ABSTRACT

LOREK, JAMES DAVID. A point-of-care diagnostic device for quantifying estradiol levels in human saliva. (Under the direction of Dr. M.K. Ramasubramanian)

This study was carried out focusing upon two primary objectives: the first being to investigate experimentally the merit of a photoelectrochemical process for quantifying estradiol by salivary assay and the second to investigate numerically a novel method of accelerated sedimentation for use in sample processing and purification as an alternative to centrifugation. The presented work began initially in pursuit of an improved means of diagnostic testing for estradiol levels in patients undergoing infertility treatment. The validity of using saliva as an alternative diagnostic medium to serum has been investigated and tested experimentally, demonstrating that saliva may be used as a means to quantify estradiol levels. Initial experimental results of the proposed assay technique hold promise, with an observable photocurrent response relative to the presence of $\text{E}_2-[\text{Ru(bpy)}_3]^{2+}$; however, further experimentation is necessary in full development of an assay. Results of a CFD analysis reveal the proposed actuation method for diagnostic sample purification to perform well in comparison to that of a centrifuge and to offer advantages in a potentially compact design well suited to a fully-integrated point-of-care diagnostic device.
A POINT-OF-CARE DIAGNOSTIC DEVICE FOR QUANTIFYING ESTRADIOL LEVELS IN HUMAN SALIVA

by

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APPROVED BY:

[Signatures]

Chair of Advisory Committee
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by
James David Lorek
DEDICATION

To my wife Sharon: finding you was the sunrise in my life.
BIOGRAPHY

James Lorek entered the world barely a year after the death of “The King”, born on October 24, 1977 to Ed and Evelyn Lorek in Wilmington, NC. James’ one sibling, Mark, arrived on July 20, 1980. James attended E.A. Laney High School and graduated in the Spring of 1996. He enrolled in the Mechanical Engineering program at North Carolina State University located in Raleigh, NC in the Fall of 1996. While completing his Bachelor of Science degree James gained experience through several summer internships and graduated Magna Cum Laude from North Carolina State University in May of 2000.

Following graduation, James began work with Corning, Inc. at their fiber optic manufacturing facility in Wilmington, N.C. where he met his beautiful wife Sharon. During his time with Corning James made several significant contributions to the company before enrolling in Graduate School at North Carolina State University, returning to pursue a Master of Science degree in January of 2003. James and Sharon were married on May 10th 2003.

During his graduate studies James taught several senior level lab sections in the Department of Mechanical & Aerospace Engineering. James graduated Magna Cum Laude in December of 2004, with a mechatronics concentration and research focused in biomedical product development.

After careful consideration of all options, including pursuit of a Ph.D. as well as the heavily favored early retirement, James plans to return to the work force to pursue a career in development of innovative medical devices and healthcare equipment.
ACKNOWLEDGEMENTS

First off I would like to thank God, whose involvement in my life remains a mystery and yet His presence remains a source of constant strength and guidance. I would also like to thank my wonderful parents, whose love and support kept me on the right path through the tough years. I would like to thank Dr. Ramasubramanian for teaching me to swim without a life preserver and for his support during the past two years. I would also like to thank my committee for the review of my thesis. I am thankful for the people I have had the pleasure of meeting and the friendships I have made in these past years. I leave North Carolina State adding two words of the Hindi language to my vocabulary and many great memories to my life. To all of those that made my time here more interesting, more fulfilling, more fun, I say thank you; you know who you are.

Finally, I would like to offer a special thanks to my amazing wife, who has been a constant source of friendship, love, humor and support in the years since we met and with whom I look forward to the wonderful years ahead.
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Figure 53. A mixture of particles initially randomly distributed throughout the tube (A) begins to sediment, with larger particles sedimenting faster than smaller particles (B). The very small particles sediment very slowly and particles less dense than the medium remain at or float to the top (70).
CHAPTER 1

Introduction
1.1 Motivation

As many as 15% of couples in the United States are considered infertile, meaning they are unable to conceive a child after one year of unprotected intercourse. Reproductive endocrinology seeks to treat infertile couples using several methods directed towards a number of possible causes. One method of reproductive therapy is in-vitro fertilization (IVF). With IVF, eggs (oocytes) are collected and fertilized in a laboratory, after which the embryo is transferred back into the uterus of the patient. This process occurs by actively stimulating the patients ovaries with menotropins in order to encourage growth of multiple follicles. Patients are given daily injections of menotropins and the response is monitored with transvaginal ultrasound scans and blood levels of estradiol. Dr. Couchman (1) of Duke University Medical Center (DUMC) states that estradiol assays are critical as they are often the deciding factor in “releasing” a patient for egg/oocyte retrieval or canceling the cycle for fear of Ovarian Hyperstimulation Syndrome (OHSS). OHSS is a syndrome in which women can be at risk for ascites, severe weight gain, stroke or renal failure.

In an ideal scenario, estradiol levels would be available immediately following the taking of the sample from the patient, while the ultrasound procedure is in progress, and the patient is with the physician. In reality, this is not the case. Serum levels drawn in the morning hours are often not available until mid to late afternoon. Patients are forced to wait anxiously all day for test results, while doctors must try to reach patients late in the day to finalize a decision on their therapy. The actual testing of the serum sample typically requires only 18 minutes using current devices; however, other factors involved tend to significantly delay the process as a whole, including: 1) drawing of serum sample from the patient, 2) transfer of
samples to and from the laboratory, 3) other obligations of presiding doctor once test results are available.

Infertile couples typically experience extreme levels of stress regarding their circumstances, levels which are heightened by the delayed process in monitoring their estradiol levels. Research pertaining to women treated for infertility shows similar, and often higher, levels of stress as those dealing with life-threatening illnesses, such as heart disease and cancer (2). Couples dealing with infertility experience stress on a monthly basis in anticipation of conception and the following disappointment when it does not occur, feeling a lack of control and helplessness in their lives (2). These factors, coupled with the coordination of numerous office visits with their doctor while trying to balance personal and professional obligations create a significantly stressful environment in which infertile couples exist.

1.2 Objectives

In this work we propose improving upon the current procedure to measure blood levels of estradiol by providing a diagnostic tool capable of immediate test results to the doctor and patient using a saliva sample for testing. The method takes advantage of current knowledge of chemiluminescence and measurement techniques regarding protein hormones and seeks to replace it with a photoelectrochemical assay method via saliva, eliminating the invasive procedure for sampling blood serum, thereby providing a quicker painless alternative. In addition, a novel method of sample processing and purification is proposed as an alternative to centrifugation, and is modeled and examined through a CFD analysis. Finally, development of a prototype point-of-care device is examined and discussed.
1.3 Outline of the Presented Work

The present study is focused on several key areas in the design of a point-of-care diagnostic device as well as the overall device design; therefore, the present work is organized as follows. First, a review of saliva, estradiol and immunoassays is presented in Chapter 2. Afterwards, a summary of various immunoassay techniques currently associated with estradiol measurement is presented in Chapter 3, along with a discussion of results from various experimentation focused on quantifying estradiol levels in human saliva. In Chapter 4, a background of photoelectrochemistry is presented along with an explanation of how it may be employed in immunoassay techniques and a discussion of experimental results for proof-of-concept of such an assay. An investigation of a novel method for purification of a saliva sample as an alternative to centrifugation is presented in Chapter 5. The overall design of the point-of-care device is discussed in Chapter 6. The present work is concluded with a discussion and review of the entire project in Chapter 7. Following the main body of the work are references and several appendices, including the formal documentation submitted to and approved by the Duke University Medical Center for collection of human saliva samples.
CHAPTER 2

A Review of Estradiol, Saliva and Immunoassays
2.1 Estradiol

Estrone, estradiol and estriol, shown in Figure 1, are known as the three classic estrogens, as they were the first to be isolated (3). As shown in the figure, oxidation of the hydroxyl group at C-17 of estradiol yields estrone, and addition of a hydroxyl group at C-16 of estradiol creates estriol.

![Estrone (E1)](image1.png)

![Estradiol (E2)](image2.png)

![Estriol (E3)](image3.png)

Figure 1. Molecular Structure of the Three Primary Estrogens.

Estradiol is the most biologically active of all naturally produced estrogens. In women estradiol is responsible for growth of the breasts and reproductive epithelia, maturation of long bones and development of the secondary sexual characteristics. Estradiol is produced mainly by the ovaries, with secondary production by the adrenal glands. Estradiol levels remain nearly constant during the early part of the menstrual cycle. This is followed by a rapid increase reaching a peak the day before or the day of Leutinizing Hormone (LH) surge.
(ovulation). It is generally believed that the rise in estradiol is the factor which triggers LH release. An increase in the anterior pituitary release of follicle-stimulating hormone (FSH) and (LH) stimulates the maturation and release of the ovum (egg) into the oviduct. Estradiol is an important factor in the reproductive cycle, thus the critical need for monitoring of estradiol levels in women undergoing treatments associated with reproductive therapy.

### 2.2 Saliva

Saliva is a valuable oral fluid critical to the preservation and maintenance of oral health that has become a useful, noninvasive sampling medium for medical diagnosis and research. Consequently, it is necessary for clinicians to have a good knowledge base concerning the norm of salivary flow and function (4). Important functions of saliva include lubrication and binding, solubilizing dry food, oral hygiene, and initiating starch digestion (5).

<table>
<thead>
<tr>
<th>Property</th>
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<tbody>
<tr>
<td>Freezing Point</td>
<td>0.07 – 0.34 °C</td>
</tr>
<tr>
<td>pH</td>
<td>5.6 – 7.6</td>
</tr>
<tr>
<td>Rate of Flow</td>
<td>1 – 1.5 L / day</td>
</tr>
<tr>
<td>Specific Gravity</td>
<td>1.010 – 1.020</td>
</tr>
<tr>
<td>Mucin Concentration</td>
<td>5.53 mg/mL</td>
</tr>
</tbody>
</table>

**Table 1.** Properties of Human Salivary Gland Secretions (4, 6, 7).

Whole saliva is a clear, slightly acidic secretion of fluids from major and minor salivary glands. The basic secretory units of salivary glands are clusters of cells called an acini. These cells secrete a fluid that contains water, electrolytes, mucus and enzymes, all of which flow out of the acinus into collecting ducts (5). The major salivary glands include the paired
parotid glands (producing a watery, serous secretion), which are located opposite the maxillary first molars, and the submandibular (producing a mixed secretion of both serous and mucus composition) and sublingual glands (producing a predominantly mucus secretion), which are found in the floor of the mouth (4, 5).

