Developing a biosensor for cervical cancer

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A Thesis Proposal by

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ABSTRACT

Human papillomavirus infection and cervical cancer are threats to women around the world. Persistent infection with a high risk strain of HPV is the overwhelming cause of cervical cancer [16]. Since over 80% of the female population will be infected with HPV at one point in their lives [2], there are millions of women currently at risk for cervical cancer. There is no cure for HPV infection [4], and the available vaccines only prevent infect ion in women who have never come into contact with the virus [16]. The Pap smear test is the most common procedure for identifying infections that have caused precancerous or cancerous cell growth. However, this method is inaccurate and expensive. Women who are considered at risk for cervical cancer must be retested at least every 6 months [4]. In 90% of all cases, the infection will clear within two years without harm to the patient [4], negating the need for the repeated tests. The United States alone spends nearly $4.6 billion on HPV care, the majority of which pays for these repeated Pap smear tests in women who will never develop cervical cancer [4]. Additionally, most of the nearly 250,000 annual deaths associated with cervical cancer occur in areas where regular medical attention is not available [4]. Clearly, there is a need for a cheap and accurate cancer screening device.

Biosensors offer a potential solution: they are accurate, sensitive and generally inexpensive [21]. It is proposed that a biosensor based on electrochemical impedance spectroscopy (EIS) will be capable of distinguishing between cancerous and normal cells . For this application, antibodies that are overexpressed on the surface of cancer cells must be identified. Indirect immunofluorescent immunohistochemistry was used to test the expression of two antibodies, Epithelial Cell Adhesion Molecule (EpCAM) and the integrin alpha V Beta 6 (aVB6), on two cervical cancer cell lines and two normal cervical cell strains in vitro. It was expected that both biomarkers would exhibit increased expression on the surface of the cancer cells. EpCAM expression followed this pattern, but aVB6 showed increased staining on the normal cells. aVB6 is both a growth marker and a cancer marker [16]. Since the cells were not tested at full confluency, it is believed that aVB6 is simply a stronger growth marker than it is a cancer marker. Once EpCAM had been identified as a potential biomarker for this application, it was necessary to develop a working cell attachment biosensor model.

The biosensor consists of a gold slide coated with antibodies to the identified biomarker. A Pt spiral counter electrode and an Ag/AgCl reference electrode are used in a three electrode configuration (the gold slide is the working electrode). The biosensor surface is interrogated with a small sinusoidal AC probe, and the current response of the system is measured. A frequency response analyzer and a potentiostat are used to record the open circuit potential and to perform voltammetry sweeps and impedance measurements on the biosensor. Impedance measurements from the biosensor and antibody layer will be compared to the impedance measurements after incubation with a sample of cells. The biosensor will be tested using both cancer cells and normal cells. It is expected that the cancer cells will attach in much larger numbers. Since the biosensor is being modeled with a Randle's circuit, this increased attachment will increase the value of Rct. This change can be visualized by examining the Nyquist plot for the system [20]. Theoretically, the percentage of cancer cells in the sample can be correlated to the impedance response of the system [13]. The biosensor's impedance response should be able to provide an accurate expression of cancer progression in cell samples similar to those collected in a Pap smear examination.

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A. SPECIFIC AIMS

A.1 Problem Statement

Human papillomavirus (HPV) infection and cervical cancer are threats to women worldwide. HPV infections are the most commonly diagnosed sexually transmitted disease globally [4]. More than half of all sexually active adults will be infected with HPV during their lifetime, and over 80% of women will acquire an HPV infection by the age of 50 [2]. There are over 120 different types of the human papillomavirus, and over 40 of these affect the urogenital tracts [4]. These 40 types of HPV are divided into two categories: high-risk and low-risk. Infection with a low-risk HPV (HPV 6 and 11, commonly) is usually accompanied by genital warts [16]. However, nearly 100% of cervical cancers contain DNA sequences from high-risk strains of HPV [16], cementing the relationship between infection with a high-risk strain of HPV and cervical cancer. HPV 16 alone is responsible for nearly 70% of cervical cancer cases [16].

Cervical cancer is the second most common type of cancer in women worldwide, and is responsible for the deaths of over 250,000 women each year [4]. Most of these deaths occur in third world countries, where medical care is limited and safe sex is not practiced [4]. Though cervical cancer can be successfully treated if it is caught early enough, there is no cure for the HPV infection that causes it [4]. The new prophylactic vaccine targets only four of the forty types of problematic HPV strains. More importantly, this vaccine is only effective if the patient receives it before coming into contact with the viruses it protects against. Considering the proliferation of HPV infections, this effectively limits the vaccine's target population to young girls who have not yet become sexually active. Thus, it will be nearly thirty years before we see a decrease in cervical cancer cases in the United States as a result of the vaccine. Also, the cost of the vaccine is currently over $350, severely limiting its use in third world countries where the need is highest [16].

The primary difficulty with HPV and cervical cancer screening is determining which infections will progress to cancer. Once a woman has been diagnosed with a high-risk HPV infection, she must be retested at least every six months to check for early signs of abnormal cell growth [4]. However, nearly 70% of infections clear within the first year, and 90% of infections clear within two years [4], negating the necessity of the frequent screenings. Currently, there is no concrete method to predict which infections will progress to cervical cancer and which will resolve without complications [4]. The current screening method, the Pap smear test, requires collecting and examining a sample of the cervical lining. This method is extremely error prone, and is one of the most common issues in medical malpractice litigation [4]. Furthermore, the United States alone spends nearly $4.6 billion annually on HPV related conditions, only 10% of which goes toward treatment of cervical cancer. The remainder of this huge sum of money pays for repeated Pap smear tests on infections that will most likely clear without ever resulting in cancerous growth [4].

Given the high cost and inaccuracy of the current HPV screening methods, an alternative method has been proposed that would reduce costs and increase accuracy of HPV screening. This new method will integrate electrochemical impedance spectroscopy (EIS) into a simple biosensor that would ideally able to distinguish between HPV infections that will progress to cervical cancer and those that will resolve without harm to the patient. Since this mechanism is still unknown, research is centered on developing a working cell adhesion biosensor that is capable of detecting the presence of cancerous cells in a sample of cervical cells.

