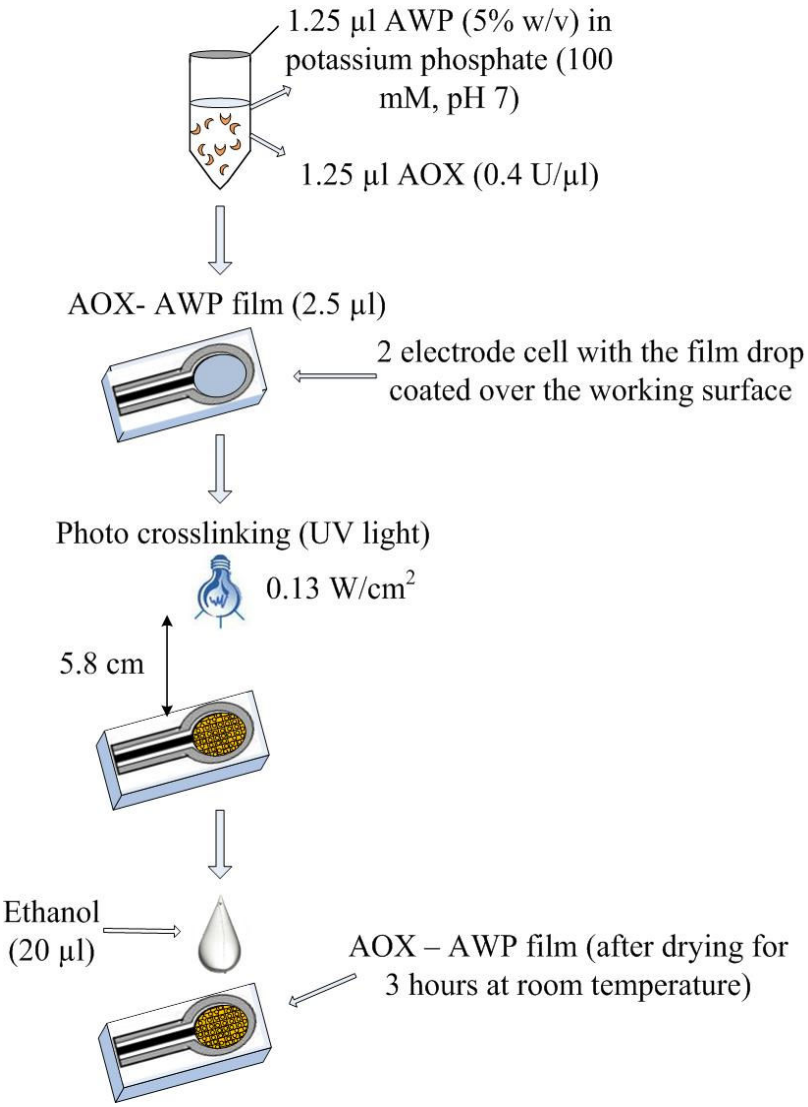

Figure 4.4. Immobilizing AOX in PVA-AWP required subjecting the film dropcoated over the working surface of the electrode to ultraviolet light for 10 minutes and drying for 3 h at room temperature.

4.2.3 Experimental Design

Effect of Thickness of Immobilized Film and UV Exposure Time

Three film thicknesses of 0.36, 0.18 and 0.09 mm were prepared by depositing 10, 5 and 2.5 µl aliquots of the BSA-AWP mixture, respectively, over the working surface of electrode and exposing them to UV light for 10 or 15 min. A bare electrode cell was used for comparison. In the hydrogen peroxide step experiment, the electrodes were submerged in a continuously stirred

10 ml potassium phosphate buffer solution. A 10 µl aliquot of hydrogen peroxide (0.3% w/w) was added to the solution at regular intervals of 3 min and the corresponding change in current response was recorded (Figure 4.5). The average current response (I_C) after each hydrogen peroxide addition was calculated and plotted against hydrogen peroxide concentration. The slope of the curve is the sensitivity of the film (Figure 4.6). The test was replicated three times. Regression analysis (R, Version 2.9.0, St. Louis, MO) was used to compare the sensitivities. Statistical significance was tested at 5% ($p < 0.05$).

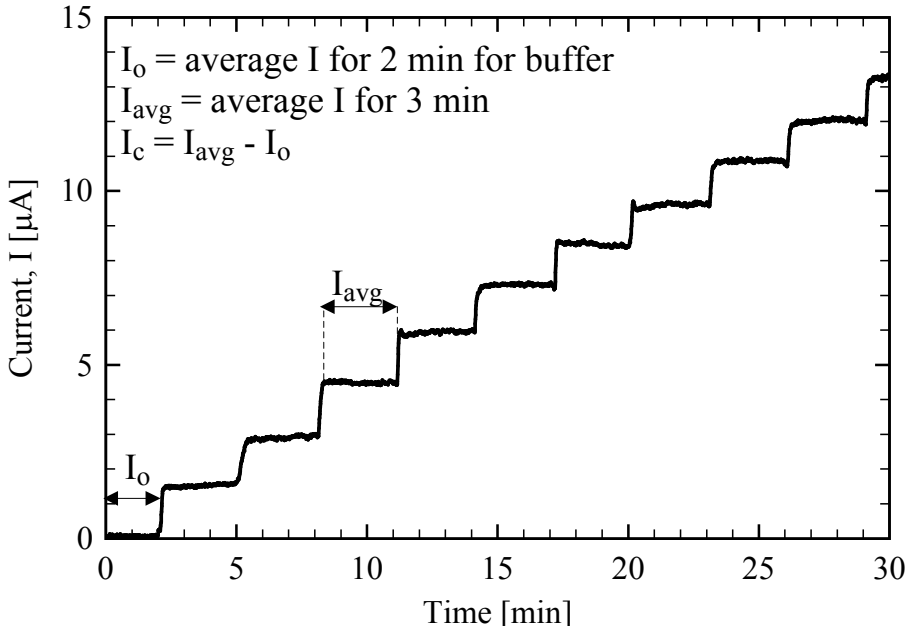

Figure 4.5. A BSA-AWP film coated electrode was expected to respond linearly to the addition of 10 µl aliquots of hydrogen peroxide (0.3 % w/w) at 3 min intervals. An initial incubation period of 2 min was allowed before the addition of hydrogen peroxide.

Effect of Immobilization Medium on Biosensor Sensitivity

An AOX-based biosensor was first exposed to ambient air for a period of 2 min prior to the addition of 20 µl ethanol sample (0.0001 - 0.01% v/v). Amperometric measurements were recorded for an additional 6 min. The current responses between 5 and 7 min were averaged (as shown in Figure 4.3 in biosensor preparation) and corrected by subtracting the average current response for deionized water, which was used as a control (Figure 4.6). All tests were replicated three times. Regression analysis (R, Version 2.9.0, St. Louis, MO) was used to compare the

linearity of the current responses to various ethanol concentrations for non-immobilized AOX, AOX-glutaraldehyde, and AOX-AWP films. The slope of the regression curve is the sensitivity of the AOX-based biosensor. Statistical significance was tested at 5% ($p < 0.05$).

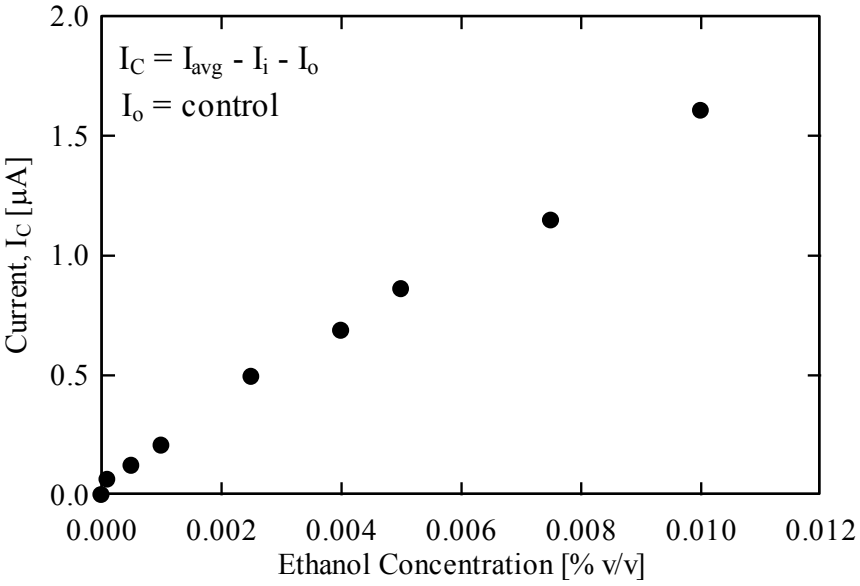

Figure 4.6. The corrected current response of biosensors with immobilized AOX was expected to respond linearly with increasing levels of ethanol in the sample.