Figure 2. Histological sections of the canine salivary gland. Cells stained pink are serous cells, which secrete a watery fluid essentially devoid of mucus, while the white, foamy cells are mucus-secreting cells that produce a very mucus-rich secretion (5).

Saliva is very dilute, composed of more than 99% water, and is composed of a variety of electrolytes as well as other compounds such as immunoglobulins, proteins, enzymes, and mucins (4).

2.3 Saliva as a Diagnostic Fluid

Reflective of the status of health in the body, salivary samples can be analyzed for: (1) tissue fluid levels of naturally, therapeutically, and recreationally introduced substances; (2) emotional status; (3) hormonal status (4) immunologic status; (5) neurological status; and (6) nutritional/metabolic influences (8). Over the past 20-30 years numerous research studies have validated the use of saliva as a diagnostic medium to measure the biologically active
fraction of steroid hormones in the bloodstream (9, 10, 11, 12, 13). A review of steroid hormones in human saliva has previously been published by Ferguson, *et. al.* The use of saliva offers many advantages to the use of blood serum. The saliva represents the biologically active (free) fraction of steroids in the bloodstream as opposed to blood or urine which measures total levels. By representing the unbound concentration of serum steroids, the saliva concentration may better measure the exposure of target organs to steroids when compared to serum concentrations (14). In addition, collection of the saliva sample is a non-invasive procedure that can be performed by patients themselves if necessary. While many patients may have an aversion to invasive blood serum sampling, the use of a saliva sample may aid in reducing stress and eliminating pain, as well as the possibility of blood contamination. Saliva hormones remain stable for at least three weeks at room temperature without a preservative (9). This provides for the possibility of patients collecting multiple saliva samples at home, thus providing more information than can be obtained from a single serum sample (14). Mailing of saliva samples to laboratories for testing is already a common practice in some cases.

The steroid hormones that have been studied most extensively in saliva include estrogens (estradiol, estrone, and estriol), progesterone, androgens (DHEA, testosterone, DHT), and cortisol (9). Interest is steadily increasing in non-invasive diagnostic testing, partially from haemophobia stemming from the AIDS epidemic and also from new developments in home based diagnostic tests (15). The medical community has rapidly begun to accept saliva as a preferred method to blood for steroid hormone testing (9). Despite very low concentrations, the quantification of a large number of hormones and drugs in saliva has become
commonplace due to highly sensitive test procedures (16). Saliva has found use as a diagnostic aid in an increasing number of clinical situations as all steroids of diagnostic significance in routine clinical endocrinology can now be readily measured in saliva (16). Saliva sampling is typically less expensive than blood sampling and often covered by insurance plans (9). The estrogen present in greatest concentration in saliva during normal menstrual cycles, and of utmost significance in the presented work, 17-β estradiol, has been shown to follow the same pattern as in plasma over the cycle based on data collected through saliva assay (15, 18, 19).

Human saliva is an excellent medium for the medical and research community in monitoring plasma steroid levels. It is evident that the ability to accurately quantify hormone levels via a medium such as human saliva offers significant advantages to serum measurements.

**Figure 3.** Salivary Estradiol Assay provided by Salimetrics, LLC.

This noninvasive method has permitted the evaluation and assessment of a multitude of endocrine studies that would have been extremely difficult, if not impossible, by more
traditional means (20). The value of saliva undoubtedly will continue to increase because it serves as an easily collected, noninvasive source of information (8).

Indeed, salivary estradiol assays, such as that shown in Figure 3, are currently commercially available through companies such as Salimetrics. While the example shown here does offer highly sensitive quantification of estradiol levels (at levels below 10 pg/mL) in human saliva, the test procedure requires several hours and use of laboratory equipment such as a plate reader and centrifuge.

2.4 Immunoassays

Immunoassays have become a standard technique in analytical chemistry, employing antibodies as analytical reagents based on the observation that in a system containing analyte and a specific antibody, the distribution between the bound and free forms is quantitatively related to the total analyte concentration (21). The use of immunoassays initially met with exceptional success in the determination of hormones levels, leading to a revolution in endocrinology and the introduction of new tests for the diagnosis and monitoring of endocrine disorders (21).

Antibodies are proteins naturally produced by the body that only bind to a specific substance, and are the body’s tool in identifying foreign substances. Generally speaking, the antibody binds to the analyte in question, such as estradiol in this case. There are various assay principles that are typically applied, such as the sandwich assay or the competitive assay. For example, in a competitive assay the analyte competes with a labeled analyte for antibody
binding sites. The labels used are typically some tag or marker which provides a direct form or measurement or whose concentration is easily quantifiable. Immunoassays typically involve some form of separation or capture, in which the bound analyte is isolated from excess reagents, such as excess antibodies or unbound labeled analyte. A common technique for capture is to conjugate the antibody with biotin and use streptavidin coated magnetic beads to bind with the biotin. By applying a magnetic field, the solid phase may be isolated from the assay.

While any binding protein may be used for determination of the analyte, antibodies are most widely used due to their exceptional specificity, stability and versatility (21). Antibodies may be produced for a wide variety of compounds across a wide range of molecular weight.

Radioimmunoassays (RIA) were the first type of immunoassay used, and their introduction (Yalow and Berson, 1960) is likely the single most important advance in biological measurement of the past three decades (22). The high sensitivity of these assays is due to the high detectability of radioisotopes and the high binding affinity of antibodies (21). As a result, they are often used to measure the quantities of hormones or drugs present in a patient's serum (23). Ag:Ab complexes precipitate from solution, and the radioactivity of the supernatant, representing the unbound radiolabeled hormone, is measured (23). The amount of radioactivity present is directly proportional to the amount of hormone in the sample (23). Due to the requirement of using radioactive substances, RIAs have frequently been replaced with other methods based on various forms of luminescence that offer similar sensitivities. Work with radioactivity is hazardous as it cannot be seen or smelled, and the effects can
manifest themselves many years after an incident has taken place. Regulations governing the
handling of radioactive materials are continually becoming more rigorous, posing serious
limitations on the analytical uses (24).

In the absence of radioisotopes, a tag or marker capable of luminescence is typically used
with immunoassays. This luminescence may be initiated by irradiation of the
immunocomplex (i.e. fluorescence), or by an emission resulting from a chemical reaction
(i.e. chemiluminescence). The luminescent intensity is dependent upon the concentration of
a substance, such as estradiol, in assay. A standard procedure exists for developing a
standard curve to use in determining unknown concentration of antigens in assay. This
procedure involves the accurate measurement of luminescent emission for several known
antigen concentrations, from which a standard curve is generated. With luminescent
applications such as fluorescence or chemiluminescence (CL) a photomultiplier tube (PMT)
is typically used to output a current proportional to the radiant input.

Enzyme-linked immunosorbant assays (ELISA) were developed extensively as a general
immunoassay method, as the enzyme-linked label provides a means for amplifying the
analyzed antigen-antibody (antigen-Ab) complex formation. Development of fluorescence,
chemiluminescence or electrode potential changes as a result of the enzyme reactions
provides means for the transduction of the formation of antigen-antibody complexes. The
salivary estradiol immunoassay provided by Salimetrics, shown previously in Figure 3, is an
ELISA.
A review of research efforts made in development of immunosensors has been summarized previously by Blonder et al., 1997. For example, capacitance changes at an electrode/electrolyte interface resulting from the formation of antigen-Ab complexes has been employed to develop a series of capacitive affinity sensors (26). Other immunosensor devices are based on microgravimetric analysis of antigens or antibodies resulting from frequency changes of piezoelectric crystals due to changes in mass as antigen-Ab immunocomplexes form on the crystal (27). Electrochemical detection of antibody-antigen interactions has been the subject of an increasingly large number of research efforts, and is perhaps the most promising, or most popular means of immunosensor development. The experimentation presented in this work is associated with electrochemical and photoelectrochemical detection of immunocomplexes.
CHAPTER 3

Quantifying Estradiol Levels in Human Saliva
3.1 Fluorescence

Spectrophotometry is any procedure that uses light to measure chemical concentrations. Fluorescence spectroscopy is one method of spectrophotometry. Fluorescence is increasingly important and widely used as a tool of investigation, analysis, control and diagnosis in many fields relevant to physical, chemical, biological and medical sciences (28). Fluorescence is defined as the radiative transition between two electronic states of the same spin multiplicity resulting in an emission of light often accompanied by deactivation of an electronically excited species (29). In other words, fluorescence is a short-lived photon-energy emission of light from an absorbing media, commonly referred to as fluorophores. Fluorescence is initialized via excitation by a high-energy photon, supplied by an external source such as an incandescent lamp or a laser and absorbed by the fluorophore, thus creating an excited electronic singlet state. This process distinguishes fluorescence from chemiluminescence, in which the excited state is populated by a chemical reaction (30). The excited fluorescent molecule re-emits the absorbed energy, $h\nu_{\text{ex}}$, as a low-energy (longer wavelength) photon, with the energy difference between the absorbed and emitted photons producing molecular vibrations, or heat (31). Typically, the absorbed photon is in the ultraviolet range, while the emitted light is in the visible range. Lifetimes of observed fluorescence for organic molecules are in the picosecond and nanosecond range (29). Fluorescence is named after the mineral fluorspar, or calcium fluoride, which is known to exhibit the phenomenon detailed here.

Fluorescence emission intensity may be measured in order to determine chemical concentrations. The correlation between fluorescence intensity and chemical concentration is
dependent upon Beer-Lambert’s Law. When light is absorbed by a sample, the radiant power of the beam of light is decreased (32). The transmittance, \( T \), is defined as the fraction of the original light that passes through the sample.

\[
T = \frac{P}{P_o}
\]  

(1)

A more useful quantity is the absorbance, \( A \), also known as optical density, which is defined as:

\[
A = \log_{10}(\frac{P}{P_o}) = -\log_{10} T
\]  

(2)

The Beer-Lambert Law states that the absorbance of a sample is directly proportional to the concentration of the light-absorbing species in the sample, thus making absorbance a very useful parameter. This relationship is expressed as follows:

\[
A = -\log_{10}(\frac{I}{I_o}) = \varepsilon cl
\]  

(3)

where \( A \) is absorbance, \( \varepsilon \) is the molar absorption coefficient, \( c \) is the concentration, and \( l \) is the path length. The molar absorption coefficient is the proportionality factor whose magnitude reflects the probability of the absorption of a photon of a given energy by the molecule (29). Similarly, the light emitted by the absorbing species is proportional to the light absorbed, as shown in Figure 4 (32). Thus, the fluorescence intensity of the species in the sample correlates directly to the concentration of the light-absorbing species in the sample.
Typically a certain process, such as binding of a fluorophore to an antigen via an antibody, is sought out so as to provide a fluorescence proportional to the concentration of the substance in question. This principal is used in a wide variety of applications, such as quantification of estradiol levels in a person's bloodstream.