The biosensor interface will consist of a thin gold slide coated with antibodies to specific cell surface proteins that are highly upregulated in cancer cells. Theoretically, the cancer cells, which will have a much higher density of these proteins, should be much more likely to attach to the biosensor surface via an antibody-antigen interaction. The gold slide, the solution containing the cells, and several electrodes form an electrical circuit. A small AC probe voltage will interrogate the gold slide, and the AC current response of the circuit can be measured. As cells (ideally only cancerous cells) attach to the slide, the response of the circuit will change. The percentage of cancerous cells in the sample can be correlated to the impedance response of the circuit [13]. Thus, the biosensor will provide an alternative, and presumably more accurate, method of diagnosing cancerous progression in cervical cell samples. When the mechanism determining which HPV infections will progress to cancer is fully understood, this knowledge can be incorporated into the biosensor, producing a simple, accurate electronic device that will eliminate the costs associated with repeated Pap smear testing.

A.2 Objectives

Developing a working cell adhesion biosensor for the detection of cervical cancer is a multistage process involving the cooperation of Dr. Woodworth's lab in the biology department and Dr. Suni's lab in the chemical engineering department. The research will focus on the following objectives.

A.2.1 Identify potential biomarkers.

For this application, it will be necessary to identify cell surface proteins that are overexpressed in cervical cancer cells. Very little research has been done in this area, since it is much more common to investigate cell surface proteins that are downregulated in cancer cells. Also, cervical cancer, although very common worldwide, is not as heavily researched in the United States as other types of epithelial cancers such as breast or lung cancers. To identify possible biomarkers for this application, a thorough literature review will be performed to generate a list of cell surface proteins that have been shown to be upregulated in epithelial cancers. Ideally, the expression of these biomarkers will have been evaluated in cervical cancer.

A.2.2 Evaluate the expression of likely biomarkers in normal and cancerous cervical cells.

Once a list of potential biomarkers has been generated, two of the most promising biomarkers will be chosen for testing. Degree and consistency of upregulation and the cost and availability of the antibodies will be taken into consideration when determining which biomarkers are the most likely candidates for this application. The expression of the two chosen biomarkers will be evaluated qualitatively using indirect immunofluorescent immunohistochemistry. Secondary antibodies will be tagged with the fluorescent dye Alexa Fluor 488. The relative brightness and staining patterns on the normal and cancerous cervical cells will be compared to evaluate the two different biomarkers' expression in these cell types.

a. Hypothesis 1: Biomarker 1 will show a surface staining pattern, and will show increased expression (brightness) on cancerous cells.

b. Hypothesis 2: Biomarker 2 will show a surface staining pattern, and will show increased expression (brightness) on cancerous cells.

A.2.3 Develop basic, working cell adhesion biosensor.

The simple gold slide and stationary fluid model that is envisioned for this biosensor has been used primarily with individual protein molecules rather than living, mammalian cells. Cells are much larger than these protein molecules, and present unique challenges for the design of a simple EIS biosensor. For example, if the cells settle onto the biosensor surface without binding, this will still affect the biosensor reading. Microfluidic-based diagnostics on cervical cancer have been performed successfully using cell surface antibody-antigen interactions [7], and in situ EIS has been used to characterize cervical tissue [8], but there is no published procedure for a stationary fluid EIS biosensor that can be used to characterize cancerous cells on the basis of cell surface attachment.

Using the available EIS laboratory equipment, cancerous cells and the two identified biomarkers, a basic model for a working cell adhesion biosensor will be developed. The sensor must be capable of identifying whether cells have attached based on the system's changing impedance response. It is expected that the testing procedure previously used for protein attachment will need to be adjusted to accommodate the size and fragility of living mammalian cells. Also, the biosensor-cell system must be characterized using basic measurements such as the open circuit potential. Ideally, antibodies for both of the identified biomarkers will be attached to the biosensor surface, but this may not be possible due to interactions between the different antibodies.

A.2.4 Test the basic biosensor model using samples of normal and cancerous cervical cells.

Once a working biosensor has been developed using cancerous cell samples (which should attach to the biosensor), the biosensor must be tested using normal cell samples. To be effective, the biosensor must be able to distinguish between normal cells and cancerous cells.

<u>a. Hypothesis 3</u>: Normal cells will show significantly less attachment to the biosensor in comparison to the tumor cell samples, as measured by the biosensor-cell system's impedance response.

Since the normal cells should have a much lower density of the chosen biomarkers, it is expected that the normal cells will show much less attachment to the biosensor. To avoid skewing the results, it will be very important to avoid allowing the cells to settle onto the biosensor surface. Less attachment will be represented by smaller impedance measurements in comparison to the impedance measurements obtained from the cancerous cell samples.

<u>b. Hypothesis 4</u>: A mixed sample of normal and cancerous cells will show an intermediate impedance response that can be correlated to the proportion of cancerous cells in the sample.

In a mixed sample containing a certain percentage of cancerous cells, it is expected that most of the cancerous cells will attach and few of the normal cells will. This should give a smaller total number of attached cells than in the purely cancerous cell sample, and a larger total number of attached cells than in the purely normal cell sample. Therefore, it is expected that the impedance response of the biosensor with this mixed sample will lie somewhere between the impedance responses of the biosensor with either pure sample. In previous protein attachment studies using this EIS technique, the impedance response of the biosensor could be directly correlated to the percentage of proteins in the sample that attached to the biosensor surface [13]. Accordingly, the percentage of cancerous cells in the sample should be directly correlated to the impedance response of the biosensor-cell circuit. Ideally, the experimental data will be robust enough to provide an approximate relationship between impedance response and the percentage of cancerous cells in the sample.