The response times were calculated for each type of film (Figure 4.7). The peak of the current response curve, i.e., deposition of ethanol sample, coincided with the time at which the first derivative was zero or changed from a positive value to a negative value. The current response curve stabilizes once the ethanol substrate has diffused through the AOX layer (non-immobilized or immobilized) and coincided when the first approximation curve was zero. The time elapsed between the peak of the current response curve and the current stabilization point was defined as the response time, t_r (Figure 4.8).

The effect of immobilizing medium on the diffusion rate was interpreted from the response times. Student's t-test (R, Version 2.9.0, St. Louis, MO) was used to determine the difference between the response times for the three types of enzyme films. The level selected to show statistical significance was 5% ($p < 0.05$). Pearson correlation test (R, Version 2.9.0, St. Louis, MO) was used to compare the effect of ethanol concentration on the response time for the three types of enzyme films.

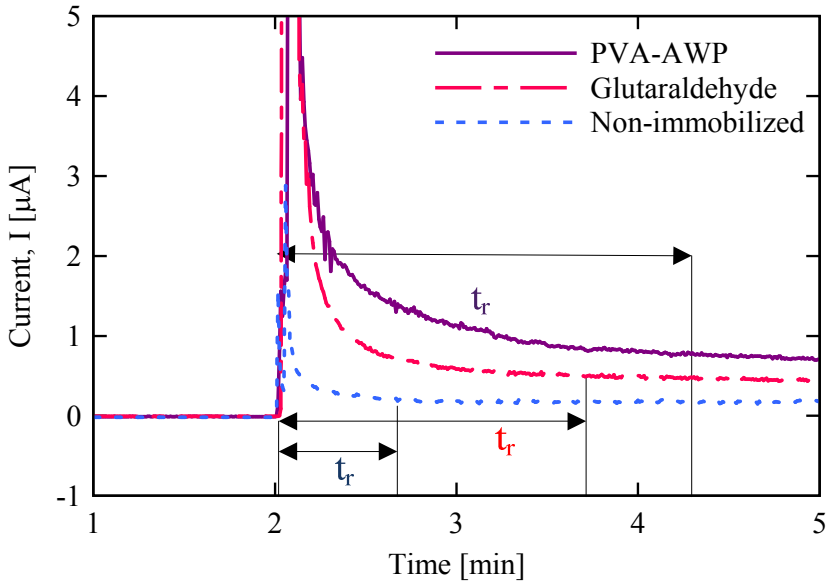

Figure 4.7. The response time, t_r, is the time it takes for the current response to stabilize after the addition of ethanol.

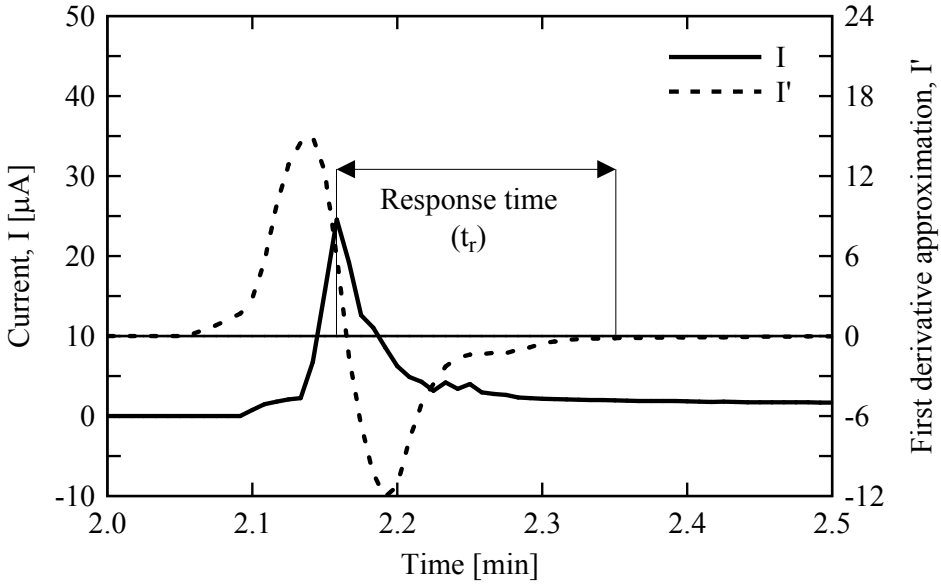

Figure 4.8. The response time, t_r, is the time between two points where the first derivative approximation of the curve is zero.

Stability of Immobilized AOX-AWP Films

The stability of AOX-AWP films was measured by comparing the sensitivity of AOX-based biosensors after a 24 h storage period at different temperatures. AOX-AWP coated electrodes were prepared as usual and packed in heat sealed aluminum packs prior to storage at

three different temperatures, -17°C, 3°C and room temperature, which was approximately 23°C. After 24 h, they were tested for current responses using 20 µl of ethanol solution of 0.005% (v/v) concentration.

4.3 Results and Discussion

4.3.1 Effect of UV Exposure Time and Film Thickness of PVA-AWP on Biosensor Sensitivity

As the volume of AOX-AWP mixtures dropcoated on the electrode increased, the thickness of the enzyme film also increased and the sensitivity decreased (Table 4.1). Increased film thickness was negatively correlated with biosensor sensitivity (Figure 4.9). Regression analyses determined that sensitivity was different ($p < 0.05$) for UV 10 and 15 min exposure times and film thicknesses 2.5 and 5 µl. Biosensors with 2.5 µl volume films showed sensitivities that were higher ($p < 0.05$) than those with 5 and 10 µl films. Based on these results, AOX-AWP film of 0.09 mm thickness (2.5 µl volume) and UV exposure time of 10 min was chosen in subsequent experiments.

Table 4.1. Sensitivity and coefficient of variation for UV 10 and 15 min exposed BSA-AWP films.

Volume of AOX-AWP film (µl)	Estimated film thickness (mm)	UV 10 min		UV 15 min	
		Average Sensitivity [a] [A/M]	Coefficient of variation [R^2]	Average Sensitivity [b] [A/M]	Coefficient of variation [R^2]
0.0	0.00	0.0158a	0.99	0.0158A	0.99
2.5	0.09	0.0111b	0.99	0.0106B	0.99
5.0	0.18	0.0092c	0.99	0.0074C	0.96
10.0	0.36	0.0068d	0.99	0.0070d	0.99

[a] Mean sensitivity values followed by the same letter in a column (abcd, ABCD) or row (abcd, ABCD) were not different ($p < 0.05$)