**Figure 4.** Excitation of a fluorophore at three different wavelengths (EX1, EX2, EX3) does not change the emission profile but does produce variations in fluorescence emission intensity (EM1, EM2, EM3) that correspond to the amplitude of the excitation spectrum (30).
Figure 5. Fluorescence detection of mixed species. Excitation (EX) in overlapping absorption bands A1 and A2 produces two fluorescent species with spectra E1 and E2. Optical filters isolate quantitative emission signals S1 and S2 (30).

A typical curve demonstrating the measurement of fluorescence relative to the concentration of a protein in solution is shown in Figure 6.

Figure 6. Example of fluorescent emission relative to protein concentration (33).
3.2 Native Fluorescence

Native fluorescence is the innate ability of a molecule to exhibit fluorescent characteristics without the use of fluorescent dyes or markers (34). A precedent exists for the native fluorescence of estradiol (35). With this in mind, experimentation was conducted to establish the measurement sensitivity associated with the native fluorescence of estradiol as the most direct means of quantifying concentrations in a human sample.

3.3 Native Fluorescence of Estradiol – Experimental Procedure

Estradiol was purchased from Sigma Aldrich (17 β-Estradiol, Product # E8875) in 1 gram quantity of white powder form. A 2.6 mg sample of estradiol was measured on a precision scale and dissolved in 50 mL of ethanol (estradiol is soluble in ethanol but not in water, as specified by the vendor), which was then mixed with 50 mL of distilled water. This resulted in a solution concentration of 26 μg/mL, or 26 mg/L of estradiol. As previously stated, saliva is comprised of better than 99% water; therefore using distilled water seemed an effective means of mimicking a saliva sample in this experimentation. A sample of this solution was placed in a quartz cuvette specifically designed for use in the UV spectrum. The cuvettes were stored in an ethanol bath prior to use to ensure cleanliness. The absorption of the sample was measured using a Hewlett-Packard HP8453E spectrophotometer with an output range set between 200 – 400 nm. The absorption of the sample demonstrated a peak at 280 nm, as shown in Figure 7. The original solution was then used to create concentrations of 80%, 60%, 40%, 20%, 10% and 5% of the original concentration, all of which were measured in the previously described manner. Absorption at 280 nm was recorded. This
data is presented in Figure 8, showing a continual drop in the absorbance level for the peak at 280nm as the concentration of estradiol in the sample decreased.

![Graph showing absorption of 17-β Estradiol in solution.](image)

**Figure 7.** Absorption of 17-β Estradiol in solution.

The addition of an alkali tablet to the sample resulted in a peak wavelength shift from 280 nm to 300 nm, and a slight increase in the absorption. This test was conducted with the 100%, 60% and 20% solution concentrations and demonstrates the effect of pH on the absorption reading. The original sample pH was neutral (~8-9) while the addition of the alkali tablet shifted the pH to basic (~14). A linear relationship is shown to exist for the data collected in this experimentation, as shown in Figure 9, where the absorption level is plotted versus the concentration of estradiol. Absorption was inconclusive below the 150 ng/mL region. While the result is encouraging it falls short of a desired sensitivity of the 5 – 4000 pg/mL range associated with state-of-the-art commercially available assays.
Figure 8. Absorption of 17-β Estradiol in solution at various concentrations; peaks in the ~300 nm range are tests conducted with an alkali tablet added.

Figure 9. Absorption at 280 nm of 17-β Estradiol in solution.
3.4 Chemiluminescence

Chemiluminescence (CL) is the emission of light resulting from a chemical reaction (36). The light emission occurs as the product of a chemical reaction relaxes from a high energy level to the ground state with the emission of photons. This is highly similar to fluorescence with the key difference being the means by which the luminescent emission occurs. The appeal of CL is that it provides the opportunity to carry out sensitive assays over a wide range of concentrations using relatively inexpensive equipment (36).

3.5 Electrochemiluminescence

Electrochemiluminescence (ECL) is a form of chemiluminescence in which the light emitting CL reaction is activated with the use of electric current flow or an applied electric field. The advantage of ECL is that it provides an opportunity for controlling the time and position of the light emitting reaction until the desired molecules attached to the CL agents are confined to a certain location, thereby increasing the detector sensitivity and the signal to noise ratio. Similar control over light emission can be attained with alternative methods, such as fluorescence; however, the required instrumentation is likely to be more sophisticated and expensive (36).
3.6 Current Immunoassay Technique for Quantifying Estradiol at Duke University Medical Center

The state-of-the-art in ECL is a technique available through Roche Diagnostics. Their method uses a human serum sample for determining the concentration of various components therein, such as estradiol. The technique is highly sensitive, providing a measurement range from a few pg/mL up to 4000 pg/mL. This competition assay technique requires two 9 minute incubations, resulting in a total time of 18 minutes. A serum sample is incubated with an estradiol-specific biotinylated antibody for 9 minutes. In this manner, an immunocomplex is formed in an amount dependent upon the analyte concentration in the sample. Streptavidin-coated microparticles and an estradiol derivative labeled with a ruthenium complex are added and the second 9 minute incubation occurs, with the estradiol in the serum sample and the ruthenium-labeled estradiol derivative competing for the antibody binding sites. The interaction of biotin and streptavidin allows the immunocomplex to be bound to the solid magnetic particles, thus allowing capture of the immunocomplex through interaction with a magnetic field. The reaction mixture is aspirated into a measuring cell with a buffer solution containing tripropylamine (TPA). The microparticles are magnetically captured onto the surface of an electrode and unbound substances are removed. A voltage is applied to the electrode thus inducing a chemiluminescent emission which is then measured by a photomultiplier tube multiple times during a 0.4 second interval. Measurements are integrated to provide a single value and then compared to a calibration curve to calculate the sample result.
Figure 10. ECL Technique used in Estradiol Assay of Human Serum (37).

The chemistry involved in a typical ECL reaction involving Ru[bpy$_3$]$^{2+}$ is demonstrated in Figure 10. The mating of Ru[bpy$_3$]$^{3+}$, a powerful oxidant, with TPA$^-$, a strong reductant produces Ru[bpy$_3$]$^{2+}$ in an excited state. This compound emits a photon at 620 nm which may be measured by a PMT. In this way the compound returns to the ground state and may begin the process anew, thus regenerating the reaction. The photoreactive properties of Ru[bpy$_3$]$^{2+}$ have been well studied and will be discussed in Chapter 4.
3.7 Quantification of Estradiol Levels in Saliva – Experimental Procedure

Experimentation was conducted to evaluate the relationship between estradiol levels quantified using a saliva sample versus those performed with a serum sample. A salivary estradiol immunoassay kit was purchased from Salimetrics, LLC. The kit is provided with an estradiol standard and a high and low control in a saliva-like matrix. These samples, along with an assay diluent, (that should contain no estradiol), were measured for estradiol using a state-of-the-art device, the Elecsys 2010 shown in Figure 11, at DUMC. It should be noted that the Elecys 2010 uses a serum sample for estradiol assays and is not qualified for measurement of estradiol levels in human saliva. The results of this experimentation are presented in Figure 12. It is evident that a near linear relationship is indeed present between the measured values and the actual concentration of estradiol in the saliva-like samples. This serves to support the arguments presented previously for the use of saliva as a diagnostic fluid.

Figure 11. Elecsys 2010 by Roche Diagnostics.
Figure 12. Concentrations of estradiol in a saliva-like matrix as measured by a CL technique for estradiol in human serum; measured concentrations are plotted relative to the corresponding actual concentration of estradiol in the saliva-like sample.
CHAPTER 4

A Novel Photoelectrochemical Approach to an Estradiol Immunoassay
4.1 An Introduction and Review of Photoelectrochemistry and Related Research

The French scientist Becquerel was the first to publish a paper on photoelectrochemistry (PEC), reporting his findings at the Academy in Paris in 1839 when he was only 18 years of age (38). Becquerel discovered the flow of a small current between two electrodes coated with silver halide immersed in electrolytic solution when exposing one of the electrodes to sunlight. The history and evolution of photoelectrochemistry since this discovery has been summarized and reported by Honda. At present, photoelectrochemistry is most commonly known as the technology used for solar energy conversion. Light energy has been converted into electrical energy with an efficiency of ~10% in systems that have been developed using sintered semiconductor nanoparticles on a transparent conducting electrode (39). Typically the nanoparticles are coated with a sensitizer dye to facilitate charge injection into the conduction band of the nanoparticles. Incident light intensity can be modulated and current resulting from this photoexcitation has been shown to be proportional to light intensity up to a saturation point. Thus, while the current from an electrochemical cell is limited by the number of redox centers, the current in a photoelectrochemical cell is enhanced by photoexcitation provided there is a sufficient concentration of sacrificial donors (40). Although organic dye sensitization has been extensively investigated, only coumarine and ruthenium bipyridyl dyes have yielded high photocurrents, as the spectral response of other dyes is not well suited to the solar spectrum (41).

An increased attention on nanostructured semiconductor thin films and their sensitized electrodes has arisen since the breakthrough of photoelectrochemical solar cells was reported
by Graetzel et al (41). A key feature of this system is a mesoporous oxide layer composed of nanometer-sized particles which have been sintered together to allow for electronic conduction to take place. The material of choice has been TiO$_2$ (anatase); however alternative wide band gap oxides such as ZnO, and Nb$_2$O$_5$ have also been investigated (43).

The use of semiconductors is prominent in PEC as they are associated with a band gap in their molecular structure (Figure 13). The high energy associated with transfer of an electron to the conduction band (CB) via photoexcitation allows the charge to move through an external circuit and thus be measured, as in chronoamperometry. This phenomenon is not present in metals as the valence band (VB) and CB overlap. The use of semiconductor electrodes and the band model is discussed in detail by Bard and Faulkner. Many PEC studies have focused on sensitized TiO$_2$ nanostructured thin films because of their stability in

![Figure 13](image-url)  

**Figure 13.** Formation of bands in solids (left) by assembly of isolated atoms into a lattice (42).
aqueous solution (41). When TiO$_2$, $E_g$ of 3.0 eV, is deposited onto a conductive substrate and annealed, it forms a porous membrane which increases the physical surface area by as much as 1000 times, thereby increasing the efficiency of electron transfer to the working electrode (44).