B. BACKGROUND AND SIGNIFICANCE

B.1 The human papillomavirus and cervical cancer

Papillomaviruses are small double-stranded DNA viruses that infect squamous epithelia and induce proliferation, producing various types of lesions. The HPV genome contains two important oncoproteins, E6 and E7, which are linked with cancerous progression. The virus targets stem cells. The viral DNA contained in these dividing cells is replicated about 75 times, and viral gene expression is very low until the human cell begins to differentiate. At this point, the viral genes become massively upregulated and prevent the cell from differentiating, which would stop cellular DNA replication. The viral genome is copied over 1000 times. The virus then continues to reactivate the host cell's DNA synthesis, since replication of the viral genome is dependant on the human cell's DNA replication machinery. As with any cell, continuous DNA replication will eventually lead to gene mutations that may endanger the cell's health or produce cancerous behavior, such as enhanced invasiveness. In a normal cell, the cell machinery would sense this DNA damage and activate apoptosis, killing the cell and removing the damaged DNA from the body. The two primary proteins responsible for this cell check system are p53 and pRb. However, the HPV oncoproteins E6 and E7 bind to p53 and pRb respectively, preventing these proteins from activating the apoptosis pathway if DNA damage is detected. Thus, an HPV infection invites cancerous development by inciting continuous cell division and preventing mutated cells from being destroyed. In low-risk infections, this leads to the development of genital warts, a type of benign lesion. In high-risk infections, however, this can lead to cervical cancer if the infection is not eradicated from the body within two years. The longer the infection lasts, the greater the chance that cancerous mutations have developed in the continuously dividing cells. High-risk strains of HPV have been implicated in nearly 100% of cervical cancer cases. [16]

B.2 Biomarkers for cancerous progression

A biomarker is any characteristic that can be used to typify a cell or living mass. It may be anything from a specific chromosomal anomaly to the expression of a particular gene [9]. For this application, it was important to identify several proteins that are upregulated or overexpressed on the cell surface of cervical cancer cells. Typical biomarkers used to classify cervical cancer include the overexpression of p16 [10] and the loss of E-cadherin. Unfortunately, neither of these biomarkers are appropriate for this application, since p16 is not a cell surface protein and an upregulated, not downregulated, cell surface protein was needed. Typical cancer properties, such as increased invasiveness, increased cell growth and division, increased angiogenesis, decreased cell adhesion, and decreased apoptosis, were used to search through the literature for possible cell surface biomarkers present in epithelial cells. Two biomarkers were chosen for further evaluation: Epithelial Cell Adhesion Molecule (EpCAM) and the integrin alpha V Beta 6 (aVB6).

EpCAM is a cellular adhesion molecule present only in epithelial tissues. Cell adhesion molecules are proteins present on the cell surface that interact with other cells and with the cellular matrix. They are typically transmembrane proteins, which means they originate within the cell, pass through the cell membrane, and extend into the surrounding environment and are capable of passing signals into the cell [11].

EpCAM is expressed in differentiated, healthy, simple epithelial tissue, which is ideal for this application [12]. Generally, a Pap smear contains only the top layer of the cervical lining, which is comprised of differentiated simple epithelia. EpCAM interacts with other EpCAM proteins on other cells, and promotes cell-cell adhesion. It also interacts with Claudin 7, another cell surface protein, forming highly metastatic complexes. Its primary oncogenic property is its interactions with E-cadherin. EpCAM acts as a functional antagonist to E-cadherin by disrupting an important link necessary to E-cadherin. It is presumed that the loss of E-cadherin is caused or at least accompanied by an increase in EpCAM expression. This is what accounts for EpCAM's overexpression on cancer cells. It is important to not that EpCAM is also expressed during the inflammatory response [12].

Overexpression of EpCAM has been demonstrated to be necessary for cancerous proliferation, and has been linked to decreased survival rates in patients with breast cancer (another epithelial cancer) [13]. EpCAM has already been shown to be overexpressed in advanced cervical cancer lesions, and expression can be correlated to the severity of the lesion [14].

The aVB6 integrin is present only in epithelial cells. Integrins are cell surface receptors that interact with the extracellular matrix and other cells, passing signals between cells. Integrins are unique in that they pass signals both into and out of the cell. They can influence cell shape, mobility and growth. Integrins are comprised of two subunits, called alpha and beta, which are noncovalently linked. There are 19 different alpha subunits and 8 beta subunits [15].

The alpha V subunit binds to several different beta subunits, but the beta 6 subunit is present only in epithelial cells. The aVB6 integrin binds to fibronectin, a component of the extracellular matrix. It is a marker of the epithelial to mesenchymal transition (EMT). EMT indicates that a cell has lost much of its potential for cell-cell adhesion and interactions and has increased its migratory capacity. These characteristics are typical of invasive cancer cells as well. An epithelial tumor containing cells that have undergone EMT is much more invasive [16]. It is uncertain why aVB6 is present in such large amounts in cells that have undergone EMT.

Integrin aVB6 has been shown to be overexpressed in both non-invasive and invasive ovarian cancers, while normal and benign tissues did not shown any aVB6 expression at all [17]. aVB6 expression has also been evaluated in cervical tissues, showing weak expression in normal cells and extreme upregulation in metastatic tumors [18]. Overexpression of aVB6 in cervical carcinomas has been linked to a poor prognosis [18].

B.3 Indirect Immunofluorescent Immunohistochemistry

Any type of immunohistochemistry relies on antibody-antigen interactions. An antigen is any protein whose expression or functionality needs to be examined. An antibody is a glycoprotein that will bind to the epitopes (binding site) of one or more antigens. The binding site on the antibody is called the variable region. Typically, the nonbinding tail of the antibody interacts with immune cells to identify invasions and wounded cells. See Figure B.1 for antibody structure. In immunohistochemistry, antibodies to antigens of interest are purified, typically from a different species. Antibodies are available in polyclonal or monoclonal form. Monoclonal antibodies consist of a single antibody that will bind to the antigen, while polyclonal antibodies contain many different antibodies that should bind to the antigen.

Polyclonal antibodies will produce a stronger signal, but the potential for binding to the wrong antigen is increased. This is called non-specific binding, or a cross reaction, and can be limited by the use of monoclonal antibodies.