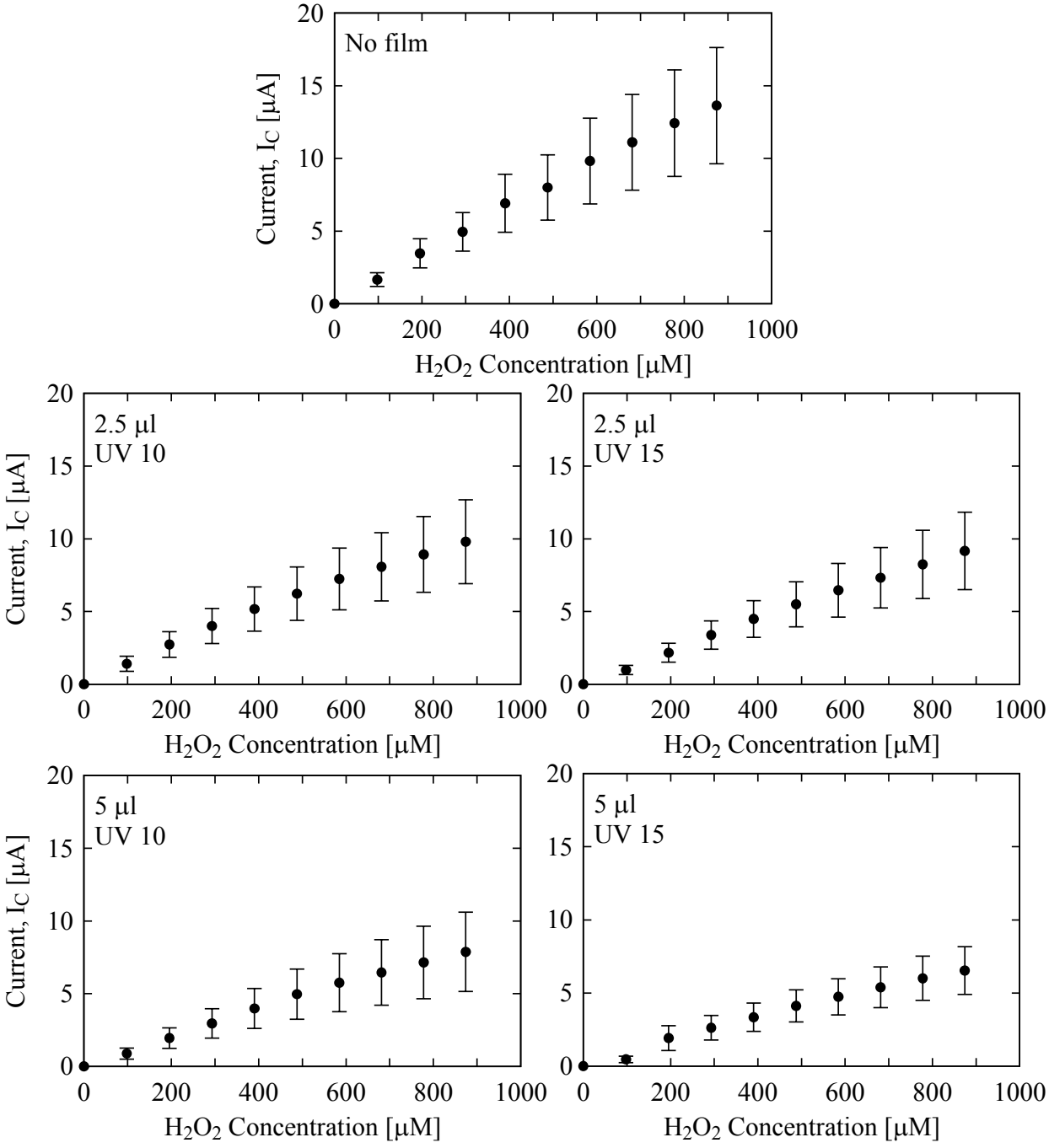

Figure 4.9. Corrected average current responses from three replications were plotted against hydrogen concentration in the buffer solution for no film, 2.5, 5 and 10 µl films exposed to UV light for 10 and 15 min. As the film thickness increased, the sensitivity decreased.

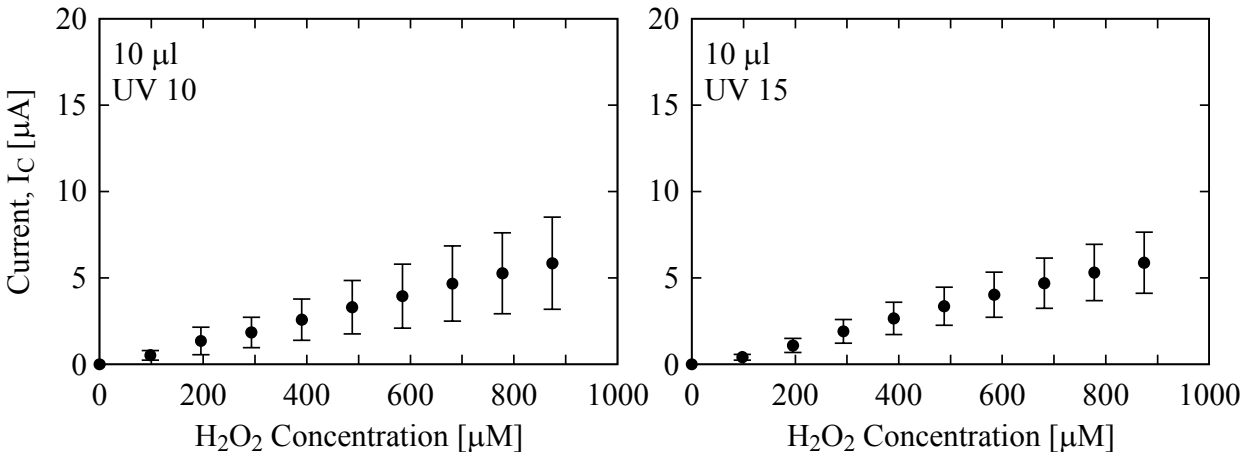

Figure 4.9. (cont.)

4.3.2 Effect of Immobilization Medium on Biosensor Sensitivity

Current responses for ethanol solutions (0.001-0.01% v/v) catalyzed in three enzyme films -- non-immobilized AOX, AOX immobilized in glutaraldehyde, and AOX immobilized in PVA-AWP films were corrected and the average current responses were plotted against ethanol concentration for each type of film (Figure 4.10). Regression analyses determined that the current responses increased linearly with increasing levels of ethanol (Table 4.2) and the linear response was higher for immobilized AOX films than non-immobilized AOX film. Biosensors with AOX immobilized in glutaraldehyde had higher sensitivity ($p < 0.05$) followed by non-immobilized AOX and AOX immobilized in PVA-AWP. Though the sensitivity in AOX-glutaraldehyde film was highest, the variability was also highest. The lower limit of ethanol detection using AOX immobilized in PVA-AWP was 171.3 µM; AOX in glutaraldehyde films was 17.13 µM; and non-immobilized AOX was between 485-625 µM.

Table 4.2. Sensitivity, coefficient of variation and lower limit of detection for each type of corrected AOX film.

Type of film	Sensitivity [a] [A/M]	Coefficient of Variation [R^2]	Limit of detection [µM]
Non-immobilized AOX	113.62a	0.84	428-625
AOX-glutaraldehyde	154.49b	0.99	17.13
AOX-AWP	77.88c	0.98	171.3

[a] Mean sensitivity values followed by the same letter in a column (abcd) were not different ($p < 0.05$)

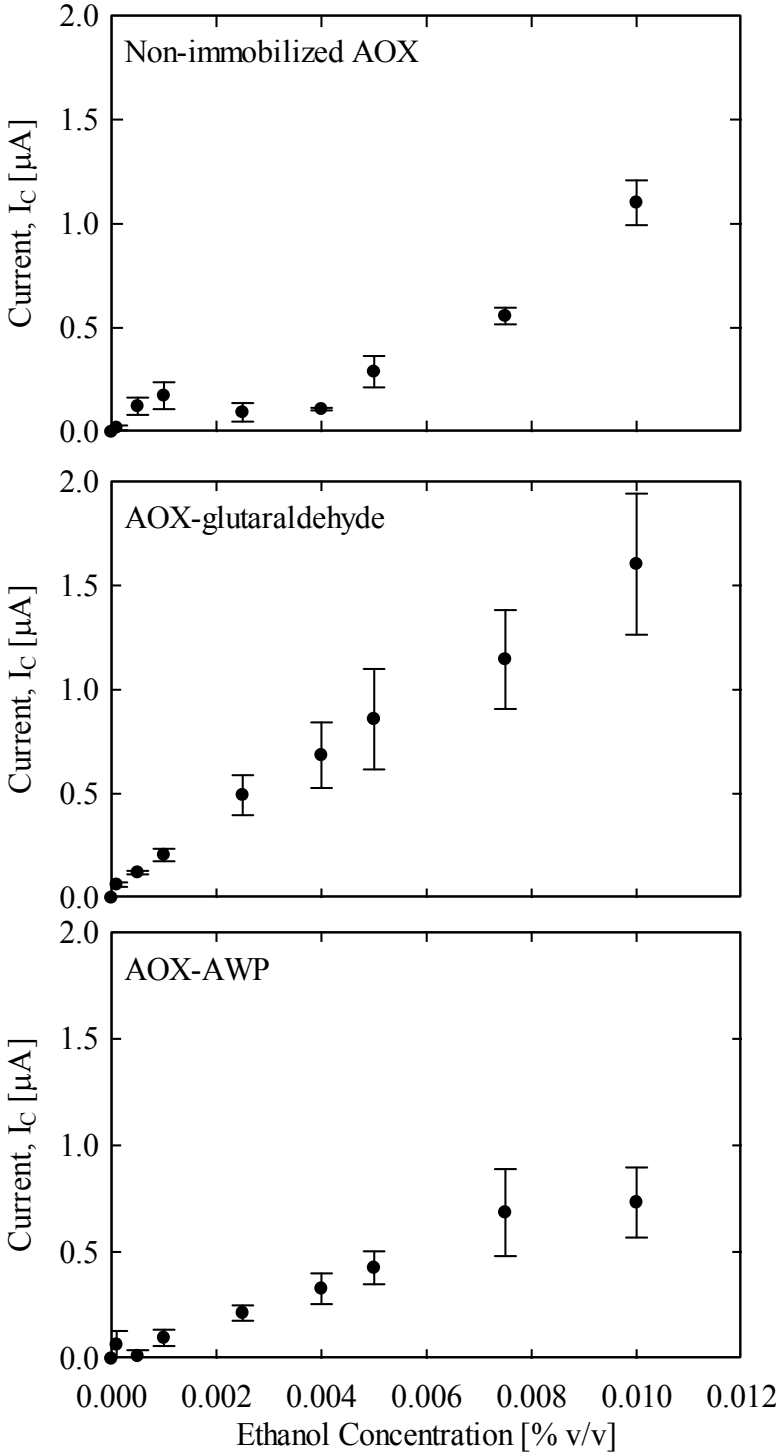

Figure 4.10. For non-immobilized and immobilized AOX, the current responses increased linearly with increasing levels of ethanol. Although sensitivity was highest with AOX-glutaraldehyde films, the variability in the response was also greatest. Error bars are ±1 standard error.