**Figure 14.** Principle of operation and energy level scheme of the dye-sensitized nanocrystalline solar cell. Photo-excitation of the sensitizer (S) is followed by electron injection into the conduction band of the mesoporous oxide semiconductor. The dye molecule is regenerated by the redox system, which itself is regenerated at the counter electrode by electrons that are passed through the load, thus completing the circuit (43).

There are a number of well-studied applications of dye-sensitized nanocrystalline titanium oxide particles for electrochemical applications. Grätzel et al has studied injection solar cells employing transition metal complexes for spectral sensitization of mesoporous TiO$_2$ films together with suitable redox electrolytes or amorphous organic hole conductors (39, 40).
many of these complexes photoinduced charge injection, shown in Figure 17, occurs quantitatively within a few femtoseconds (45, 46). Additionally, a combination of a ruthenium trisbipyridine and methylviologen has been used to create a supramolecular read-write-erase device (47). Successful detection of cancer cells via CL has recently been reported by Weizmann, et al. The investigation of photochemically activated electrodes applied to the design of a reversible immunosensor have been studied and reported by Blonder, et. al.

**Figure 15.** Dye sensitized electrode. A photoexcited ruthenium complex attached to the surface of a TiO\(_2\) electrode is capable of injecting charge into the conduction band. The Ru is regenerated by an electrolyte in solution that diffuses to the gold counter electrode. Recombination processes can occur that diminish the efficiency of the device for energy producing applications.

Photoelectrochemical detection for DNA hybridization assays have previously been demonstrated (49). This concept is demonstrated in Figure 16. A nanoparticle label was bound to the surface by DNA hybridization or another biomolecular binding event. Light
can excite molecular labels on the surface of a nanoparticle which then can transfer electrons to the surface (40). The dye molecule used in the experiment was $[\text{Ru(bpy)}_2(\text{bpySH})]^{2+}$. The $[\text{Ru(bpy)}_3]^{2+}$ dye molecule was electronically excited by irradiation, and the excited electron injected into the electrode. The electron was collected as a current spike, signifying hybridization (49). Figure 17 demonstrates a periodic step current that occurs as the light is switched on.

![Diagram of photoelectrochemical detection](image)

**Figure 16.** Illustration of the principle of photoelectrochemical detection.

![Graph of anodic current vs. time](image)

**Figure 17.** Anodic current vs. time for an ITO electrode in 0.3 M phosphate buffer/0.05 M EDTA following absorption of 10 nm diameter gold particles to the electrode via DNA hybridization. The potential was held at 0.3 V vs. Ag/AgCl. ssDNA/gold nanoparticle conjugates were hybridized from a 1pM solution. Arrow indicates light on (49).
4.2 Ruthenium (II) trisbipyridine

Ruthenium is a rare transition metal of the platinum group with an atomic number of 44, found in the periodic table with the element symbol Ru. The reactivity involving the excited state of Ru(bpy)$_3^{2+}$ is perhaps the most well studied transition metal compound in the world. As shown in Figure 12, the compound is composed of a ruthenium metal center chelated by three 2,2’-bipyridine ligands in an octahedral geometry.

![Ruthenium trisbipyridine: Molecular Structure.](image)

Ruthenium (II) polypyridine complexes have been extensively investigated in the last two decades because of their remarkable chemical, and photophysical properties (50). As a result, ongoing research in electrochemistry (ECL) and photoelectrochemistry (PEC) is often involved with ruthenium complexes. One of the most common ruthenium complexes used is ruthenium (II) trisbipyridine, Ru(bpy)$_3^{2+}$, known as an organometallic, or a molecule containing a metal-to-carbon bond. In particular, complexes containing bipyridine ligands with carboxylate substituents at 4,40-positions have received special attention due to their great facility to adsorb onto nanocrystalline TiO$_2$ particles, enabling them to be used in high performance solar cells (50). The metal-to-ligand charge transfer, (MLCT), excited states have also found practical applications in sensors, displays, and photovoltaic devices (50).
Upon light absorption, an electron is formally transferred from the Ru (II) metal center to one of the bipyridine ligands. As determined experimentally, shown in Figure 20, excitation of $[\text{Ru(bpy)}_3]^{2+}$ at 450 nm results in the promotion of an electron from the ruthenium atom to the bipyridil ligands. As shown in Figure 20, a broad range of wavelengths appear in the peak associated with the MLCT for $[\text{Ru(bpy)}_3]^{2+}$. The excited states of $[\text{Ru(bpy)}_3]^{2+}$ and the related derivatives may undergo a variety of electron and energy transfer processes.
Figure 20. Absorbance spectrum of [Ru(bpy)$_3$]$^{2+}$ 2Cl$^-$ (in water) (51).

Figure 21. Formation of deactivation pathways for the $^3$MLCT excited state of [Ru(bpy)$_3$]$^{2+}$ (52).
Bioorganometallic chemistry incorporates organometallic compounds in life science, with research efforts focused at the interface between organometallic chemistry and biological chemistry. The basis of bioorganometallic chemistry involves the addition of an organometallic functional group onto a biological target, such as a biomolecule, a protein, or DNA, so as to modify its properties (53). Labeling of an antibody, hormone or protein is a common practice in ECL and/or PEC applications of immunoassay. Conjugation of a ruthenium complex, such as \([\text{Ru(bpy)}_3]^{2+}\), to estradiol provides a bioorganometallic molecule capable of the same attractive chemical features as \([\text{Ru(bpy)}_3]^{2+}\). Investigation of estradiol derivatives labeled at the 17α position with various organometallics, such at ruthenium complexes, and their influence on hormone-receptor affinity have been studied and summarized by Top, et. al.

### 4.3 A Proposed Novel Assay Technique

In the present work it is hypothesized that the technology developed for solar energy conversion using PEC may be used to detect binding events between estradiol and an anti-estradiol antibody through photoexcitation of a photosensitizer, thus initiating charge injection. The photoexcitation will occur via a laser, used in place of the sun, as an intense and focused source of light. It is theorized that the desired detection range of 1 pg/mL – 4000 pg/mL may be achieved through the use of a \([\text{Ru(bpy)}_3]^{2+}\) dye and semiconductor nanoparticle labels and surfaces composed of TiO₂ semiconductor nanoparticles.
A conceptual outline of the described process may be seen in Figure 22. As shown, the irradiation provided by a laser beam would excite the \([\text{Ru(bpy)}_3]^{2+}\) resulting in an electron being transferred to the electrode. From here the electron would pass through a completed circuit where it may be measured as a photocurrent.

**Figure 22.** Concept for the proposed PEC assay of estradiol.
4.4 Photoelectrochemical Experimentation

4.4.1 Introduction

Electrochemical experimentation is conducted at a homogenous reference electrode held at a constant potential. As such, any changes in the experimental cell result from changes at the working electrode (54). The potential at the working electrode is measured with respect to that of the reference electrode. The energy of the electrons within the working electrode is controlled by controlling the potential of the working electrode (55, 56). The energy of the electrons at the working electrode may be raised by applying a more negative potential to the electrode; likewise, the energy of the electrons may be lowered as more positive potentials are applied. If electron energy is raised adequately, a flow of electrons to solution occurs, known as a reduction current; likewise, if the electron energy is lowered sufficiently, the electrons may flow from solution to the electrode, also known as an oxidation current. The oxidation or reduction peaks could occur at different potentials. The potential at which these reactions occur is of significance as the current levels are at an optimal level for measurement. These currents are affected by variance at the working electrode, for instance the capture of a solid-phase immunocomplex containing Ru[bpy3]2+ from solution onto the electrode surface could enhance the resulting current levels which can then be measured and may correlate to the amount of compound added to the surface of the working electrode.

Such is the case with PEC as well, with current flow initiated and measured as the chemical cell is irradiated with light. As explained previously, a photocurrent is produced through the absorption of light by an electrode or by electron injection from a photoexcited dye via
irradiation by a laser, etc. The resulting photocurrent is dependent on several factors including wavelength, electrode potential, and solution composition (54). The dye used in this experimentation was [Ru(bpy)$_3$]$^{2+}$. Two possible sacrificial donors were examined for this experimentation, tripropylamine (TPA) and disodium ethylenediaminetetraacetic acid (EDTA). These were selected due to their common use in CL and PEC systems for [Ru(bpy)$_3$]$^{2+}$ (51). The working electrode used was indium-tin-oxide (ITO) coated with a sintered layer of TiO$_2$.

4.4.2 Capture of Magnetic Particles / Immunocomplex

The immunocomplex used in this experimentation is captured to the solid phase by applying a magnetic field, thus capturing magnetic microparticles to the surface of an electrode. Permanent magnets were tested for use in this step for experimentation with photoelectrochemistry. A sample of the magnetic microparticles suspended in solution is shown in Figure 23. An NdFeB permanent magnet (National Imports, Magnetic Products Division) was adhered to the side of test tube housing the sample. The magnetic particles were captured to the center of the magnet contact with the tube within a matter of seconds, as shown in Figure 24, leaving a clear solution in the test tube.
**Figure 23.** Magnetic microparticles suspended in solution.

**Figure 24.** Magnetic particles captured from solution using a permanent magnet.
4.4.3 TiO$_2$ Electrode Preparation

The TiO$_2$ electrodes used in this work were prepared using a variation of a published technique (57, 58). Finely divided TiO$_2$ particles were prepared by grinding 1.2 g of TiO$_2$ powder (Degussa, P25) in 0.4 mL of H$_2$O combined with 0.04 mL of acetylacetone and 0.02 mL of Triton X-100 surfactant with a mortar and pestle. An additional 1.6 mL of water was slowly added with continued grinding for 10 minutes. Indium tin oxide (ITO) coated glass electrode substrates were secured, conductive side up, to a piece of white paper with scotch tape. Several drops of the TiO$_2$ solution were distributed about the electrode surface and then spread uniformly by rolling a 1 cm diameter test tube across the electrode surface. Uniform spreading was not easily achieved, and multiple applications and rolling of the test tube were necessary. The scotch tape served to determine the thickness of the TiO$_2$ coating as well as to mask an area of the electrode for ohmic contact to the conductive glass. The electrodes were then allowed to air dry for 30 minutes, followed by heating to 500 °C for 30 minutes in a furnace. Samples, shown in Figure 25, were stored in a descanter until use.
Figure 25. TiO$_2$ coated ITO electrodes as prepared using the described technique.

