

An Infrared Approach to Thin-Layer Based Biosensors

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ABSTRACT: Biosensors exploit the theoretical and instrumental approaches of analytical chemistry and the unique properties of biological molecules. They utilize the intermolecular interactions and specificity found in systems such as hormone-receptor, antigen-antibody, pathogen-host and substrate-enzyme. Manipulation of the structural and energetic factors that guide formation of these macromolecular complexes may provide a rational basis for the development of new sensor technology. To this end we have investigated myoglobin thin films for their ability to bind ligands. FTIR spectroscopy has been chosen as the transduction methods and has been shown to discriminate between several analytes.

Abbreviations: DCDI, N,N'-Dicyclohexylcarbodiimide; DPPE, 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine; ECDI, 1-Ethyl-3-(3-Dimethylaminopropyl)Carbodiimide; ATR, Attenuated Total Reflectance; IRE, Internal Reflection Element. *To whom correspondence should be addressed.

INTRODUCTION

Biosensors

Biosensors are analytical devices which use biological interactions with molecules to provide qualitative and quantitative results that are specific for the molecule being analyzed (1). These analytical devices incorporate a biological material or a biomimic (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids etc.), intimately associated with or integrated to a transducing system. The advantage of using a biological sensing element is their remarkable ability to distinguish between the analyte of interest and similar substances. Another advantage is that biosensors are quick at providing information and they are simple to use. Their simplicity results from the incorporation of the biological element and transducer into one unit so the analysis can be essentially completed in one step. The most notable advantage of biosensors is the immobilized biological element can be regenerated and reused. By contrast, other bioanalytical assays, such as ELISA (enzyme linked immunosorbent assay) result in irreversible binding to the assay and are thus used once and discarded.

The science of biosensors has evolved significantly in the last several years. The first biosensors, called enzyme electrodes, were developed to detect glucose (1). Research efforts for developing glucose biosensors have opened the way for the application of biosensors to other molecules. Other types of biosensors include the construction of an optical biosensor for the detection of insulin (2) and a fluorometric biosensor for the detection of fermentation activity (3).

Biosensors are finding use in increasingly broader ranges of application. For example, their use has dramatically increased in clinical diagnosis and

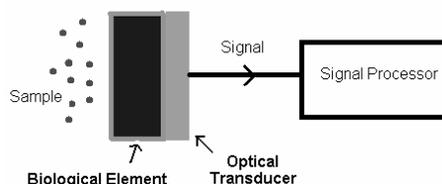


Figure 1: *General schematic representation of a biosensor.* The components of a biosensor include a biological element to which the analyte being analyzed binds. The biological element is attached to a transducer that transforms the chemical interaction of the biological element with the analyte into an electrical signal that is displayed onto a computer or other form of signal processor.

biomedicine, pharmaceutical and drug analysis, pollution control and monitoring, and detection of industrial and toxic gases (4-5). To be commercially successful, a biosensor has to follow general requirements such as it has to provide good resolution, sensitivity, accuracy, quickness of identifying the ligand, and insensitive to electrical and other environmental interference. Other factors also contribute to the requirements a biosensor must meet before it can be commercially available.

In the construction of a biosensor there are several considerations that must be made in the selection of a suitable biological element, a suitable immobilization method, and in the selection of a suitable transducer.

The selection of the biological element involves searching for a biological element that binds efficiently to the analyte or analytes being studied. The immobilization method should be carefully chosen so that the biological element remains functional after its immobilization. Finally, the transducer should be chosen so that the chemical interaction of the biological element with the sample is converted into an electrical signal that can be processed and displayed. The Figure 1 depicts the general arrangement of a biosensor and its components.

There are a variety of transducing systems that can be used such as optical, electrochemical, thermometric,

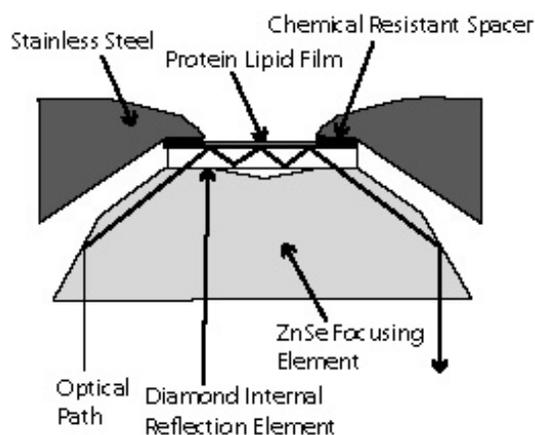


Figure 2: *Attenuated Total Reflectance Accessory.* In this example the biological component is a protein with a supporting lipid on a diamond internal reflection element (see Figure 3 for an enlarged view of the film).