The diffusion rate of the substrate into the three different enzyme films was assessed by comparing the response times in the films for different ethanol concentrations (Table 4.3). The t-test showed that all the response times were different ($p < 0.05$). The response times for non-immobilized AOX were lower ($p < 0.05$) than those for immobilized AOX films. This is due to the thickness of the immobilized AOX films. The response times for AOX-glutaraldehyde films were lower than AOX-AWP films for most of the ethanol concentrations. The viscosity of 10% (w/v) AWP is 2825 cP/ 25°C (Ito et al., 2005). The AOX-AWP films were based on 5% (w/v) PVA-AWP solutions which, even after a 1:1 dilution with aqueous AOX solution, were more viscous than 1% (w/v) glutaraldehyde in water. The viscous nature of PVA-AWP resulted in denser enzyme films than glutaraldehyde films, and the density and thickness of the films slowed down the diffusion of substrate to the electrodes.

Table 4.3. Average response times from three replications for the three enzyme films.

Ethanol Concentration [%v/v]	Average Response Time [min]		
	Non-immobilized AOX	AOX-glutaraldehyde	AOX-AWP
0.0001	0.26	0.47	0.59
0.0005	0.24	0.57	0.62
0.0010	0.42	0.56	0.60
0.0025	0.13	0.43	0.70
0.0040	0.16	0.44	0.38
0.0050	0.29	0.39	0.43
0.0075	0.31	0.43	0.42
0.0100	0.29	0.44	0.46

Response times for AOX-glutaraldehyde and AOX-AWP films had a moderately decreasing linear correlation relationship with increasing ethanol concentrations (Figure 4.11, Table 4.4) but the response time for non-immobilized AOX film was not affected by ethanol concentration. As the concentration of ethanol increased, the number of ethanol molecules

available for reaction increased and the time taken for them to reach the sensor surface which had the immobilized enzyme decreased.

Table 4.4. Pearson correlation coefficients for the three enzyme films.

Type of film	Correlation Coefficient
Non-immobilized AOX	0.07
AOX-glutaraldehyde	-0.62
AOX-AWP	-0.67

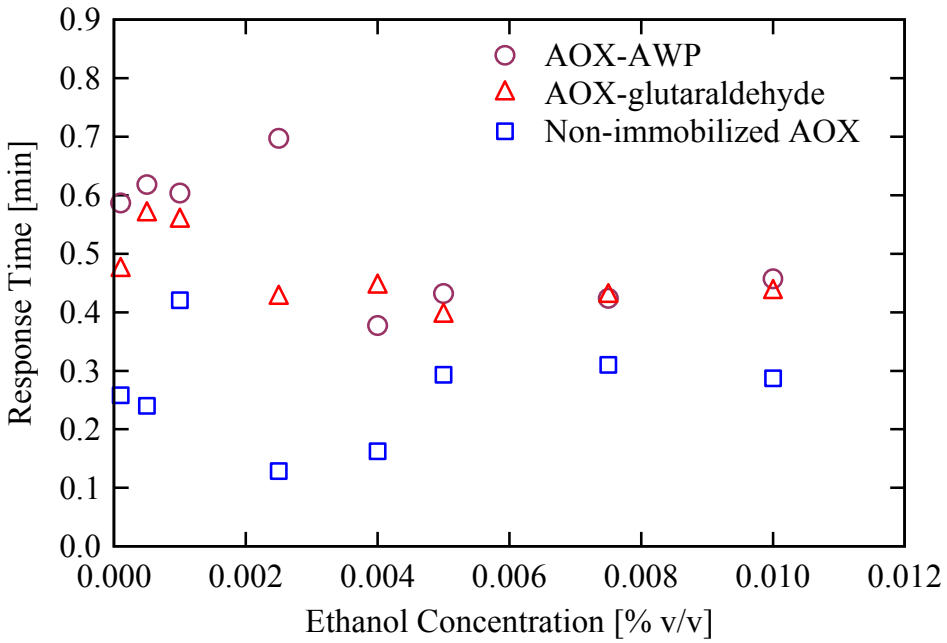

Figure 4.11. The average response times decreased moderately with increasing levels of ethanol for AOX-AWP and AOX-glutaraldehyde films while there was no specific trend for non-immobilized AOX film.

4.3.3 Stability of Immobilized AOX-AWP Films

Temperature and time had an effect on the current response given by immobilized AOX-AWP films. Ethanol concentration of 0.005% (v/v) was used for comparison. Current response

decreased by 7%, 36% and 40% for films stored at -17°C, 3°C and 23°C, respectively (Figure 4.12, Table 4.5).

Table 4.5. Decrease in current response was calculated for AOX immobilized in PVA-AWP films stored for 24 hours at -17°C, 3°C and 23°C.

Method	Storage Temperature [°C]	Current [µA]	Decrease in current response [%]
1	No Storage	0.82	-
2	-17	0.76	6.99
3	4	0.53	35.72
4	23	0.49	40.29

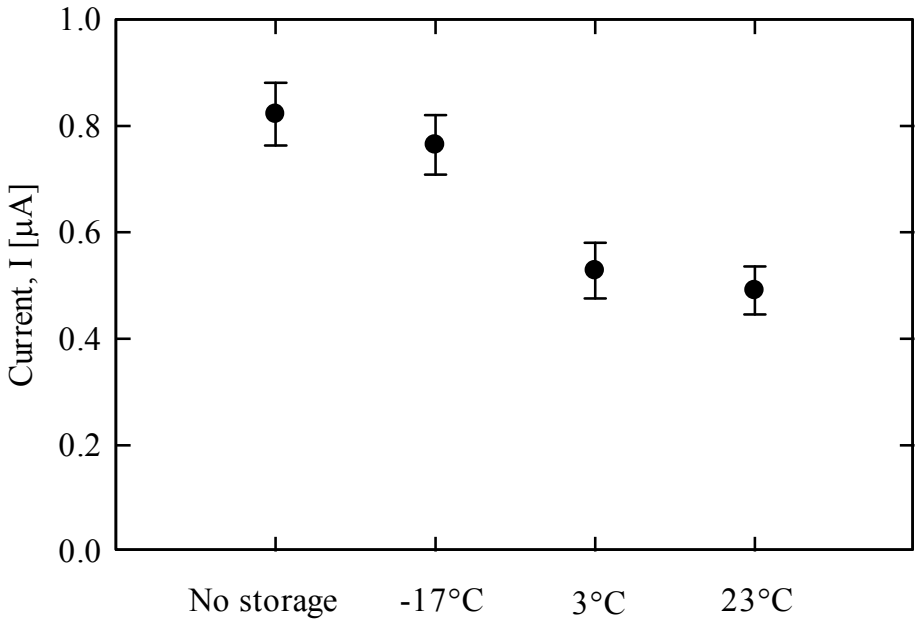

Figure 4.12. Average current responses from three replications are reported for stability of AOX immobilized in PVA-AWP films. Films which were not stored were compared with films stored at three different temperatures for 24 h and tested using ethanol solution of 0.005% (v/v) concentration. Error bars are ±1 standard error.

4.4 Conclusions

The current response decreased ($p < 0.05$) with increasing thickness of PVA-AWP films on the electrode and UV exposure 10, 15 min. The current response of the AOX-based biosensor varied with type of immobilization medium of the enzyme. Immobilized AOX films gave higher current responses compared to non-immobilized AOX film for lower concentrations of ethanol solution. The linear responses of sensors with immobilized AOX were higher than non-immobilized AOX sensors. Biosensors with AOX immobilized in glutaraldehyde had higher sensitivity ($p < 0.05$) followed by non-immobilized AOX and AOX immobilized in PVA-AWP. The lower limit of ethanol detection using AOX immobilized in PVA-AWP was 171.3 μM; AOX in glutaraldehyde films was 17.13 μM; and non-immobilized AOX was between 485-625 μM. The rate of diffusion of ethanol into the films was lower for immobilized AOX films compared to non-immobilized AOX film. The rate of diffusion for immobilized AOX films had a moderately decreasing linear relationship with increasing concentrations of ethanol. Current response decreased by 40%, 36% and 7% when AOX immobilized in PVA-AWP films were stored for 24 h at 23°C, 3°C and -17°C, respectively.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

Immobilization in PVA-AWP does not change the conformation of HRP and it has higher mechanical stability as compared to glutaraldehyde. Thickness of PVA-AWP film layer and UV exposure time negatively affected the sensitivity of detection. Sensors with immobilized alcohol oxidase had more linearity than those with non-immobilized AOX. The rate of diffusion of ethanol into the immobilized enzyme films decreased with increase in thickness of film on the electrode and had a moderately decreasing linear relationship with increase in concentration of ethanol. Current response decreased when AOX immobilized in PVA-AWP films were stored at 24 h at different temperatures.