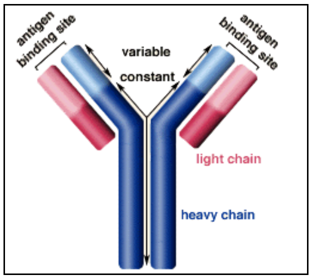

Figure B.1: Antibody structure [http://www.biology.arizona.edu/IMMUNOLOGY]

Indirect immunofluorescent immunohistochemistry is a technique for evaluating the expression of a particular protein (antigen) on the basis of antibody-antigen interactions. The cells being tested must first be fixed. This procedure kills the cells and utilizes chemical cross linkers to hold the cells in place so that a consistent expression pattern can be observed. To evaluate the expression of proteins in the cell's interior, the cells must also be lysed. For this application, the antigen of interest was on the cell surface, so formaldehyde was used to fix the cells without lysing them. Care must be taken to avoid altering the structures of the proteins in the cell with a powerful fixing agent. A primary antibody, which will bind to the antigen of interest, is chosen. This antibody will have been produced in another species, typically mouse, rabbit or goat. A serum of sterilized blood from that species is obtained. This serum contains a variety of proteins and other molecules typical of that species, and is used to block any nonspecific binding that may occur between the two species' proteins. After blocking, the primary antibody is added and will bind to the protein of interest. Next, a secondary antibody tagged with a fluorescent molecule is added to the cells. This antibody comes from yet another species, and is designed to attach to anything from the species that produced the primary antibody. In this experiment, mouse primary antibodies were used and a secondary goat anti-mouse antibody was added in this step. Using a secondary antibody tagged with a fluorescent molecule is generally more successful than using a tagged primary antibody. Multiple secondary antibodies often attach to a single primary antibody (and hence antigen), multiplying what might otherwise have been a weak signal. The fluorescent tags can be easily viewed using light at the wavelength specific to that particular tag. [19] See Figure B.2 for a pictorial representation of this process.

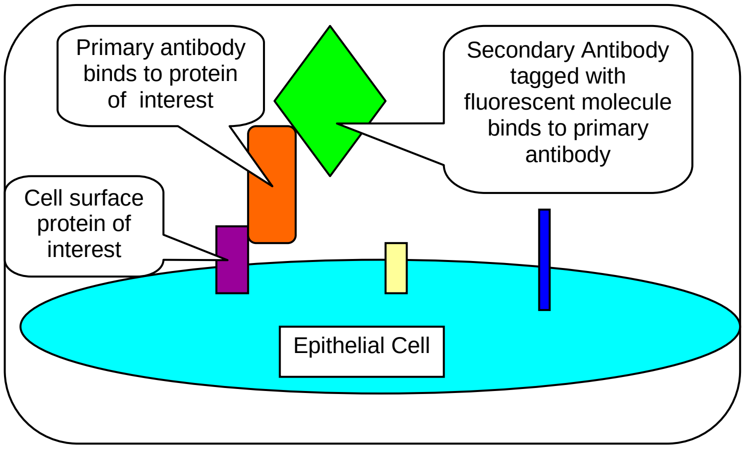

Figure B.2: Indirect Immunofluorescent Immunohistochemistry

B.4 Electrochemical Impedance Spectroscopy

Electrochemical impedance spectroscopy (EIS) has been used to characterize electrochemical systems for years. To obtain an impedance measurement, a small sinusoidal AC voltage probe is applied to the system, and the current response is measured. The probe must me small enough to obtain a linear system response. The in-phase current response can then be used to calculate the real, resistive component of the impedance. Impedance methods can be used to study a wide array of processes, and are generally both accurate and sensitive.

The impedance values calculated from the system's current response are typically fitted to an equivalent circuit containing resistances and capacitors. This equivalent circuit represents the system in electrical terms. A common equivalent circuit is the Randle's circuit, containing two resistances, one impedance and one capacitor (Figure B.3). A plot of the imaginary and real parts of the impedance will give the Nyquist plot. This plot provides a simple, visual method of comparing different systems. In the Randle's circuit, Rct and Cd are the two values most applicable for biosensor technologies.

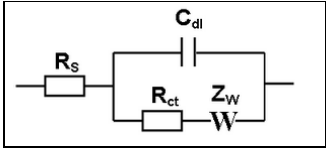

Figure B.3: Randle's Circuit [http://en.wikipedia.org/wiki/Randles_circuit]

Cd, the measured capacitance, arises from a series combination of elements. In this application, these elements include the gold slide, the self assembled monolayer (SAM), the attached antibody and the analyte, or attached cells. Cd can be represented mathematically as shown in Equation 1.

$$\frac{1}{C_d} = \frac{1}{C_{Au}} + \frac{1}{C_{SAM}} + \frac{1}{C_{AB}} + \frac{1}{C_{cell}} \qquad \text{Eq.1}$$

Rct provides a more understandable relationship, in that Rct = Rau + Rsam+Rab+Rcell. As more cells attach to the biosensor surface, the increase in mass will cause an increased resistance to electrical flow across the biosensor surface. Consequently, the value of Rct will go up, changing the shape of the Nyquist plot (Figure B.4) and producing a visible measure of cell attachment. Impedance spectroscopy is most applicable for systems in which the sensing layer (SAM and antibodies) is significantly disturbed, which makes it ideal for a cell-based biosensor. [20]

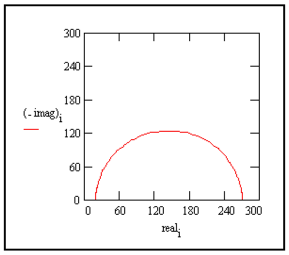

Figure B.4: Nyquist Plot [http://www.gamry.com/App_Notes/EIS_Primer/k.gif]

EIS technology has been used to identify higher concentrations of certain cancer-linked proteins in cells using enzyme labels [21], but this method is cumbersome and would not be viable in a medical setting. The electrical impedance of cancerous and normal breast tissues has also been studied [22], and significant differences existed between the tissues. Also, biosensor impedance measurements have been used to determine the concentrations of peanut proteins (allergens) in solution [23]. This experiment was very similar to this research project. However, very little work has been done involving impedance classification of living cell attachment to a biosensor surface.

B.5 Microfluidic Cancer Screening

Although little work has been done on cell attachment using EIS technology, there has been a significant amount of work on microfluidic cell attachment for cancer diagnostics. Microfluidic screening utilizes antibody-antigen interactions to separate infected and normal cells in a suspension. The cells are floated over a surface coated with an antibody to a cell surface protein. Typically, a cell surface protein which is overexpressed on the infected cells is used. The infected cells will attach to the surface and the normal cells will continue to flow out of the test chamber. Another fluid can then be passed down the chamber to remove the infected cells, and a measurement of the concentration of cells in the two washes will provide an approximate measure of the percentage of infected cells in the sample. This method has been successfully applied to cells infected with HPV, which overexpress the integrin subunit a6. [7] These microfluidic experiments demonstrate that, at the very least, cells overexpressing a particular protein are more likely to attach to a surface coated with the appropriate antibody.