piezoelectric or magnetic systems. Ultraviolet/Visible spectroscopy and infrared internal reflection spectroscopy have gained increased interest in the development of biosensors. These analytical photometric tools provide a method to record changes in biosensor activity and to identify the molecule attached to the biosensor. In this research, experiments were conducted using a UV/Visible apparatus and Attenuated Total Reflectance Fourier Transform Infrared Spectrometer (ATR-FTIR). Both of these techniques are capable of providing analytical information on the changes occurring due to analyte binding to the biological element.

ATR-FTIR spectroscopy has been applied to several proteins including rhodopsin, bacteriorhodopsin, the nicotinic acetylcholine receptor, and to generate redox difference spectra of the unligated and cyanide bound states of bovine cytochrome c oxidase (6-7). When using an ATR apparatus, the sample is placed

onto an internal reflection element (e.g. diamond) (Figure 2). Infrared radiation is focused on the end of the internal reflection element. The infrared radiation enters the internal reflection element and reflects down the length of the diamond. At each internal reflection the infrared radiation actually penetrates a short distance into the sample as discussed above. An infrared spectrum is obtained through this process.

Attenuated Total Reflectance(8)

The path of light will change as it passes through a medium with refractive index n_1 and enters a medium with refractive index n_2 , assuming $n_1 \neq n_2$. The extent of refraction is described by Snell's law:

$$\frac{\sin \alpha_1}{\sin \alpha_2} = \frac{n_2(\bar{\nu})}{n_1(\bar{\nu})}$$

where α_1 is the angle of incidence and α_2 is the angles of refraction.

Reflection also occurs at the interface between media 1 and 2. Furthermore, the portion of the radiation that is reflected becomes larger as the difference in refractive index increases. The fraction of light reflected, R , for a beam traveling normal to the interface is given by

$$R = \frac{I_R}{I_O} = \frac{(n_2(\bar{\nu}) - n_1(\bar{\nu}))^2}{(n_2(\bar{\nu}) + n_1(\bar{\nu}))^2}$$

where I_0 and I_R are the intensities of the incident and reflected radiation, respectively.

Total internal reflection occurs when light traveling in a medium of high refractive index impinges on an interface with a less dense medium (i.e. one with a low refractive index). When the angle of incidence α_1 equals the critical angle θ_c all the light is reflected, and Snell's law becomes

$$\theta_c = \arcsin\left(\frac{n_2(\bar{\nu})}{n_1(\bar{\nu})}\right)$$

This relationship provides the basis for fiber optics and total internal reflection spectroscopy. As light propagates through an internal reflection element (IRE) a portion of the electric field extends beyond the surface and into the sample. This is known as the evanescent wave and it decays exponentially as distance from the surface increases. In the case of a sample placed on the IRE surface the evanescent wave will have a penetration depth as described by d_p .

$$d_p = \frac{\lambda}{2\pi [n_1^2 \sin^2 \alpha_1 - n_2^2]^{1/2}}$$

Note that d_p is wavelength dependent. The depth of penetration for typical IRE materials ranges from a couple hundred nanometers to several microns. Therefore, the electromagnetic field can potentially interact with and be attenuated by a sample placed on the IRE surface. This technique is known as Attenuated Total Reflectance spectroscopy (ATR).

EXPERIMENTAL PROCEDURES

Spectroscopy

All UV/Vis spectroscopy was carried out using an Ocean Optics USB2000 spectrophotometer equipped with an without a fiber optic probe. Fourier Transform Infrared Spectroscopy was conducted using a Digilab FTS 7000 spectrometer equipped with a SensIR DurasamplIR II 9 reflection diamond ATR.

To conjugate Myoglobin to DPPE the intermediate N-glutarylphosphatidylethanolamine was synthesized as described in Kung, et al (9):

Formation of glutaraic acid anhydride.