From these results, the effects of different parameters on the activity of HRP and sensitivity of AOX immobilized in PVA-AWP based biosensor can be further characterized:

1. The effect of enzyme to polymer ratio on the activity of HRP immobilized in PVA-AWP can be characterized to determine increased entrapment of enzyme.
2. A wider range of film thickness and UV exposure times could be used to determine the effect on the AOX based biosensor immobilized in PVA-AWP.
3. The effect of pH, temperature and storage conditions in buffer on the sensitivity of the AOX-AWP biosensor can be characterized.
4. The effect of lower UV exposure times on the sensitivity of the biosensor can be analyzed.

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APPENDIX A

A.1. Soret Band

Soret bands were observed in HRP-agarose and HRP-AWP (3.8 and 5% w/v) films. BSA-agarose and BSA-AWP (3.8 and 5% w/v) films were used as control. Absorbance values of the films were corrected ($Absorbance_{corrected}$) by subtracting the absorbance values of control (Figures A.1, A.2, and A.3).

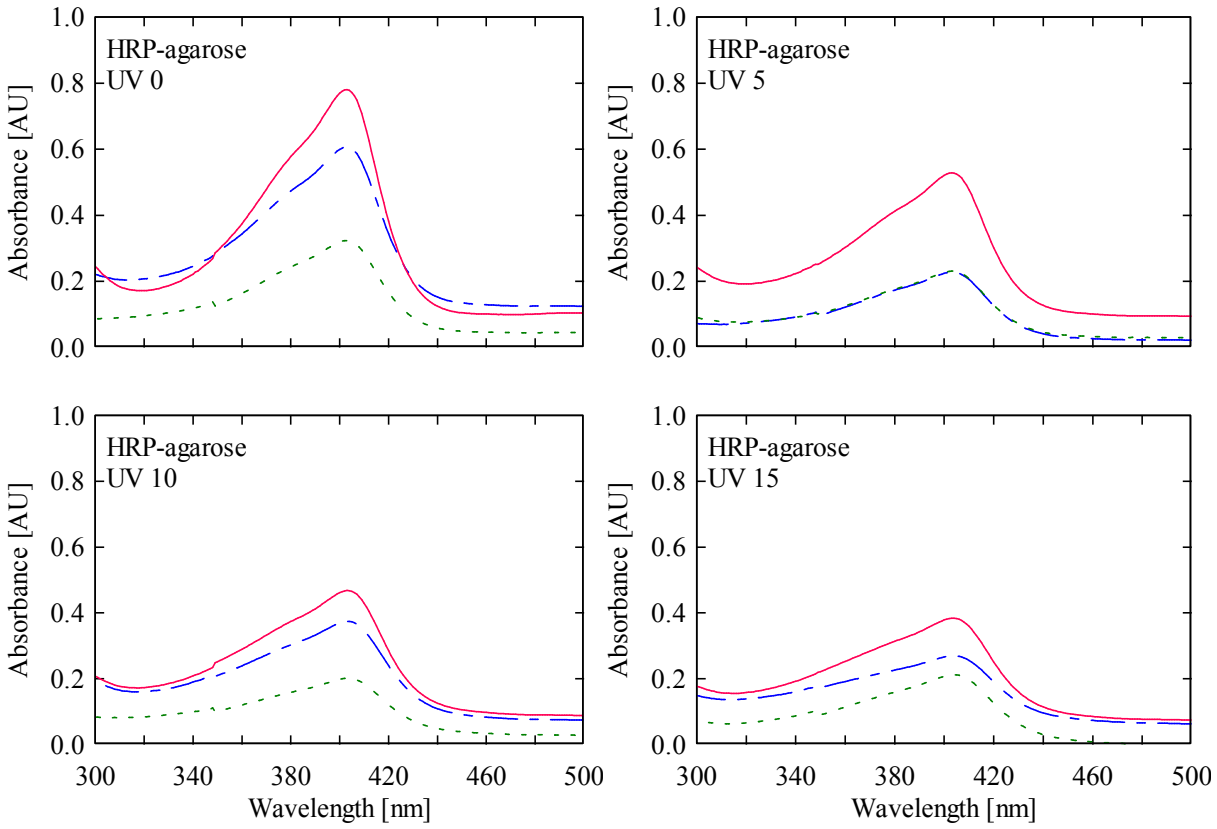

Figure A.1. Three replications of uncorrected Soret absorption band are reported for HRP-agarose films exposed to UV light for 0, 5, 10 and 15 min.

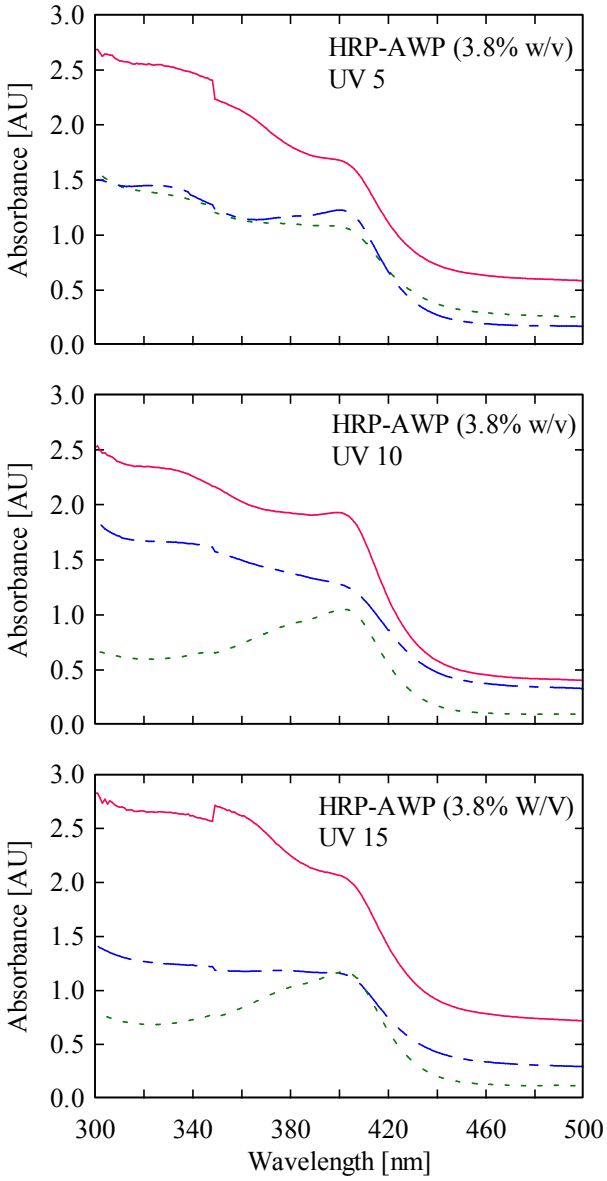

Figure A.2. Three replications of uncorrected Soret absorption band are reported for HRP-AWP (3.8% w/v) films exposed to UV light for 5, 10 and 15 min.