Pieces of the TiO$_2$ coated electrodes were cut from those shown in Figure 26 and used as the working electrode in the photoelectrochemistry experimentation.

Figure 26. Working electrode of TiO$_2$ coated ITO.
4.4.4 Experimental Apparatus

A schematic of the apparatus used for generating experimental data is shown in Figure 28. The photoelectrochemical cell was connected to an EG&G Princeton Applied Research Potentiostat/Galvanostat, Model 273A equipped with EG&G Instruments, Inc. Model 270/250 Research Electrochemistry Software 4.00. The optical window and working electrode each have an area of 0.38 cm$^2$. A Verdi –V10 10W, all-solid-state, frequency doubled Nd:YVO$_4$ laser by Coherent with single frequency green output at 532 nm. This laser was used in line with a MIRA 900 Ti:Saphire to expand the wavelength range and an Inrad frequency doubler/tripler. This setup provides a tunable wavelength range of 400 – 500 nm.

**Figure 27.** Verdi Laser System.

**Figure 28.** Scheme for the photoelectrochemical cell.
Figure 29. PE Cell Assembly - Front View.

Figure 30. PE Cell Assembly - Top View.
Figure 31. PE Cell Assembly - Rear View.

Figure 32. PE Cell Assembly Components.
4.4.5 Dark Current versus Light Current

In the experimentation the voltage of the working electrode was held constant while the current was monitored. The anodic current increases upon illumination of the system, thus the photocurrent, shown in equation 1, is defined as the absolute value of the difference in the current value when the system is illuminated versus a lack of illumination (dark current).

\[
I_{\text{PC}} = |I_{\text{Light}} - I_{\text{Dark}}| \tag{1}
\]

This terminology is perhaps better explained in Figure 34, which demonstrates a typical photoresponse expected from a PEC system.
4.4.6 Laboratory Experimentation

Photoelectrochemical testing was conducted using components provided for a typical state-of-the-art ECL immunoassay. Components used for initial testing consisted of 50 µL of streptavidin-coated magnetic microparticles (SCMM), 60 µL of biotinylated anti-E2 antibody (Anti-E2-biotin) and 60 µL of E2-[Ru(bpy)$_3$]$^{2+}$. These concentrations were chosen for initial tests based on ratio for the amounts of each component supplied and the number of tests the provided quantities should provide, as listed by the manufacturer of the components. The term “sample” will refer to a mixture of 50 µL of SCMM, 60 µL of Anti-E2-biotin and 60 µL of E2-[Ru(bpy)$_3$]$^{2+}$.

Samples were prepared for testing by pipetting appropriate volumes from the kit containers into test tubes and incubating for 9 minutes. After the incubation period, an NdFeB
permanent magnet was attached to the bottom of the test tube to capture the solid phase from solution. The excess solution was carefully removed using a pipette, leaving an adequate amount of solution to remain in the tube covering the solid phase. The magnet was then removed from the test tube and approximately 1.5 mL of the sacrificial donor added. The sample was added to a photoelectrochemical cell containing a TiO$_2$/ITO working electrode. A permanent magnet was attached to the back of the cell so as to capture the solid phase onto the working electrode, as shown in Figure 31.

Cyclicvoltammetry (CV) and chronoamperometry (CA) tests were conducted on various samples with varying sacrificial donors, excitation wavelengths, optical intensity and applied voltage to the working electrode. CV tests are used to study the redox process of a system involving organics or organometallics, and typically demonstrate a peak in oxidation or reduction at a particular voltage. This optimal voltage may then be used at the working electrode in CA tests as a light source is switched on and off of the system, from which a current response is expected.

4.5 Results and Discussion

4.5.1 Sacrificial Donor

It is desirable in a PEC system to use a sacrificial donor with a low dark current, one quickly reaching a steady state at a potential optimal for an oxidation current at the working electrode. As previously stated, two possible sacrificial donors were examined for this
experimentation, tripropylamine (TPA) and disodium ethylenediaminetetraacetic acid (EDTA), shown in Figure 35. These were selected due to their common use in CL and PEC systems for $[\text{Ru(bpy)}_3]^{2+}$ [51]. While TPA is the sacrificial donor commonly used in ECL systems with $[\text{Ru(bpy)}_3]^{2+}$, specifically with E2-$[\text{Ru(bpy)}_3]^{2+}$, the optimal donor for this immunocomplex used in a PEC system is not known. Thus experimentation is needed in order to evaluate this factor.

![Diagram of EDTA and TPA](image)

**Figure 35.** Common Sacrificial Donors used with $[\text{Ru(bpy)}_3]^{2+}$ in Photoelectrochemistry.

Cyclicvoltammagrams run for both TPA and EDTA on an TiO$_2$ coated ITO electrode are shown together in Figure 36. As seen the, TPA reaches a much higher current level, with oxidation increasing drastically as it passes 1.5 V. The CV response of EDTA shows approximately the same slope as that of TPA initially, but flatlines just before 1.0 V, with no dramatic oxidation apparent. Chronoamperometry for both donors is shown in Figure 37. TPA has a much higher slope initially than that of EDTA, continually increasing over the entire time period shown, whereas EDTA reaches a steady-state almost immediately, with little or no slope present.
Figure 36. CV comparison of TPA and EDTA on ITO with TiO$_2$.

Figure 37. CA of EDTA and TPA on ITO/TiO$_2$. 

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4.5.2 Photoelectrochemical Experimentation with TPA

PEC experimentation with the previously described assay components was performed first with TPA, despite EDTA appearing to be a more favorable option, as it is the donor proven with several ECL systems which use the same or similar assay components. Initial experiments were run using a 1X concentration of the previously described “sample”. No peaks or changes in the response of the system to CV were seen with this sample concentration. The most probable cause for this would be the low level of concentration of the sample present.

![Graph](image)

**Figure 38.** Photocurrent of E2-[Ru(bpy)₃]²⁺ on ITO/TiO₂ @ 1.5V : 405nm in TPA.
The data in the following figures has been manipulated to place the data sets approximately aligned for an easier graphical comparison. The relative current is the important factor here, as the absolute current values are not represented.

Figure 39. Photocurrent of E2-[Ru(bpy)$_3$]$^{2+}$ on ITO/TiO$_2$ @ 1.5V : 405nm in TPA - Close-up.

A 1X sample in TPA was analyzed using CA and an excitation wavelength of 405 nm. As shown previously, MLCT of [Ru(bpy)$_3$]$^{2+}$ occurs over a wide wavelength range, and 405 nm was used initially because it is the primary wavelength of the tunable laser used, and thus the easiest to achieve a stable setup. As shown in Figure 38, a photoresponse is indeed apparent.
for the system, and increases as a result of higher laser power applied to the chemical cell. A closer analysis is obtained in Figure 39, where a photocurrent of approximately 4 μA may be observed for a beam power of 20 mW and a photocurrent of approximately 9.5 μA for a beam power of 45 mW. This initial data was compared with control tests to observe the photoresponse of TPA alone on the TiO₂ coated ITO electrode, shown in Figure 40.

A closer analysis is obtained in Figure 41, where a photocurrent of approximately 3 μA may be observed for the control experiment with a beam power of 20 mW.

![Figure 40. Photocurrent of TPA on ITO/TiO₂ @ 1.5V : 405nm.](image-url)
Comparing the results of the control experiment to the photocurrent results with the sample in TPA, a difference of approximately 1 μA is apparent. This data seemed rewarding at first but was reconsidered after data stemming from further experimentation was obtained, as discussed here.

A 1X sample was run using CA with TPA as the sacrificial donor on a TiO₂ coated ITO electrode. The excitation wavelength was varied at a beam power of 20 mW to determine the response of the sample. Since MLCT occurs at a peak of 450 nm for [Ru(bpy)₃]²⁺ (see

**Figure 41.** Photocurrent of TPA on ITO/TiO₂ @ 1.5V : 405nm in TPA - Close-up.
Figure 20) it was expected that the photocurrent response would increase as the excitation wavelength approached this value; however, the opposite effect is shown in Figures 42 and 43.

**Figure 42.** Photocurrent of E2-[Ru(bpy)$_3$]$^{2+}$ in TPA on ITO/TiO$_2$.
A working electrode voltage of 1.5 V was used in the experimentation. This value was chosen based on supporting experimentation [10] with ECL for [Ru(bpy)$_3$]$^{2+}$ in TPA with an ITO working electrode, showing an oxidation peak at $\sim$1.5 V (59). This was necessary due to the lack of a visible peak in the CV experimentation for the sample in TPA. It was later realized that an error most likely existed in this data collection by choosing such a high voltage. In measuring the photoresponse of the system, energy is added by exposing the sample to intense irradiation provided by a laser beam. This energy addition should allow for oxidation to occur more easily. When viewing the ECL response of the same system, the

**Figure 43.** Photocurrent of E2-[Ru(bpy)$_3$]$^{2+}$ in TPA on ITO/TiO$_2$ - Close-up.
oxidation peak exposes the voltage needed at the working electrode to result in maximum current flow. With PEC, the addition of high-energy light should result in a lower voltage necessary at the working electrode for oxidation to occur. Based on Figure 36, oxidation of TPA increases at a constant rate until approximately 1.5 V, at which point it accelerates. If a voltage of 1.5 V is applied to the working electrode and the sample is then exposed to a high-energy light source, the level of oxidation should increase due to the added energy. This is most likely the phenomenon which appears in Figure 43. As seen, the increase in current when the laser is cycled on and off is highest at 405 nm and decreases approximately in a linear fashion as the excitation wavelength is increased. This experimentation is still useful in understanding the response of the system as a whole, including the working electrode and the sacrificial donor. Assuming the lack of a substantial photocurrent response at 450 nm is due to the low concentration of sample present, the lack of a photocurrent response from the other components is advantageous since the presence of a photocurrent at 450 nm could then be attributed to a change in the PEC system, such as the presence of a photoresponsive element, such as [Ru(bpy)₃]²⁺.