B.6 Significance

The primary significance of this work is to act as a stepping stone for further research. HPV infection and cervical cancer are some of the most common threats to women across the world, with over 250,000 women dying each year [4]. Vaccination is appropriate only in very young girls [16], and there is no way to cure existing HPV infections [4]. Since nearly 80% of the female population will be infected with HPV at one point in their lives [2], there are millions of people who may currently be at risk for cervical cancer. Consistent Pap smear testing should catch early signs of cervical cancer, but the procedure is inaccurate , lengthy and must be repeated at least once a year [4]. Cervical cancer deaths generally occur in areas where regular medical attention is not available [4]. There exists a need for a cheap, quick and accurate test for precancerous developments.

Biosensors offer a potential solution. They are small, usually inexpensive, and highly accurate [21]. It is proposed that an EIS biosensor will be able to distinguish between cancerous and normal cells on the basis of antibody-antigen interactions. The impedance response of the biosensor should be related to the percentage of cancerous cells in the sample or the degree of cancerous progression [13], and will give a much faster and more accurate result that a Pap smear test. The results from the current research could be developed into an alternative method for cervical cancer screening, but there are much broader implication of this work as well.

In the future, this basic biosensor model may be expanded to detection of premalignant changes as well. In fact, it can be used to classify any cell population that exhibits differential attachment to a particular antibody. More importantly, it may eventually be used to identify HPV infections that are likely to progress to cancer. Nearly 90% of all HPV infections clear within two years [4], and rarely result in cancer. However, high-risk infections that persist longer are the culprit in 99% of cervical cancers [16]. It is not currently understood why some infections clear and others do not [4]. When this mechanism is understood, however, it could easily be incorporated into the biosensor. This would produce a fast, accurate and inexpensive method of identifying those women who do actually need repeated testing. A biosensor capable of this would eliminate nearly 90% of the $4.6 billion dollar budget that is spent on continuous retesting of women infected with high-risk strains of HPV that will clear without threat to the patient [4]. Hopefully, the current work will provided a solid foundation for future work on EIS biosensors for cervical cancer screening.

C. PRELIMINARY STUDIES

As of now, the first phase of research, identifying, choosing and testing the expression of the two biomarkers has been completed. Initial work has begun on the development of a working cell adhesion biosensor.

C.1 Optimization of Indirect Immunofluorescent Immunohistochemistry Procedure

Any experimental procedure must first be optimized before being put into practice. This was especially true in this case, since this particular technique had not been used in this laboratory before. Generally, indirect immunofluorescent immunohistochemistry is performed on lysed cells. However, since expression of cell surface proteins was being tested, intact cells were a necessity. Formaldehyde fixation was chosen to allow for testing on whole cells. Also, it was necessary to determine which materials to use in this procedure. Antibodies are very expensive, so minimizing antibody use was a priority. The surface the cells were plated on was an important variable. Four well nonfluorescent glass slides from Nunc laboratories were chosen to minimize antibody use (smaller well volumes) and experimental error. Most importantly, both the primary and secondary antibody concentrations had to be optimized. Too much antibody would give high background staining, and too little antibody would not provide enough staining to distinguish between cell types. Antibody concentrations and experimental procedures were optimized based on cancerous cell staining in CXT2 cells. Finally, the type and quantity of medium to set the stained slide had to be determined.

Initially, the immunohistochemistry experiment was attempted with two well glass slides. A grease pencil was used to circle a section of slide, and a specified volume of the antibody solutions was placed in the circle. However, the circles often flooded, and it was very difficult to hand draw consistent circles, so each sample was receiving different concentrations of the antibody solutions. Also, the grease circles lifted the cover slips up off of the slide, so the stained cells quickly dried and produced unusable pictures. Examples of bright frame (BF) and FITC pictures of the same dried cells are shown below in Figure C.1

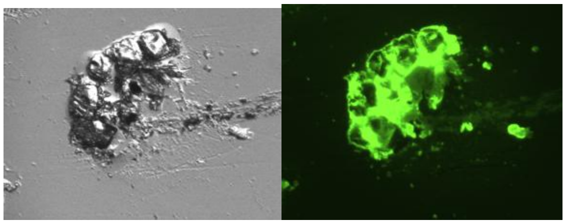

Figure C.1: BF (left) and FITC (right) images of dried cells.

To counteract the drying problem, 2 drops (instead of the recommended 1) of Vectashield H-1400 hardset medium containing DAPI was placed on the stained cells before laying down the cover slips. This produced images that were entirely saturated with DAPI staining, to the point that FITC pictures could not even be taken. However, the slides did not dry out.

After experiencing these difficulties, four well nonfluorescent Nunc Lab-Tek II slides were ordered. These slides had much smaller wells, so that the grease circles could be eliminated. One large cover slip was used, rather than individual cover slips for each well. Several drops of Vectashield hardest medium without DAPI were used to set the cover slips over the stained cells. This first experiment with the new slides produced a haze across most of the pictures, though there was very little drying. BF and FITC pictures were taken in clear regions on the slides. Figure C.2 provides an example of the haze visible across the slides. It was concluded that the pictures needed to be taken immediately after the slides had set (three days had elapsed) and that fresh Vectashield needed to be ordered (it had expired).

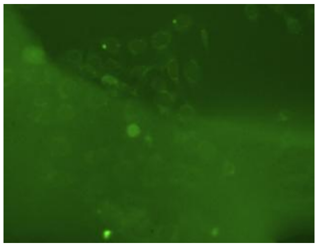

Figure C.2: Haze visible in the FITC images of optimization round 4.