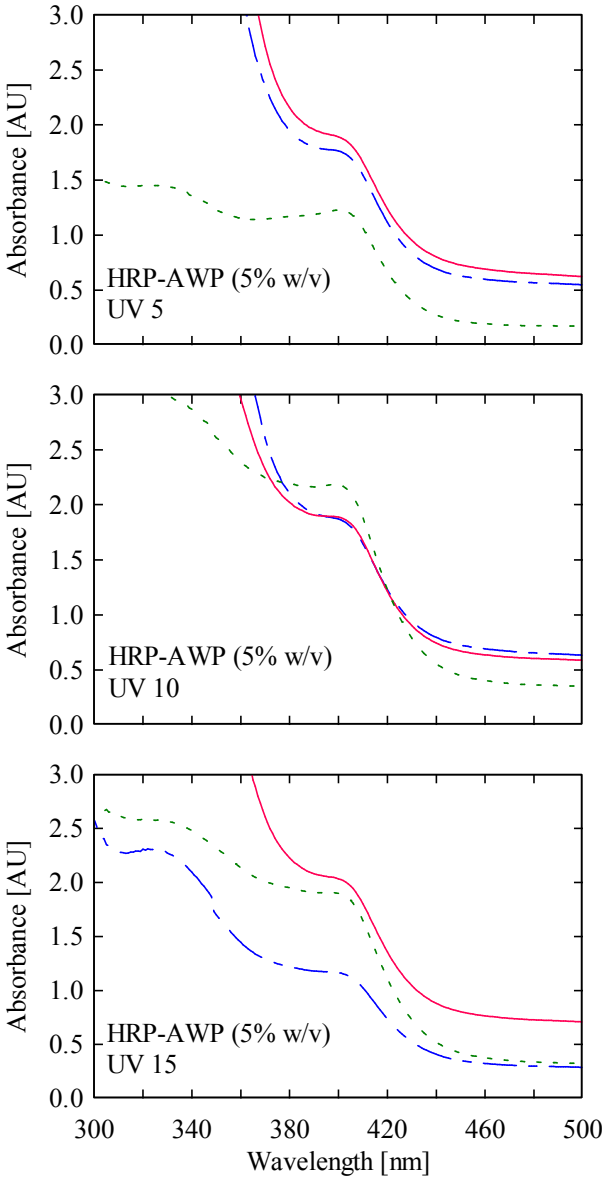

Figure A.3. Three replications of uncorrected Soret absorption band are reported for HRP-AWP (5% w/v) films exposed to UV light for 5, 10 and 15 min.

A.2 Determination of Activity of HRP Immobilized in Glutaraldehyde and PVA-AWP

Activity of HRP (Activity units / min) immobilized in glutaraldehye and PVA-AWP (3.8 and 5% w/v) was determined by observing the rate of change of absorbance of the films over a period of two minutes. BSA-glutaraldehyde and BSA-AWP (3.8 and 5% w/v) films were used as control. The slope of the curve gave the activity of the enzyme. Data was corrected by subtracting the activity values of control (Tables A.1 and A.2).

Table A.1. Three replications of activity (AU/min) of HRP and BSA determined in HRP-AWP and BSA-AWP films respectively, are reported.

Immobilization medium	UV exposure time (min)	BSA			HRP		
		1	2	3	1	2	3
PVA-AWP (3.8% w/v)	5	0.00	0.00	0.00	0.33	0.26	0.42
	10	0.01	0.00	0.00	0.53	0.26	0.21
	15	0.00	0.00	0.00	0.29	0.27	0.38
PVA-AWP (5% w/v)	5	0.00	0.02	0.02	0.13	0.41	0.50
	10	0.00	0.04	0.03	0.29	0.50	0.49
	15	0.00	0.00	0.00	0.32	0.63	0.59

Table A.2. Six replications of activity (AU/min) of HRP and BSA determined in HRP-glutaraldehyde and BSA-glutaraldehyde films respectively, are reported.

Immobilization medium	BSA						HRP					
	1	2	3	4	5	6	1	2	3	4	5	6
Glutaraldehyde (1% v/v)	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.09	0.19	0.11	0.11	0.09

A.3 Determination of activity of HRP Leached from HRP-Glutaraldehyde and HRP-AWP films

HRP-glutaraldehyde and HRP-AWP (3.8 and 5% w/v) films were washed with buffer and the wash was observed for enzyme activity. Activity of HRP (Activity units / min) leached from HRP immobilized in glutaraldehye and PVA-AWP (3.8 and 5% w/v) films was determined by observing the rate of change of absorbance of the films over a period of two minutes in the wash. The slope of the curve gave the activity of the enzyme (Tables A.3 and A.4).

Table A.3. Three replications of activity of leached HRP (AU/min) from HRP-AWP films are reported.

Immobilization medium	UV exposure time (min)	Concentration (% w/v)					
		3.8			5		
		1	2	3	1	2	3
PVA-AWP	5	0.46	0.46	0.46	0.44	0.61	0.57
	10	0.51	0.01	0.22	0.50	0.57	0.19
	15	0.55	0.62	0.18	0.39	0.36	0.40

Table A.4. Four replications of activity of leached HRP (AU/min) from HRP-glutaraldehyde films are reported.

Immobilization medium	1	2	3	4
Glutaraldehyde (1% v/v)	0.42	0.96	0.75	0.69

APPENDIX B

B.1 Effect of Film Thickness and UV Exposure on Sensitivity

The hydrogen peroxide step experiment was performed thrice on each BSA-AWP film of volumes 2.5, 5.0 and 10.0 µl for UV exposure times 10 and 15 min (Figures B.1 and B.2). A bare electrode was treated as control.

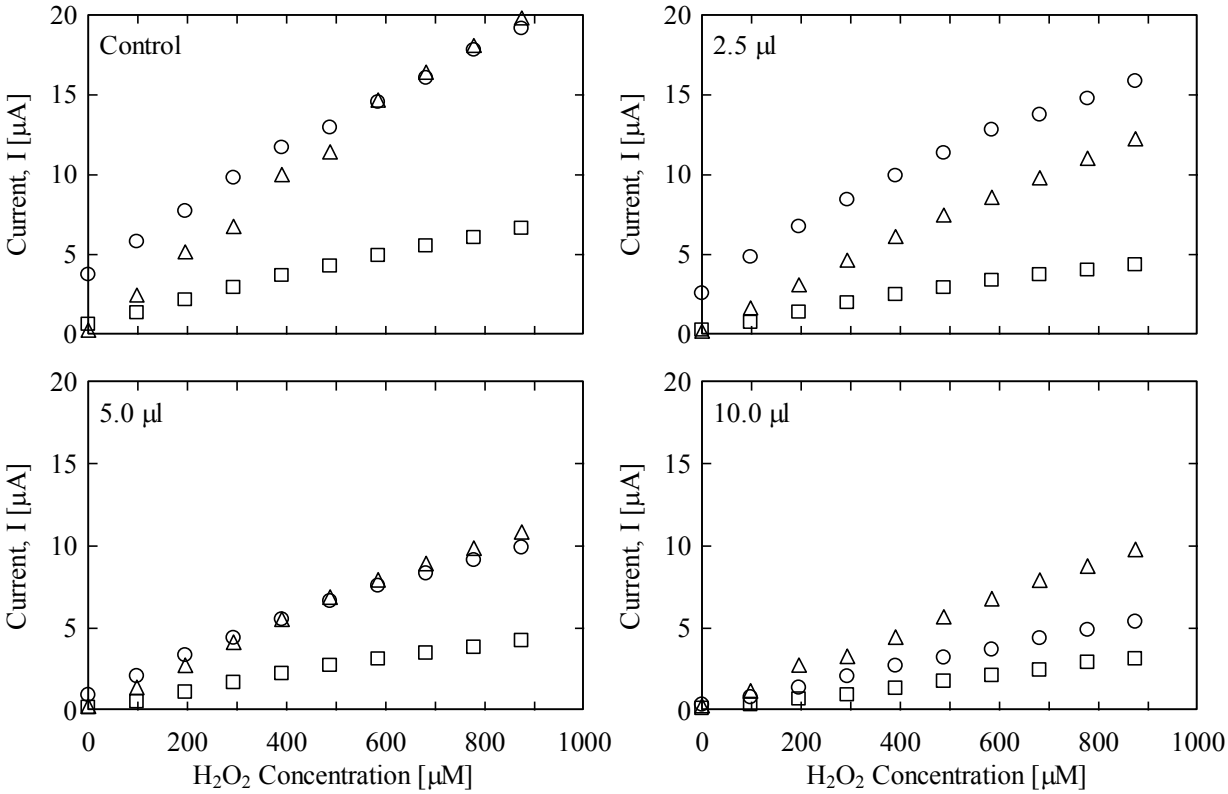

Figure B.1. Uncorrected current responses from three replications for varying hydrogen peroxide concentrations in the buffer solution for UV 10 min exposure are reported.