An isolated view of the photocurrent response for E2-[Ru(bpy)₃]²⁺ at 450 nm is shown in Figure 44, with a closer view demonstrated in Figure 45. While an oscillating step in the current response for both data series is faintly visible, the signal to noise ratio is far from desirable. There is no difference demonstrated between the response of the TPA alone compared to the of the E2-[Ru(bpy)₃]²⁺ in TPA.
Figure 44. Photocurrent of E2-[Ru(bpy)_3]^{2+} compared with TPA on ITO/TiO_2 @ 1.5V: 450nm: 20mW.
Figure 45. Photocurrent of E2-[Ru(bpy)$_3$]$^{2+}$ compared with TPA on ITO/TiO$_2$ @ 1.5V : 450nm : 20mW.
4.5.3 Photoelectrochemical Experimentation with EDTA

Cyclicvoltammograms of the estradiol assay components were also run using EDTA as a sacrificial donor. This data is shown in Figure 46. For this experimentation a sample concentration 3 times (3X) that of previous runs with TPA was used, and an excitation wavelength of 440 nm was used (use of 450 nm with the laboratory equipment resulted in complications). An oxidation peak is visible at 0.234 V, with no reduction peak present, indicating a non-reversible reaction. Two data series are shown in the figure, one with the laser illuminating the sample (shown in red) and the second without the laser illumination.

Figure 46. CV of E2-[Ru(bpy)$_3$]$^{2+}$ in EDTA on ITO/TiO$_2$. 
A shift in the oxidation peak is apparent in the CV as the laser is switched on to illuminate the sample. The peak becomes more negative (as does the entire data series) and moves slightly to the right (a higher voltage level). This is currently unexplained, as a lower voltage needed for an oxidation peak is expected with the sample illuminated.

The 3X sample was then tested for a photoresponse using CA, the results of which are shown in Figure 47.

**Figure 47.** Photocurrent of E2-[Ru(bpy)$_3$]$^{2+}$ in EDTA compared with EDTA on ITO/TiO$_2$. 
Two data series are displayed in the Figure 46, the photoresponse of EDTA alone (shown in red) and the photoresponse of E2-[Ru(bpy)$_3$]$^{2+}$ in EDTA (shown in green). A clear shift in the photocurrent is present. A photocurrent of approximately 0.25 µA is present for EDTA alone, with the photocurrent increasing to approximately 0.75 µA with the E2-[Ru(bpy)$_3$]$^{2+}$ added.

4.6 Review & Future Work

While these results seem promising, further investigation is needed to determine the contribution of each assay component to the observed phenomenon. Experimentation should be conducted to measure the photoresponse of a sample containing only SCMM and Anti-E2-biotin to ensure that indeed the presence of the E2-[Ru(bpy)$_3$]$^{2+}$ is the driving force in establishing the photocurrent increase. Additionally, samples of 3X, 6X and 9X should be measured to observe an expected increase in photoresponse relative to the amount of E2-[Ru(bpy)$_3$]$^{2+}$ present in the test sample. The work demonstrated here should also be conducted several times so as to demonstrate the repeatability of the PEC response.

It should be noted that the data presented is important in laying the groundwork for the proposed assay development. From the data presented herein, much has been learned about the proposed PEC system and additionally of TPA and EDTA as sacrificial donors. Due to continual equipment problems and time constraints further collection of data was impossible. It is the opinion of the author that the experimental results in this work seem to demonstrate that the concept indeed has merit and warrants further investigation.
The proposed long term assay technique would involve something similar to that shown in Figure 48. The advantage to this technique is the increase in the number of photoexcitable $[\text{Ru(bpy)}_3]^{2+}$ molecules that may be displaced by an estradiol molecule in the saliva sample. The current techniques employed displace a single $[\text{Ru(bpy)}_3]^{2+}$, resulting in a varying

![Diagram](image)

**Figure 48.** Proposed long-term PEC assay development.

photoresponse related to the concentration of the estradiol in assay. By labeling TiO$_2$ nanoparticles with $[\text{Ru(bpy)}_3]^{2+}$ and displacing an increased number of photoexcitable molecules a larger signal change should be evident based on concentration levels as they are measured, thus resulting in a more robust means of hormone detection.
CHAPTER 5

Investigation of a Novel Alternative to Centrifugation
5.1 Introduction and a Review of Related Research

In efforts to adapt to the ever-changing health care environment, hospitals have altered the focus of care to place emphasis more on acute and critically ill patients, thus resulting in a new paradigm of point-of-care testing currently emerging from the clinical laboratory (60). This shift has stimulated a large effort in development of new testing platforms to bring central laboratory capabilities to the bedside (60). The development of a novel point-of-care device involves many different areas, ranging from immunochemistry to automated fluid handling to circuit design. Diagnostic testing of biological fluids from a human typically requires some method of processing in order to provide a sample suitable for testing. Two of the most widely used and accepted diagnostic fluids are blood and saliva, both of which require centrifugation prior to diagnostic tests. Blood consists of plasma and serum with millions of red and white blood cells in suspension. As serum is the key component required for many diagnostic tests, the blood must be centrifuged in order to isolate the plasma, as shown in Figure 49. Similarly, saliva is a clear, dilute fluid composed of better than 99% water; however, it may be contaminated with mucus, blood or air bubbles that must be removed in order to isolate the clear extract for diagnostic tests. It is common practice in salivary research to freeze saliva samples and store them frozen until analysis. Thawing these samples almost always

![Figure 49. Blood Post Centrifuge Processing.](image-url)
demonstrates the presence of a precipitate (62). The freeze thaw process eliminates any problems with mucins as they are broken down and are easily centrifuged into a pellet leaving a clear water-like oral fluid (60). Omission of this step allows for wide variation in the viscosity of the saliva, some of which could be impossible to pipette (60).

The presence of blood components in the oral mucosa can seriously compromise quantitative estimates of salivary hormone concentrations (63). As a result, contamination of saliva with blood is of concern in diagnostic testing. Serum levels of estradiol are often 10 to 15 times greater than that of saliva, and can dramatically shift the reading of estradiol levels present in a sample (60). Blood can leak into the oral mucosa as a result of micro injuries such as burns or abrasions, the probability of leakage increasing with poor oral health, stemming from lack of care, smoking, or infectious disease (63). A recent study by Schwartz, et al., reports that transferrin levels in saliva may be used to quantify levels of blood contamination; however it is most likely preferred to eliminate the presence of blood from a sample so that it may be tested and not lost.

Currently, centrifugation and filtration are among the techniques most widely employed for suspension clarification (64). Centrifuge of saliva samples before freezing in order to remove oral debris and/or after thawing of a saliva sample to remove the resulting precipitate remains a common practice (62). Centrifuges are limited in that they are not readily compatible with in-line systems or small sample volumes, while solid filters can suffer from blockage (64).
An alternative method that has received much attention is the application of ultrasonic standing waves to isolate microparticles from solution. The ability of ultrasonic standing waves to concentrate cells and microparticles at sub-millimeter distances in megahertz-frequency non-cavitating ultrasonic standing waves has been widely studied in recent years and recently reviewed by Coakley, et. al. Ultrasonic frequencies of more than 1MHz are preferred for particle or cell manipulation, so that high-pressure amplitudes can be employed without inducing ultrasonic cavitation, with its associated vigorous order-disrupting bubble activity (64). A particle, cell or droplet, in a phase in which there is a standing wave, acquires a position-dependent acoustic potential energy such that suspended bodies experience a force which tends to concentrate them at axial half wavelength intervals, i.e. at positions of minimum particle acoustic potential energy (64). The application of ultrasonic standing waves offers some advantages over centrifugation, including the ability to be employed in-line without physical blockage (64).

In the presented work, a novel technique to accelerate the process of natural sedimentation is proposed and analyzed. This method serves as an alternative to centrifugation as well as ultrasonic standing wave isolation of cells or particles. As the proposed diagnostic device discussed in this work deals with a human saliva sample, the isolation or rapid sedimentation of salivary mucins from a saliva sample is discussed and analyzed.
5.2 Salivary Mucins

Mucins are complex protein molecules that are predominantly present in two molecular weight types, and formed by polypeptide chains that stick together (65). These mucins, while having substantial properties of lubrication, also have properties of low solubility, high viscosity, high elasticity, and strong adhesiveness. Among the various mucins known to be present in different parts of the body, salivary mucins are biologically important because of their immediate exposure to various environmental agents (66). Mucins perform an antibacterial function by selectively modulating the adhesion of the microorganisms to oral tissue surfaces, which contributes to the control of bacterial and fungal colonization (65).

![Diagram of salivary mucin structure]

**Figure 50.** Hypothetical model for the salivary low molecular weight mucin, MG2 (69).
Two mucins exist within submandibular-sublingual saliva; a high-molecular-weight mucin, glycoprotein 1 (MG1) and a low-molecular-weight mucin, glycoprotein 2 (MG2) (66). Recent studies have estimated the concentration of mucin in adult human saliva to be, on average, approximately 5.53 mg/mL (7).

The buoyant densities of isolated mucin species from human whole saliva has been determined experimentally to be 1520 kg/m$^3$ (67). The buoyant density is a terminology typically associated with large molecules and is measured via density-gradient ultracentrifugation. The buoyant density is determined relative to the necessary density of the suspending fluid medium for a given molecule to float. Assuming that human salivary mucin begins to float at a density of 1520 kg/m$^3$ for the suspending medium, the buoyant density is then the density of the molecule in question. This assumption neglects the specific geometry of the molecule in question.

### 5.3 Sedimentation

Sedimentation is the process by which particles suspended in a fluid move naturally to the bottom of the container they are held in. A simple analysis of a spherical particle reveals the governing equation for the movement of a spherical particle in a suspending fluid medium.

Consider a sphere with a diameter $d$ and specific weight $\gamma$, falling at a terminal, or constant velocity $V$ through a liquid with viscosity $\mu$, specific weight $\gamma_l$, and density $\rho$ (68). The forces acting on the sphere are shown in Figure 51.
The expression given in Figure 51 for the drag force is derived from Stoke’s Law and is only valid for small Reynolds numbers. The theoretical results presented in this section are based on the common assumption that the particle Reynolds number is small compared with unity: \( \text{Re}_p << 1 \) (69).