Finally, an optimization round went well. Using fresh, water-based hardest Vectashield without DAPI and the four well glass slides, clear images were obtained for the integrin alpha V Beta 6 staining pattern on CXT2 cells. A variety of different primary and secondary antibody concentrations were tested. The results are shown in Figure C.3. A primary antibody concentration of 1/500 and a secondary antibody concentration of 1/1000 were chosen for the evaluation of the expression of aVB6 in normal and cancerous cells. The same experiment was repeated for Epithelial Cell Adhesion Molecule (EpCAM), the other biomarker, using CX16-2 cells. The results are shown in Figure C.4. A 1/150 concentration of primary antibody and a 1/1000 concentration of secondary antibody were chosen as the best combination to test the expression of EpCAM in normal and cancerous cells. A complete, detailed description of the optimized experimental procedure for indirect immunofluorescent immunohistochemistry is provided in section D.2.

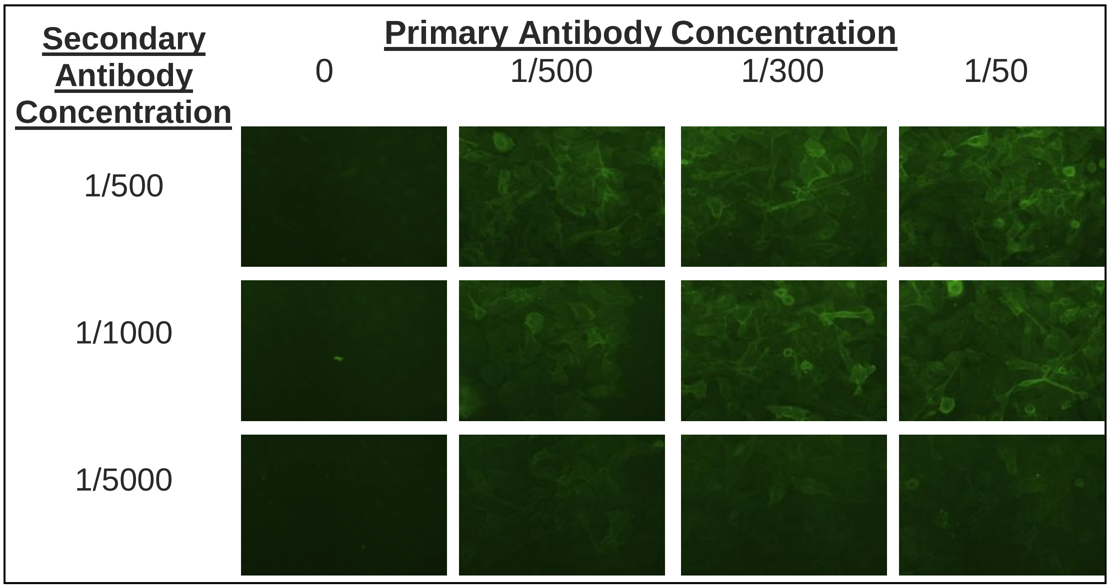

Figure C.3: Optimization results for aVB6 using CXT2 cells.

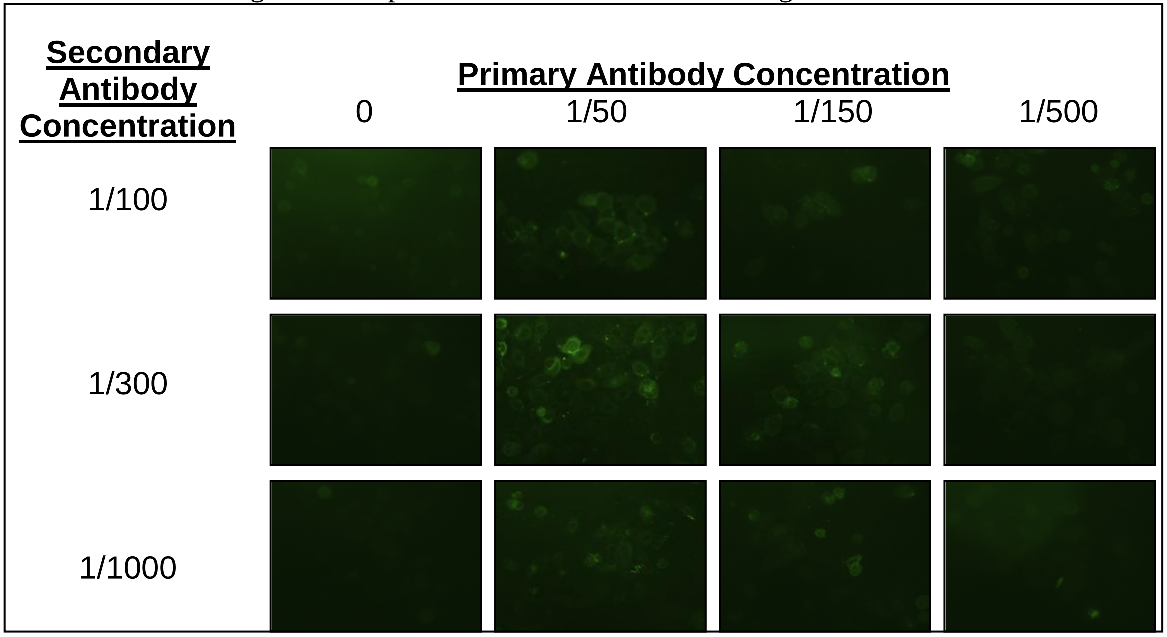

Figure C.4: Optimization results for EpCAM using CX16-2 cells.

C.2 Evaluating the expression of Epithelial Cell Adhesion Molecule in normal and cancerous cervical cells.

Once a concrete immunohistochemistry procedure was developed, testing the relative expressions of EpCAM in normal and cancerous cervical cells was straightforward. Two cancerous cell lines and two normal cell strains were tested using the optimized procedure and antibody concentrations for EpCAM. The results are shown below in Figure C.5. The ring-like staining pattern and lack of nuclear staining indicated that the cells were intact and that the EpCAM was present on the cell surface. Clearly, the cancerous cells showed much higher fluorescence, which indicated that the expression of EpCAM on the surface of the cancerous cells was much higher. However, the normal cells did show some light staining, especially when they lay at the edges of a colony. This was attributed to EpCAM's role in normal cell processes. EpCAM is part of the inflammatory response, and when cells in culture are not fully confluent, they view empty patches on the slide surface as a wound. Therefore, normal cells at the edges of the colonies may have had their immune responses activated, causing increased expression of EpCAM. Testing expression on fully confluent cells may alleviate this issue and further decrease EpCAM staining on normal cells. Regardless, hypothesis 1 has been substantiated: EpCAM does exhibit a surface staining pattern, and the cancerous cell staining was much brighter.