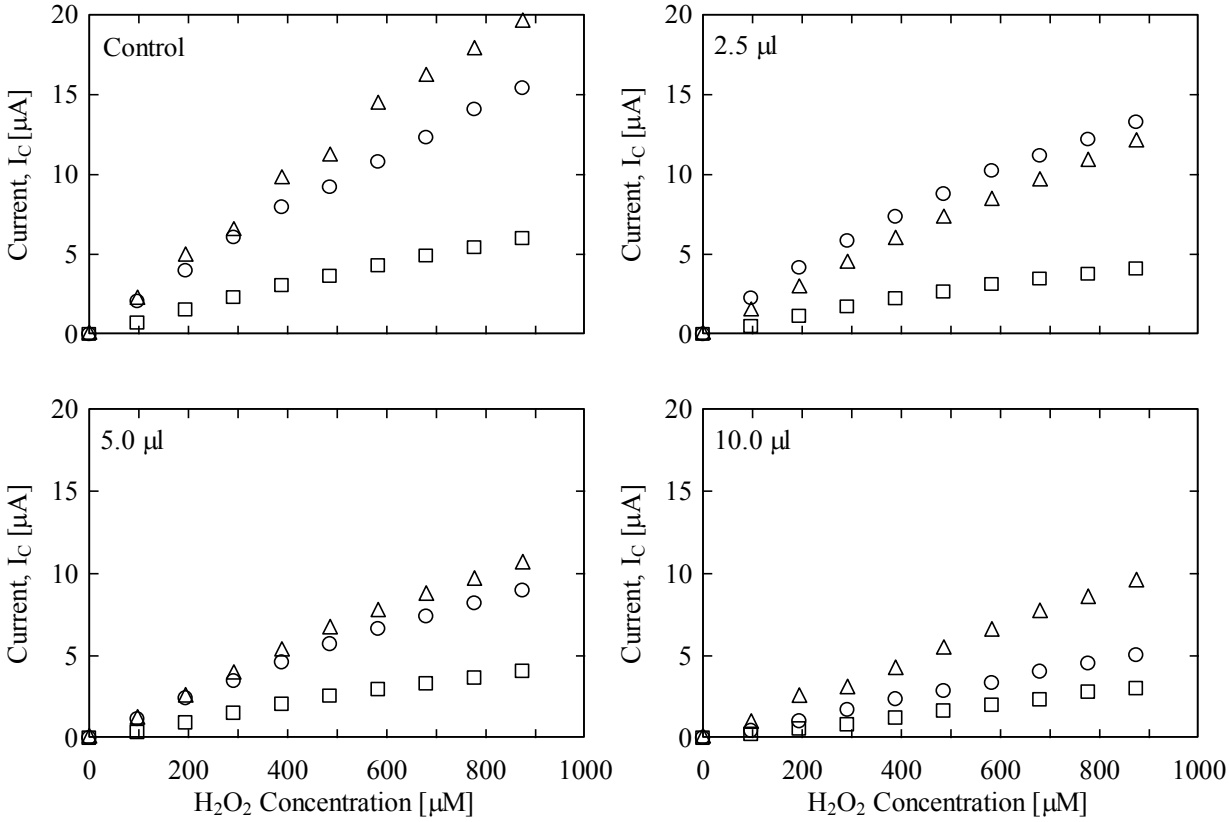

Figure B.2. Corrected current responses from three replications for varying hydrogen peroxide concentrations in the buffer solution for UV 10 min exposure are reported.

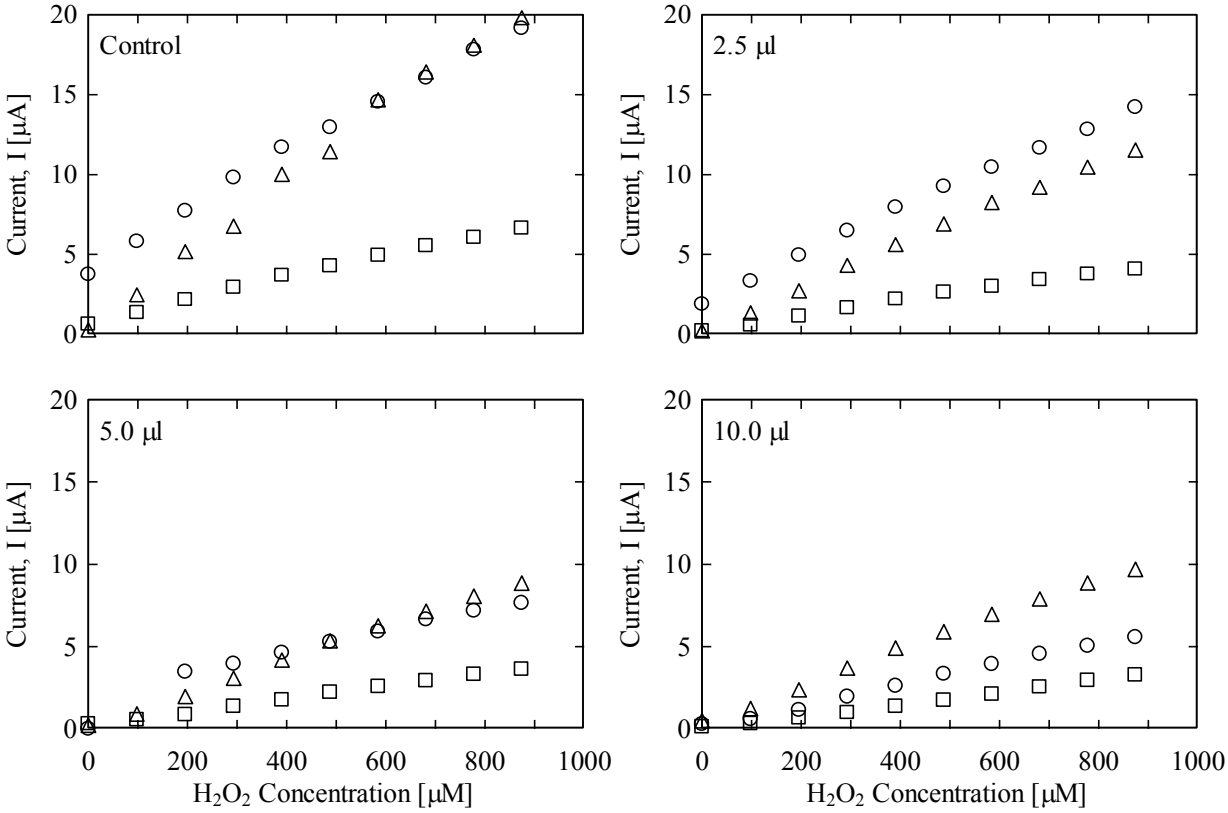

Figure B.3. Uncorrected current responses from three replications for varying hydrogen peroxide concentrations in the buffer solution for UV 15 min exposure are reported.

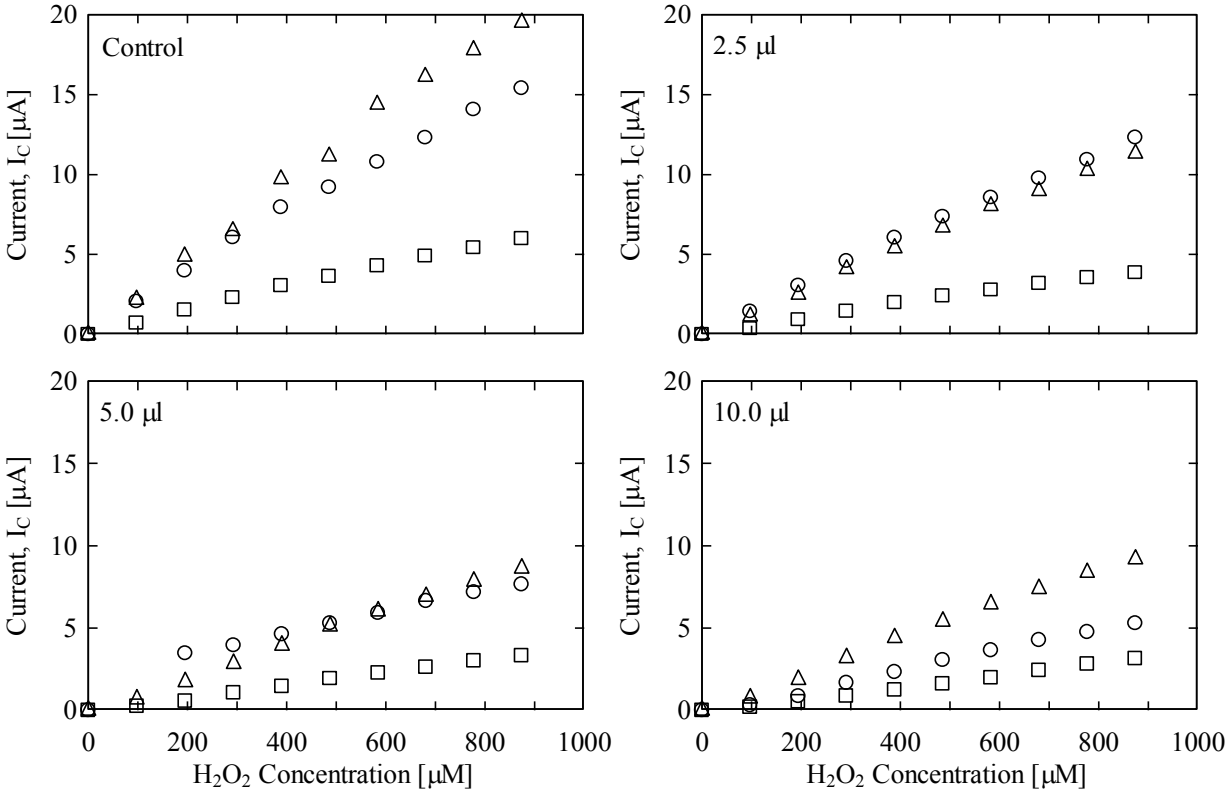

Figure B.4. Corrected current responses from three replications for varying hydrogen peroxide concentrations in the buffer solution for UV 15 min exposure are reported.