The sphere may be modeled according to Newton’s Second Law in the following manner, as the sphere is not accelerating:

\[
\sum F = 0
\]  
(1)

\[
F_D + F_B - W = 0
\]  
(2)

\[
F_D + F_B - W = 0
\]  
(3)

\[
3\pi\mu Vd + \gamma_l \frac{\pi d^3}{6} - \gamma_s \frac{\pi d^3}{6} = 0
\]  
(4)

**Figure 51.** Free body diagram of falling particle sphere in suspending fluid.
Algebraic manipulation of (4) yields an expression for $\mu$ in terms of $\gamma_s$, $\gamma_r$, $d$ and $V$:

$$\mu = \frac{d^2(\gamma_s - \gamma_r)}{18V} \quad (5)$$

Equation (6) is valid for a sphere falling far from a wall. The “wall effect” occurs when the falling sphere is close to a wall and affects the sphere when the following condition is true:

$$\frac{\text{sphere diameter (d)}}{\text{tube diameter (D)}} > \frac{1}{3} \quad (6)$$

The observed fall velocity $V_o$, must then be corrected using:

$$\frac{V}{V_o} = 1 + \frac{9d}{4D} + \left(\frac{9d}{4D}\right)^2 \quad (7)$$

In the fluid simulation modeled here, a diameter, $d$, of 2.75 μm is used, relative to a tube diameter, $D$, of 17 mm. This results in the following dimensional analysis:

$$\frac{0.00275}{17} = 1.618 \times 10^{-4} \ll \frac{1}{3} \quad (8)$$

As demonstrated by equation (9), correction of the fall velocity is not necessary in this case.

### 5.4 Centrifugation

A centrifuge is a machine that uses centrifugal (moving away from the center) forces for separating substances. The main principle of centrifugation is that particles suspended in a fluid move under gravity towards the bottom of the containing vessel at a rate generally
dependent on their size and density (70). The weight of the spherical particle, as shown in Figure 51, is dependent on the force of gravity which is naturally applied by the Earth’s gravitational field. This force is commonly expressed in units of gravity, where the natural gravitational force of the earth is equivalent to $1\, g$. The centrifugation technique uses centrifugal forces which are greater than the force of gravity and thus speed up the sedimentation rate of the suspended particles (70).

**Figure 52.** Top View of Centrifuge Operation
The centrifugal forces are achieved by spinning the sample at a velocity, demonstrated in Figure 52, so as to apply a force on the sample radially outward from the center axis of rotation. This force is known as the relative centrifugal field (r.c.f) and is measured in multiples of the Earth’s gravitational field, g-force or g’s.

Rearranging equation (5) and substituting \( \rho_f \) g and \( \rho_s \) g for the specific weights of the fluid and sphere yields an expression for the sedimentation velocity of a particle sphere in a suspending fluid (70, 71):

\[
V = \frac{d^2 (\rho_s - \rho_f)g}{18 \mu}
\]  

(9)

From equation (9) it can be seen that the sedimentation rate of a particle is proportional to its size, the difference in its density and that of the suspending fluid medium, and the applied centrifugal force. When the particle density equals that of the fluid, the rate of sedimentation is equal to zero. If the density of the particle is less than that of the fluid the sedimentation rate becomes negative and the particles move towards the top of the container as opposed to the bottom. The sedimentation rate increases as the viscosity of the surrounding medium decreases.

As stated, the benefit of centrifugation lies in the ability to increase the g-force applied to the suspended particles by rotating the sample about a central axis. The r.c.f. generated by a rotor is dependent upon two central factors: the speed of the rotor in revolutions per minute (r.p.m.) and the radius of the rotation, or the distance from the axis of rotation (70). Therefore, particles experience increasing sedimenting forces with greater angular velocity of
the rotor and greater distance from the rotor axis. Thus, the effectiveness of the centrifuge increases as particles move further and further towards the bottom of the container.

The following equations may be used in determining the r.c.f. for a given r.p.m and radius of rotation, or vice versa:

\[
\text{r.c.f.} = 11.18 \times r \left( \frac{\text{r.p.m.}}{1000} \right)^2
\]

\[
\text{r.p.m.} = 299.07 \left( \frac{\text{r.c.f.}}{r} \right)^{1/2}
\]

where \( r \) = radius in cm.

![Figure 53](image)

**Figure 53.** A mixture of particles initially randomly distributed throughout the tube (A) begins to sediment, with larger particles sedimenting faster than smaller particles (B). The very small particles sediment very slowly and particles less dense than the medium remain at or float to the top (C) (70).
5.5 A Discussion of the Proposed Alternative

In order to develop a compact, simple, and safe method of separation of biological fluids for point-of-care applications, a novel method as an alternative to a centrifuge is proposed and evaluated here. Clearly the advantage of the centrifuge method is the constant application of a large g-force to the suspension to provide rapid sedimentation. The proposed method was devised to provide sedimentation as an alternative to centrifugation using linear actuation instead of high speed spinning, which is large and cumbersome, and can be dangerous in a non-laboratory environment.

Linear actuators are commercially available in a wide variety, such as voice coil actuators or piezoelectric actuators. The important aspect with regards to a means of sedimentation is the path that the displacement of the actuator would follow. While sinusoidal and step functions, or a mixture of the two, may serve well for mixing, the inverse is of concern if sedimentation is desired. The desired effect is to mimic the operation of a centrifuge if possible, as it is the standard for sedimentation. With this in mind, a displacement curve, shown in Figure 54 with the resulting velocity and acceleration profiles, was devised for the linear movement of the actuator. As shown, the displacement curve comprises one cycle in the actuator movement, with acceleration upwards, a deceleration to a stop, and a return to the home position at a constant velocity.

The advantage of the proposed method of actuation is evident in the acceleration profile. As shown in the figure, the g-force begins very high and decays exponentially to approach zero. By moving in this manner a large sedimenting force may be applied to the fluid domain in a
Figure 54. Example Displacement, Velocity and Acceleration Profiles of Fluid Domain.
cyclic fashion. While this is in contrast to centrifugation, where the centrifugal force is applied constantly, the advantage of the proposed method lies in the possible use of a small actuator, such as a piezoelectric, for sedimentation and purification of fluids, thereby eliminating the need for centrifugation. The key aspect of the actuator displacement is in the lack of symmetry in the cycle, thus allowing for high g-forces in the beginning and little or no acceleration as the cycle progresses (see Figure 55).

Figure 55. Profile View of Proposed Actuation Method.
The application of little or no acceleration in reversing the cycle and returning to the home position theoretically eliminates re-sedimentation of the suspended fluid particles. If the displacement curve were symmetric, such as a sin wave, this would not be the case, as the acceleration used to drive the actuator upward would be equal to that used to return it to the home position, thus providing cyclic sedimentation and re-sedimentation.

In this work, simulations, using CFX-5 Computational Fluid Dynamics Software, were conducted for sedimentation of fluid particles in a suspending fluid medium with the proposed acceleration curves applied to the fluid domain. The results were evaluated and compared to similar simulations modeled to represent a centrifuge method as well as linear actuation in a sinusoidal fashion.

### 5.6 Computation Fluid Dynamics & CFX-5

Computational Fluid Dynamics (CFD) has become a widely used tool in the world or research and engineering. CFD is a computer-based tool for simulating the behavior of systems involving fluid flow, heat transfer, and other related physical processes (72). This is accomplished by solving equations describing fluid flow in a particular region with known flow and boundary conditions. The continual advances in computational power have made common the use of CFD as a standard tool, made easier to understand and analyze with robust graphical interfaces.
Partial differential equations known as the Navier-Stokes equations are used to approximate a numerical solution for models of any number of fluid problems set up by the user of the program. The most common means in obtaining a solution in CFD is the finite volume technique, which is used in CFX-5. This is based on the assumption that any small volume element of the fluid is so large that it contains a very large number of molecules and also assuming that the fluid is a continuous medium, the dependent variables describing the fluid motion are as follows (73):

\[
\begin{align*}
\text{Pressure} & \quad p = p(x,y,z,t) \\
\text{Density} & \quad \rho = \rho(x,y,z,t) \\
\text{Velocity} & \quad q = q(x,y,z,t) = (u,v,w)
\end{align*}
\]

The set of equations solved by CFX-5 are the unsteady Navier-Stokes equations in their conservation form. The instantaneous equations of mass, momentum and energy conservation can be written as follows in a stationary frame:

The Continuity Equation

\[
\frac{\partial \rho}{\partial t} + \nabla \cdot (\rho U) = 0 \tag{4}
\]

The Momentum Equations

\[
\frac{\partial \rho U}{\partial t} + \nabla \cdot (\rho U \otimes U) = \nabla \cdot \left( -\rho \delta + \mu \left( \nabla U + (\nabla U)^T \right) \right) + S_M \tag{5}
\]

The Energy Equation

\[
\frac{\partial \rho h_{\text{tot}}}{\partial t} + \frac{\partial \rho}{\partial t} + \nabla \cdot (\rho U h_{\text{tot}}) = \nabla \cdot (\lambda \nabla T) + S_E \tag{6}
\]
Specific Total Enthalpy, \( h_{\text{tot}} \), is given in terms of Specific Static (thermodynamic) Enthalpy, \( h \), for the general case of variable properties and compressible flow by (74):

\[
h_{\text{tot}} = h + \frac{1}{2} U^2
\]

where:

\[
h = h(p,T)
\]

The diagram shown in Figure 56 describes the process by which CFX-5 initiates and solves a CFD problem, hence providing a graphical interface between the user and the solution as an end result.

A solid geometric model of the fluid domain to be analyzed is created and meshed to create an appropriate number of finite volumes. The physics of the problem to be solved are then defined by the user. From this a solver file is created and a solution found through typical CFD analysis as described previously. The results are then analyzed by the user via the graphical interface. Contour surfaces, charts and animations may be created from from the results using the post-processor.

![Flowchart of CFX-5 Process for Obtaining CFD](image)

**Figure 56.** Flowchart of CFX-5 Process for Obtaining CFD.
In this work, saliva was modeled as water at standard temperature and pressure containing mucus, modeled as a dispersed fluid with spherical geometry, a diameter of 2.75 µm (75) and density of 1520 kg/m$^3$. A mesh was created for the fluid domain, shown in Figure 57, using ICEM CFD 4 from CFX. This domain represented a volume of approximately 4.75 mL, typical of a small centrifuge tube. Continuity, Momentum and Energy equations, shown as 4 – 6 previously, were computed at each time step to evaluate convergence. Convergence was attained with an rms residual value of 1x10$^{-4}$ or less. The solid particle phase was allowed to slip along the wall. Accumulation of the mucus volume fraction via centrifugation and the proposed actuation method was evaluated from the simulation results at the bottom-center of the fluid domain, shown as the tracking point in Figure 55.
5.7 Results and Discussion

Acceleration profiles of the fluid domain for the proposed actuation method are shown in Figure 58. The fluid domain was subjected to accelerations of 1, 10, 100 and 1000 g’s at frequencies of 10, 100 and 1000 Hz. The results of these simulations were analyzed specifically for the change in the volume fraction of mucus at a point in the bottom-center of the fluid domain. The performance of the proposed actuation method was evaluated relative to that of centrifugation.