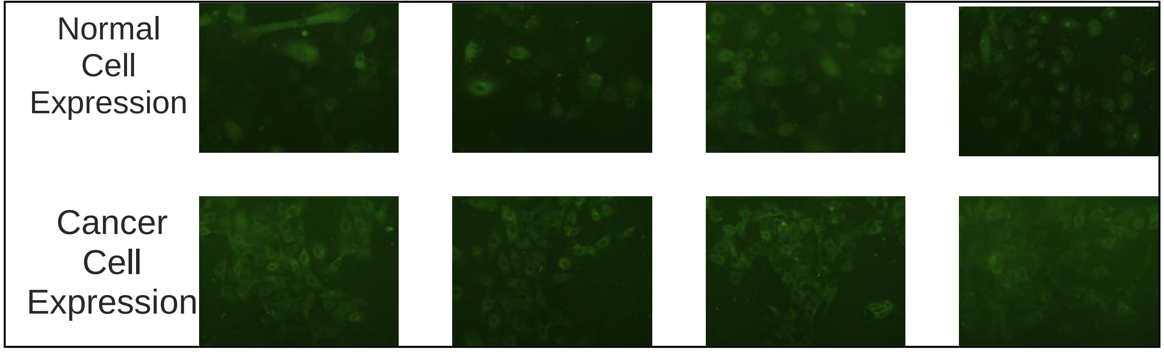

Figure C.5: Cell surface expression of EpCAM on normal and cancerous cervical cells.

C.3 Evaluating the expression of alpha V Beta 6 integrin in normal and cancerous cervical cells.

The same experimental procedure was utilized for alpha V Beta 6, along with the optimal antibody concentrations for aVB6 identified in the optimization rounds. The same two cancerous cell lines and the same two normal cell strains were used to evaluate the relative expression of aVB6. The results are shown below in Figure C.6. Once again, the overall staining pattern was consistent with surface expression: bright rings at the cell boundary and a haze over the cell body. However, some of the normal cells exhibited very bright nuclear staining. This is most likely caused due to some amount of primary and secondary antibody slipping through the cell membrane, since it appeared that the cells in question were still intact. More troubling, the normal cells exhibited much brighter staining than the cancerous cells. This indicated that the aVB6 was expressed in larger numbers on the normal cells, rather than the cancerous cells, refuting Hypothesis 2. This unexpected result can be attributed to a number of different factors. Most importantly, aVB6 is a marker for the epithelial to mesenchymal transition. As with the EpCAM trial, these cells were not confluent and were dividing to fill the empty spaces on the slide. The cells might have undergone the epithelial to mesenchymal transition to enable increased mobility and fill in the “wound” on the slide. This staining pattern may simply indicate that aVB6 is a stronger growth and migration marker than a cancer marker. Allowing the cells to reach confluency and turn off their growth and wound responses may reverse the staining pattern and produce the expected result.

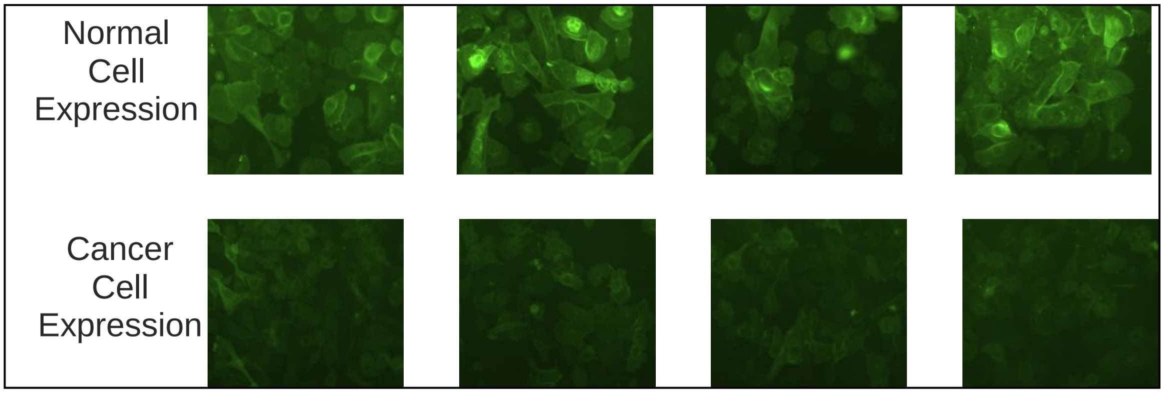

Figure C.6: Cell surface expression of aVB6 on normal and cancerous cervical cells.

C.4 Preliminary biosensor testing

To begin the second phase of research, the basics of the EIS equipment and the biosensor system must be determined. As of now, two test runs have been performed using only the activated self assembled monolayer (SAM) in an effort to avoid wasting antibody. Programs for running an open circuit potential test, voltammetry sweep and impedance measurements have been set up on the laboratory computer. A simple procedure has been outlined, and variables that need to be optimized have been identified. During the first test trial, the SAM was unstable and the wrong concentration of ferric cyanide solution was used, producing the impedance response shown in Figure C.7. In the second test trial, the open circuit potential did not stabilize and the voltammetry sweep revealed that almost no current was running through the circuit. This is most likely due to a loose connection, and a different method for connecting the alligator clips will be tested.

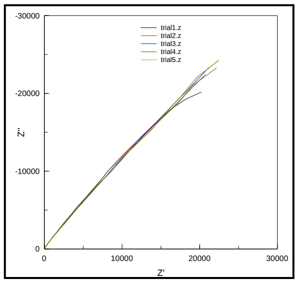

Figure C.7: Incorrect impedance response from first test trial

D. RESEARCH DESIGN AND METHODS

D.1 Research Schedule

March-May 2009: Develop and optimize basic biosensor model using cancer cells, EpCAM and the vertical electrochemical cell. Devise an alternate method (stirring, agitation, etc.) if cell settling is a problem.