10.6 mg (0.08 mmol) of glutaric acid and 8.7mg (0.042 mmol) DCDI were combined in 2ml methylene chloride in a screw-cap tube. The tube was capped and the mixture stirred under nitrogen at 23°C

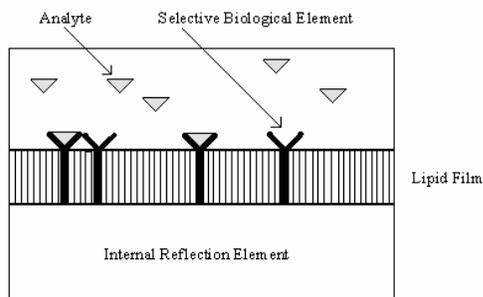


Figure 3. An enlarged view of the thin film shown in Figure 2. The presence of the lipids allow for formation of a stable film when the biological element is a membrane associated

for 48 hours with a magnetic stirring bar. A solution of DPPE (0.038 mmol) in 2 ml chloroform and 15 μ L of triethylamine (0.108 mmol) were added to the glutaric anhydride/ DCDI solution. The reaction mixture was acidified by adding 5 ml chloroform and 4 ml of 0.02 M phosphate/ 0.02 M citrate buffer (pH 5.5) with vigorous shaking. The aqueous phase was separated by low-speed centrifugation and discarded. The organic phase was dried of anhydrous sodium sulfate. The desired N-glutaryl-DPPE was purified by

silica-gel column chromatography. The dried chloroform solution was introduced into 1 x 20 cm Silica-gel column and fractions were eluted by passing through the column 50 ml chloroform effluent solutions containing successively, 0, 10, 20, 30 and 50% methanol. The fractions eluted at each of the five different methanol concentrations were analyzed by TLC on silica gel plates developed with chloroform/methanol/water (65:25:4, v/v). The presence of N-glutaryl-DPPE was detected by black light absorption. The product reagent was found in the 30% methanol effluent. N-Glutaryl-DPPE has an R_f value of about 0.3.

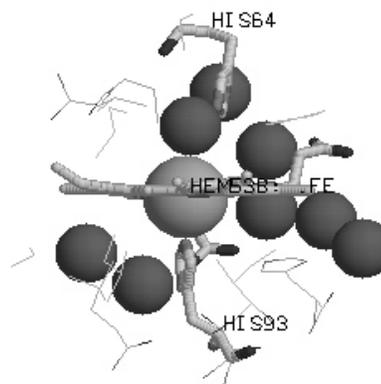


Figure 4: The myoglobin heme site. At the center of the heme is an Fe(II) atom. Four of the six coordination sites around this atom are occupied by nitrogen atoms from a planar porphyrin ring. The fifth coordination site is occupied by a nitrogen atom from a histidine side chain on one of the amino acids in the protein. No ligand is bound on the distal side of the heme. Blue spheres are waters.

Conjugation of Myoglobin to liposomes.

Liposomes were activated by water-soluble (EDCI) at room temperature for 1 hour. The liposome concentration was 1 μ mol/ml and EDCI was 2.5 mg/ml in a buffer containing 10 mM NaH_2PO_4 /0.15 M NaCl (pH 5) during the activations. Then 1.5 ml of the liposome/ EDCI mixture was added with 75 μ L of 10 mg/mL bovine myoglobin and 75 μ L of 1 M NaCl; the pH was adjusted to 8 with NaOH. The conjugation reaction was carried out overnight at 4°C.

Thirty μ L aliquots of a 9mM DPPC / 1mM myoglobin-conjugated DPPE were used during this set of experiments. Each aliquot was deposited and dehydrated with $\text{N}_{2(g)}$ to form a thin layer on the IRE. Once dried, 30 μ L of a solution containing one type of

ligand was added to rehydrate the thin layer. The solutions used were as follows: 10mM Na₂PO₄/150mM NaCl and one of the following: 20 mM potassium fluoride, 20 mM sodium cyanide, 20 mM sodium azide, 20 mM ascorbic acid. A spectrum of a rehydrated thin layer was used to obtain difference spectra containing each ligand.

Polystyrene

A thin polystyrene film adhered to the ATR crystal was developed by drying 25 µl of a 0.25 mg/ml polystyrene solution in toluene on the diamond surface. 25 µl of a 50 mg/ml myoglobin solution in Tris buffer were dried onto the film. Tris buffer was then run across the sample with a flow cell (flow rate ~10 µl/sec) to remove excess of myoglobin.