Based on the above results, average of the three replications of sensitivity or slope of the curve in each film was calculated and plotted against volume of the dropcoated BSA-AWP film or estimated thickness of the film.

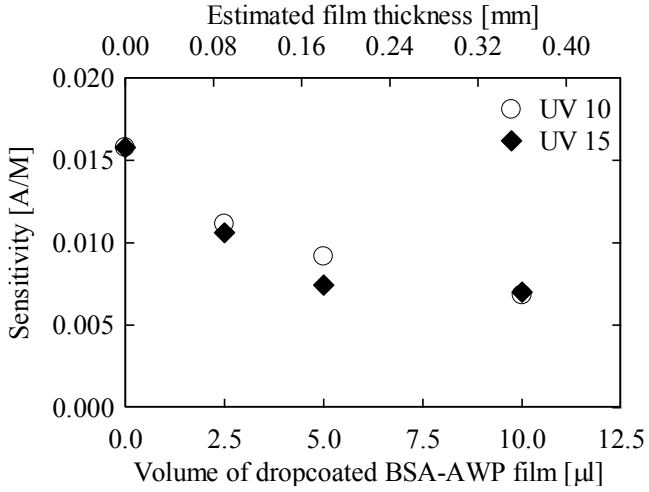

Figure B.5. Sensitivity of film was plotted against volume of thickness of BSA-AWP film. Sensitivity decreased with increase in film thickness.

B.2 Effect of Immobilization Medium on Sensitivity of Biosensor

Average current response was calculated after the addition of 20 µl of ethanol in non-immobilized AOX, AOX-glutaraldehyde and AOX-AWP films. Three replications were performed on each film (Figure B.6).

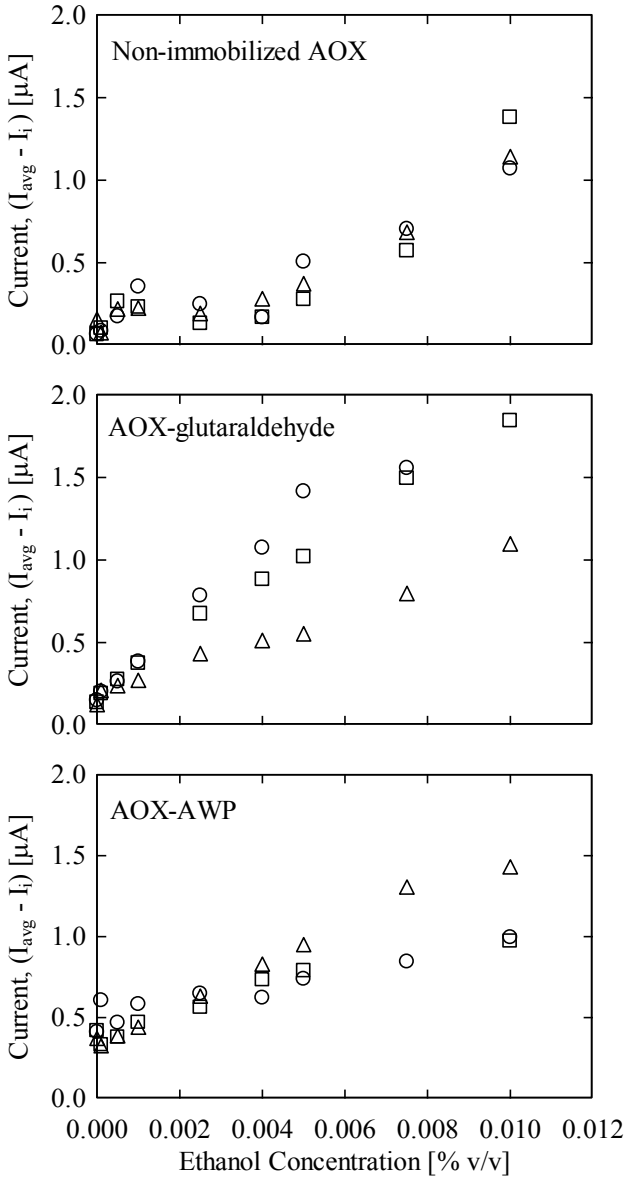

Figure B.6. Three replications of uncorrected average current responses in AOX films for varying ethanol concentrations are reported.

Regression analysis was performed on above data after correcting, by subtracting the control, for each replication in the three films. The slope of the curves was the sensitivity of the AOX biosensor (Figure B.7).

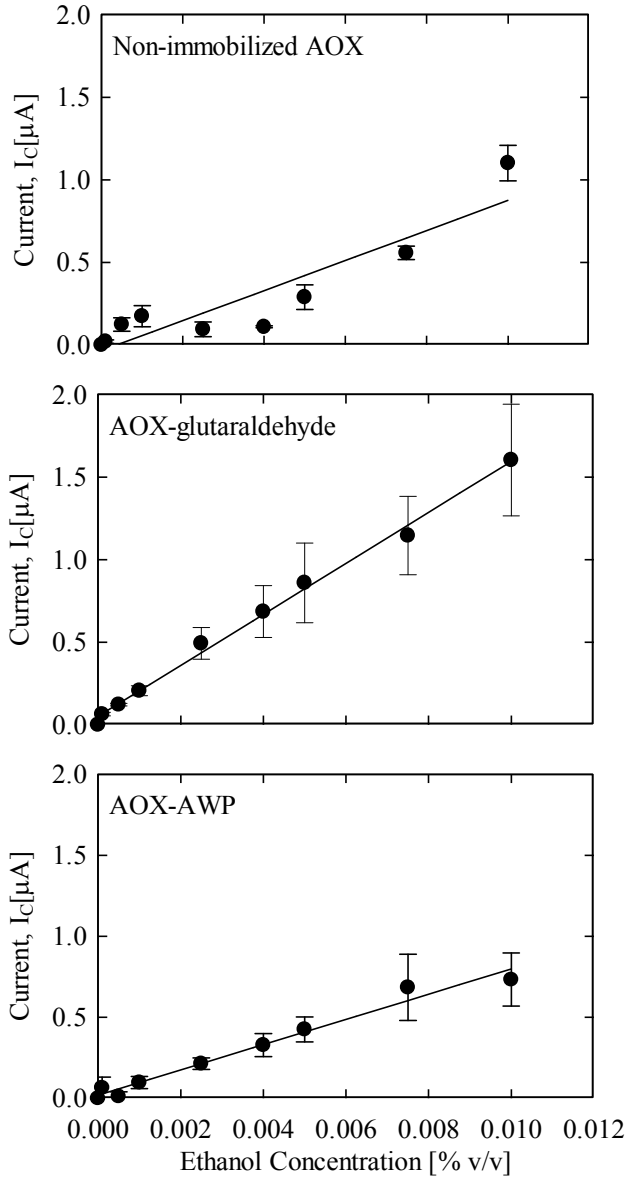

Figure B.7. Linearity of current responses with change in ethanol concentration was more for immobilized films. Though sensitivity of AOX-glutaraldehyde films were higher, variability was also higher.

B.3 Response Times

Response times were calculated in non-immobilized AOX, AOX-glutaraldehyde and AOX-AWP films. Three replications were performed on each film and the response times calculated in each replication are reported (Table B.1).

Table B.1. Three replications of response times measured in each type of AOX film for different ethanol concentrations are reported.

Ethanol Concentration [% v/v]	Response Time [min]								
	Non-immobilized AOX			AOX-Glutaraldehyde			AOX-AWP		
	1	2	3	1	2	3	1	2	3
0.0001	0.13	0.45	0.20	0.48	0.53	0.41	0.45	0.70	0.62
0.0005	0.20	0.21	0.31	0.50	0.49	0.72	0.54	0.76	0.56
0.0010	0.32	0.61	0.33	0.41	0.47	0.80	0.52	0.66	0.63
0.0025	0.16	0.13	0.10	0.51	0.41	0.36	0.39	0.77	0.94
0.0040	0.24	0.12	0.13	0.45	0.42	0.47	0.36	0.46	0.32
0.0050	0.25	0.48	0.15	0.46	0.30	0.42	0.34	0.35	0.61
0.0075	0.11	0.13	0.69	0.46	0.41	0.41	0.30	0.56	0.52
0.0100	0.11	0.62	0.13	0.46	0.45	0.40	0.43	0.45	0.49