August-October 2009: Test biosensor using EpCAM and pure samples of cancer cells and normal cells. Compare impedance response; adjust procedure and concentrations if necessary.

October-November 2009: Test biosensor using EpCAM and at least 3 different mixed cancer/normal cell samples. Compare to pure normal and cancer cell impedance measurements.

December 2009 (if there is time): Test biosensor using aVB6 and pure samples of normal and cancer cells.

D.2 Optimized indirect immunofluorescent immunohistochemistry procedure.

The chosen cell sample was split at confluency from culture dishes and re-suspended at 2x concentration in KFSM growth media. Two drops of the cell suspension were placed into each well in the four well Nunc Lab-Tek II glass slides. 1 mL of KFSM growth media was added to each well, and the cells were allowed to settle for four days. The growth media was removed, and 1 mL of 3.7% formalin was placed in each well. The cells were fixed for 10 minutes, and the formalin was removed. The plastic divider was stripped from the slides, and the slides were washed in a 1x phosphate buffered solution (PBS) bath. 100 uL of the antibody diluent was then placed in each well and allowed to sit for 20 minutes (blocking step). The slides were again washed in PBS, and 100 uL of the primary antibody at the specified concentration was added to each well. Incubation time for the primary antibody was one hour. The slides were washed once more in PBS, and then 100 uL of the secondary antibody at the specified concentration was added to the wells and incubated for 30 minutes. The slides were washed a final time. 12.5 uL of hardest Vectashield without DAPI was spread over each slide, and a single cover slip was laid down. Care was taken to avoid trapping air bubbles underneath the slips. BF and FITC pictures were taken immediately.

The variables that were optimized included antibody concentrations, antibody and blocking solution volumes, formalin volume and fixing time, cell confluency, setting medium type and volume, and the time elapsed between the experiment and the pictures.

D.3 Details of evaluating biomarker expression using indirect immunofluorescent immunohistochemistry.

Mounting Medium: VECTASHIEL Hard-Set, H-1400
EpCAM Primary Antibody: Monoclonal mouse antibody, sc-25308 from Santa Cruz Biotechnology
    Concentration: 1/150, in antibody diluent
aVB6 Primary Antibody: Monoclonal mouse antibody to B6, MAB4155 from R&D Systems
    Concentration: 1/500
Secondary Antibody: Alexa Fluor 488 goat anti-mouse, A11017 from Invitrogen
Antibody Diluent: 100 mL
    1 mL normal goat serum (Invitrogen)
    1 g albumin bovine serum
    Fill to 100 mL with 1x sterile PBS
Normal Cell Strains: HCX 235 ecto, HCX 238 ecto
Cancerous Cell Lines: CXT1, CXT2

D.4 Optimizing the EIS procedure for the biosensor

The basic procedure for the EIS procedure utilizes a vertical virgin Teflon electrochemical cell with a 5 mL capacity and an exposed electrode area of .32 cm2. Any electrical measurements are made using a three electrode configuration. A Pt spiral counter electrode and an Ag/AgCl reference electrode are used. The gold surface acts as the working electrode. A Solarton 1250 frequency response analyzer and an EG&G PAR 263A potentiostat are used to measure the system's response. A computer program, ZView 3.0a, is used to analyze the signals and produce Nyquist plots. A 5mM solution of Fe(CN)6 in 50 mM PBS solution is used as the background test solution during all electrochemical measurements. All other solutions are dissolved in 50 mM PBS as well.

A thin glass coated slide is cut to size so that it will fit in the electrochemical cell slot, and is fixed into the slot using an O-ring and screws. The gold electrode surface is cleaned with ethanol and water, to remove fingerprints and contaminants, and is allowed to dry. The test solution is added to the cell, and the open circuit potential (OCP) of the system is measured and a voltammetry sweep is performed. The test solution is then replaced with 5 mL of a 1 mM solution of 11-mercaptoundecanoic acid (11-MUA), and allowed to sit, covered, for 17-18 hours. The 11-MUA solution is then replaced with the test solution, and impedance and voltammetry sweeps are performed. This 11-MUA layer is the self assembled monolayer. The test solution is then replaced with 5 mL of an EDC/NHSS solution and allowed to sit. This EDC/NHSS solution activates the monolayer, so that it is more reactive towards the antibody. After one hour, the activating solution is replaced with 5 mL of an antibody solution and allowed to sit for 30 minutes. The antibody solution is then replaced with 5 mL of the test solution and impedance and voltammetry sweeps are performed. These measurements provide the base impedance values for the bare biosensor. After removing the test solution, 5 mL of a cell suspension is added to the cell and allowed to sit for 15 minutes. The cell solution is then carefully poured out and replaced with test solution. Impedance and voltammetry sweeps are repeated to allow for characterization of cell attachment to the biosensor surface.

Since this is still a rough procedure, several values still need to be optimized. Most importantly, a method must be devised to prevent cells from settling without binding to the electrode. For now, a vertical electrochemical cell will be used to prevent gravitational settling during measurements, and will simply be covered and turned sideways during cell attachment so that the cells will come into contact with the biosensor surface. (The electrode surface is normally perpendicular to the ground in a vertical electrochemical cell) Other methods, including stirring of the cell solution during cell attachment, may need to be utilized as well. Also, the optimal concentrations of the antibodies and cells must be determined. Antibody cost is always an important factor. Other issues, like incubation times and methods to attach the alligator clips to their respective electrodes, also must be considered and optimized.
EDC: N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, 75 mM

NHSS: N-hydroxysulfosuccinimide sodium salt, 15 Mm

D.5 Collecting EIS data

Collecting useful EIS will involve a number of different experiments. Once a basic biosensor model has been developed using cancer cells, the biosensor must be tested using normal cells. If the impedance results are as expected, the biosensor will be tested using normal cell solutions containing various percentages of cancer cells. The Nyquist plots from these trials will be compared to determine if the biosensor can distinguish between normal and cancer cells, and also to determine if a correlation between the impedance of the system and the percentage of cancer cells in a cell sample can be developed. Both antibodies should be tested, and the biosensor may also be attempted using both antibodies at once.

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