RESULTS AND DISCUSSION

Myoglobin

Myoglobin was used in this project as the biological element in the development of the biosensor. One advantage of myoglobin is its activity is easily measured due to the presence of a light absorbing group (the heme) in its structure. Myoglobin is a protein that plays a significant physiological role as a result of its ability to bind oxygen. It is found mainly in muscle tissue where it serves as an intracellular storage site for oxygen when the oxygen content of the cell is high and to release the oxygen when the oxygen content of the cell is low.

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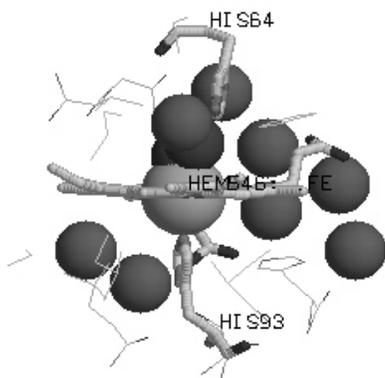
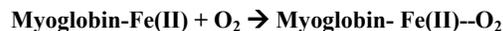
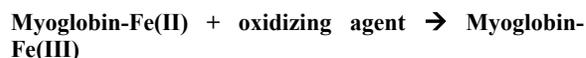


Figure 5: The myoglobin heme site. The sixth coordination site is occupied by bound oxygen.

three dimensional structure of myoglobin heme site is depicted in Figure 4. The heme prosthetic group with oxygen bound is diagramed in Figure 5. The iron in the heme is physiologically present in the +2 oxidation state (the ferrous state) which is the state that binds gases like oxygen and carbon monoxide.



Although myoglobin is physiologically present in the Fe(II) state which binds to gaseous diatomic molecules, the study of other ligands are also of interest. In the oxidized form, Fe(III) (ferric state), iron can bind to ligands such as fluoride, cyanide, and azide.



The three dimensional structure of myoglobin transforms upon ligand binding to the iron (Figure 4 and 5). For example, oxygen binding to the iron is accompanied by shortening of the Fe-N bonds, a shift in the iron out of the heme plane, and the movement of the helix F toward the heme plane (10,11). The changes in the myoglobin protein upon ligand binding can be detected by spectrometric techniques.

Biosensor

In this project, the ability to successfully couple the myoglobin biological element to a lipid thin layer (or a polystyrene matrix) and to determine whether the protein maintained its function was explored. Two types of analysis were used to study the activity of myoglobin: UV/Visible spectroscopy and Infrared spectroscopy (ATR-FTIR). These spectrometric methods provided information related to changes in

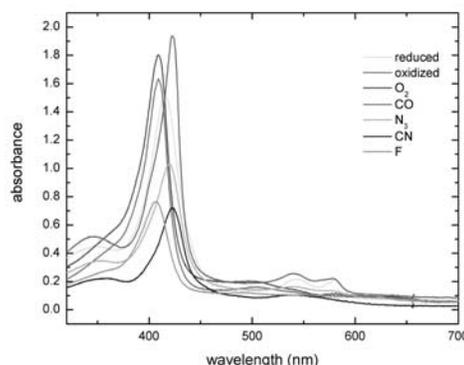


Figure 6: Myoglobin reduced and oxidized with various bound ligands. UV-Vis spectra.

the chemical structure of myoglobin upon binding to different ligands. Shifts in the frequency of absorption bands and changes in the relative band intensities indicated changes in the chemical structure of myoglobin that was specific for each ligand.

Myoglobin was exposed to different ligands (fluoride, carbon monoxide, oxygen, azide and cyanide) and studied using these spectrometric techniques. The spectral results were analyzed to determine whether the myoglobin biosensor system could be used to distinguish between the ligands.

The research presented in this paper was conducted in order to attest the ability of developing a biosensor using UV/Visible spectroscopy and ATR-FTIR spectrometry after conjugation of the protein to a lipid film and other support matrices. The experiments also provide a comparison of the efficiency of each type of analytical technique in differentiating between the ligands.

Ultraviolet and visible spectroscopy can provide information on the absorbance of the heme around 409 nm for the oxidized state of the heme and approximately 423 nm for the reduced state (Figure 6). The presence of bound ligand also alters the absorbance spectrum. Figure 7 illustrates that a thin film of myoglobin on reflective aluminum retains behavior similar to myoglobin in solution.

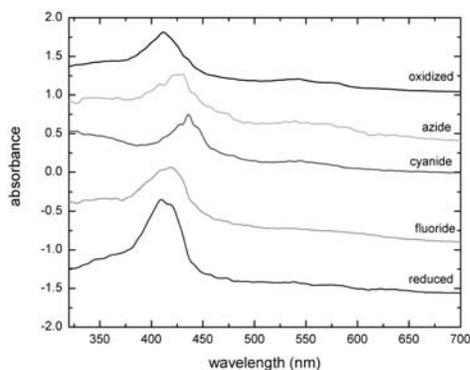


Figure 7: Myoglobin immobilized on aluminum reduce and oxidized with various bound ligands.

The infrared region between 1150 and 1180 cm^{-1} provides a rich amount of information about the Fe(III)- porphyrin complex influence on the vibration of carbon-carbon and carbon-nitrogen bonds in the histidine and the region between 1050 and 1150 cm^{-1} provides information on the influence of the histidine on the absorbance of the Fe(III)-porphyrin complex (12-14). These absorbance regions of myoglobin can be efficiently observed using ATR-FTIR spectrometry (Figure 8). Myoglobin's porphyrin component contains both skeletal and vinyl modes in this region of the infrared spectrum (12-14).

After generation of the myoglobin-lipid conjugate the protein lipid vesicles were fused to the diamond surface Figure 8 is the result of experiments with

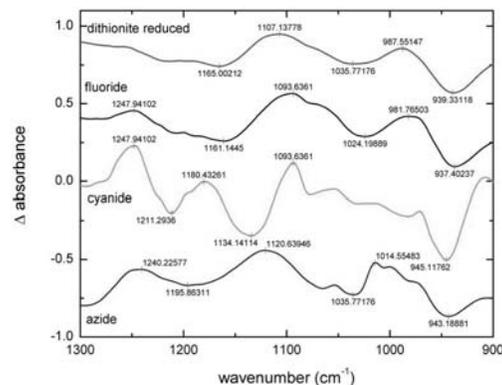


Figure 8: Myoglobin conjugated to DPPE. The protein-lipid vesicles were fused by dehydration onto the diamond.

redox states and ligand binding. At this time we are not attempting to specifically assign peaks in the difference spectra presented here. The spectra are differences with the oxidized state of the heme subtracted from them. Although all ligands studied perturb the infrared spectrum of the Fe(III)-porphyrin and Fe(III) histidine complex, cyanide produced the most noticeable changes within the difference spectra (Figure 8). Note the differential peak at about 1100 cm^{-1} . Both fluoride and azide binding result in a shift in the peak near this region. Reduction of the heme iron from Fe(III) to Fe(II) also results in a perturbation of the myoglobin heme spectrum. However, cyanide and azide binding to the heme can be clearly distinguished from each other and from the redox experiment.

It is clear that sodium cyanide provides the most distinguishable changes in absorbance when it is bound to lipid-conjugated myoglobin. Furthermore cyanides binding to the heme can be clearly observed in the polystyrene based films (Figure 9)

Conclusion

These experiments prove that infrared biosensors are indeed plausible. There is still a matter of being able to distinguish between some of the more subtle results. Perhaps not just local maximum positions and absorbance intensities can be used to identify future unknown ligands, but the ratios of different wavenumbers on the same spectrum could prove useful. Furthermore regions of the infrared spectrum

above 1900 cm^{-1} will be useful, but an IRE other than a nine reflection diamond will be needed.

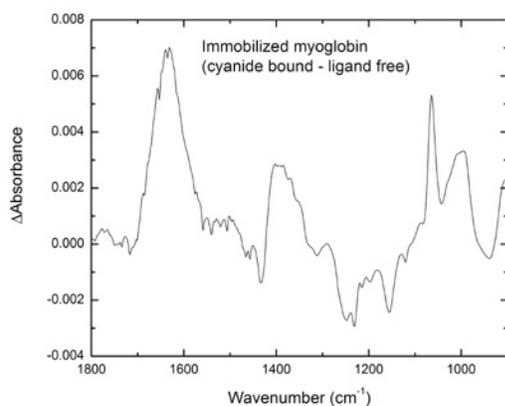


Figure 9: Myoglobin-polystyrene. Myoglobin adsorbed on to a polystyrene thin film with cyanide bound.

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