Figure 58. Acceleration Profiles of Fluid Domain at 1Hz over 5 seconds.
An example of the accumulation of volume fraction for the proposed actuation method operating at 1000 g’s and 100 Hz is shown in Figure 59. As shown, the volume fraction increases dramatically as the fluid domain moves through each acceleration cycle. A point of interest in the figure is the shape the plot follows in each cycle. The acceleration cycle, as described previously, decreases as an exponential decay to approximately zero, changes direction and then returns to the home position (at a constant velocity, or with the acceleration remaining zero). The cyclic shape of the curve shown through each cycle of

![Figure 59. Accumulation of Mucus Volume Fraction by Proposed Actuation Method operating at 1000g, 100Hz.](image-url)
the domain movement seems to demonstrate an increase in volume fraction that follows an exponential decay as it increases rapidly and slowly trails off to zero. A vector plot of the mucus velocity at the beginning of a cycle is shown in Figure 60. The figure demonstrates the velocity arrows pointing downward, indicating the mucus particles sedimenting to the bottom of the fluid domain. The velocity is shown to be approximately 24 - 37 mm/s, which can be compared to a 30 mm length for the fluid domain from top to bottom.

![Mucus Velocity Vector Plot](image)

**Figure 60.** Mucus Velocity Vector Plot: Close-up view demonstrates the velocity pointing the mucus particles towards the bottom of the container during the actuation upstroke.
Figure 61. Snapshots of the mucus velocity vector plot through the proposed actuation cycle. High velocities are initially present during the upstroke but quickly fall of the nearly zero.
Several snapshots of the mucus velocity are shown in Figure 61. These figures demonstrate the unique effectiveness of the proposed actuation scheme. In slide 1, the velocity is shown to be high, with the onset of an actuation cycle. Slide 2 shows an appreciable velocity still present; however, slides 3, 4 and 5 demonstrate the absence of a mucus velocity during the remainder of the actuation cycle, including the return to the home position (lack of re-sedimentation). Slide 6 demonstrates the initialization of the next actuation movement upwards, away from the home position.

Data for each combination of g-force and frequency of operation was collected in efforts to attain as much real-time simulation at possible. A curve fit was then used for each resulting data set that could be drawn out to a specific time value for all data sets. In this way all combinations could be normalized and evaluated over the same time scale. Both linear and quadratic curves were used to fit to the data sets in order to attain an optimal representation of the performance. A summary of this data may be viewed in Appendix C of the presented work. Additionally, simulations were run in increments of 10, 20 and 30 cycles and compared for changes in performance, as shown in Figure 62. In this figure a quadratic fit is used for the data sets, and indeed there exists a difference using a 30 cycle data set rather than 10 or 20 cycles. As a result, the 30 cycles of data were used in evaluating the performance of the simulations.
Figure 62. Quadratic curve fits of 10, 20 and 30 cycles of the proposed actuation method operating at 1000 g, 100 Hz.

The most probable explanation of the non-linear performance of the proposed actuation method would be that as time progresses more and more particulate is closer to the tracking point, thus allowing for a greater amount to sediment during actuation. Slight differences did exist between the linear and quadratic curve fits. The normalized accumulation of volume fraction for the simulated combinations of g-force and operation frequency are presented for both linear and quadratic curve fits in the surface plots shown in Figure 63 and Figure 64. Centrifugation performance is also present in the figures, represented by “constant”, meaning a constant acceleration, as opposed to a cyclic one, was applied.
Fig. 63. Surface plot of performance for the proposed actuation scheme; linear fit used here for displaying data.

An anomaly was found in the simulated data for a combination of 1000 g’s at 1000 Hz. This data set did not provide enough points of reference for an accurate curve to be fit. As a result the performance at these parameters could not be simulated and compared with the other data collected in this study.

The final data, as shown by the surface plots in Fig. 63 and Fig. 64, is also summarized and presented in Table 2. As shown, the optimal settings in the proposed actuation scheme exist at 1000 g’s operating at a frequency of 100 Hz. These operating settings produce
Figure 64. Surface plot of performance for proposed actuation scheme; quadratic fit used here for displaying data.

Table 2. Actuation Combinations of g-force and Frequency, Ranked by Performance as Compared with Centrifugation.

<table>
<thead>
<tr>
<th>Rank</th>
<th>g’s</th>
<th>Frequency [Hz]</th>
<th>Increase in Vol. Fraction [Multiples of 100%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>10</td>
<td>10</td>
<td>1.043</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>100</td>
<td>1.075</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>10</td>
<td>1.19</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>1000</td>
<td>1.425</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>100</td>
<td>1.438</td>
</tr>
<tr>
<td>3</td>
<td>1000</td>
<td>10</td>
<td>2.613</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>1000</td>
<td>5.12</td>
</tr>
<tr>
<td>**</td>
<td>1000</td>
<td><strong>100</strong></td>
<td><strong>5.28</strong></td>
</tr>
<tr>
<td>**</td>
<td>1000</td>
<td><strong>1000</strong></td>
<td><strong>-10.505</strong></td>
</tr>
</tbody>
</table>

**Centrifuge**

<table>
<thead>
<tr>
<th></th>
<th>Frequency</th>
<th>Increase in Vol. Fraction [Multiples of 100%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Constant</td>
<td>1</td>
</tr>
<tr>
<td>100</td>
<td>Constant</td>
<td>2.893</td>
</tr>
<tr>
<td>1000</td>
<td>Constant</td>
<td>12.523</td>
</tr>
</tbody>
</table>
results that outperform centrifuge at 10 and 100 g’s and account for approximately half of the performance of centrifuge at 1000 g’s.

An alternative view for comparing the performance of the proposed scheme with that of centrifugation is shown in Figure 65. The accumulation of mucus volume fraction was normalized to the initial uniform concentration present throughout the fluid domain, or the initial concentration is taken to be a value of 1. The final data is presented as a percent

**Figure 65.** Performance of Centrifuge and Proposed Actuation Method
increase in volume fraction shown in hundreds, meaning that for a number of 2, for example, the volume fraction present at a specific location in the domain has doubled. The performance of centrifugation is shown for 1, 10, 100 and 1000 g’s, and that of the proposed actuation method shown for 1000g, 100 Hz operation. There is little difference in 1 and 10 g’s, however the efficacy of the method is obvious as the acceleration increases to 100 and 1000 g’s, with the peak performance at 1000 g’s showing that the volume fraction increases to 13 times that of the original concentration. Relative to this benchmark, the proposed method provides an increase in the volume fraction to approximately 5 times that of the original concentration, surpassing the performance of centrifugation at 100 g’s or less.

Slides of the mucus volume fraction distribution before and after a steady-state run are shown in Figure 66 on the following page. The top slide shows the initial setting of a volume fraction uniformly distributed throughout the fluid domain. The bottom slide demonstrates an accumulation of the volume fraction to bottom of the fluid domain via the proposed actuation method, providing further evidence of its efficacy.
Figure 66. Before and after views of the mucus volume fraction distribution throughout the fluid domain. Initially the mucus is uniformly distributed throughout the domain (top), but is concentrated in the bottom regions of the domain after a steady-state run has completed (bottom).
For purposes of comparison, simulations of the fluid domain previously described were run using a sinusoidal acceleration profile at 10, 100 and 1000 g amplitude at 10 Hz. The resulting data is shown in Figure 67.

![Figure 67. Sin Oscillation of Fluid Domain at 10g, 100g, 1000g at 10Hz.](image)

The data in Figure 67 demonstrates a sinusoidal response in the mucus volume fraction present in the fluid domain. Interestingly, this again demonstrates a movement of the volume fraction that appears to mirror the applied acceleration. Indeed, the sinusoidal method of acceleration does not exhibit an enhanced performance that would present an alternative to
centrifugation. The sinusoidal acceleration may be more suited for mixing applications and should be investigated as such in the future.

5.8 Review & Future Work

The purpose for this section of work was to evaluate the efficacy of the proposed method in sedimenting fluid particles suspended in a medium of differing fluid, as is the case with mucus and saliva. The viscoelasticity and strong adhesive properties of mucus as well as aggregation of the mucus molecules were not modeled here as these effects are beyond the scope of this research. Based on the work reported here, a more accurate model may be created to more adequately reflect the true behavior of mucus and saliva when subjected to the proposed sedimentation process. Nonetheless, a novel process for sedimentation of fluid microparticles in a suspending medium has been modeled and demonstrated as unique and worthy of further investigation and verification through analytical and experimental methods.

Using the proposed actuation scheme a large sedimenting force may be applied to the fluid domain in a cyclic fashion. While this is in contrast to centrifugation, where the centrifugal force is applied constantly, the advantage of the proposed method lies in the possible use of a small actuator, such as a piezoelectric, for sedimentation and purification of fluids, thereby eliminating the need for centrifugation. This method holds large potential in a wide variety of fluid handling applications but has been demonstrated here relative to a diagnostic application where biological fluids are involved.
It should be noted that the data presented in this study is acquired through simulations over a time scale limited by computational time and power. The data was acquired for a number of settings and then normalized over a specific time scale for evaluation. All resulting conclusions demonstrate the efficacy of a particular method relative to the others evaluated, but not necessarily represent their actual performance in a real-world setting.

The next step is to build a device that can be used to experimentally evaluate the proposed method of actuation and compare to the simulation data presented here. A piezoelectric actuator, etc. may be used to manipulate a volume of fluid suspension and the change in concentration of suspension at a point in the sample evaluated through turbidity measurements. Turbidity is a measure of the cloudiness of water and is related to the transmission of light through a fluid sample. If indeed the actuation described in this work accelerates the sedimentation of suspended particles in a fluid, then the turbidity of the fluid should demonstrate this, as more light will be transmitted as the sedimentation occurs.

![Figure 68. Experimental Apparatus to Evaluate Proposed Actuation Technique for Accelerated Sedimentation Through Turbidity Measurements.](image-url)
The turbid measurements of the fluid undergoing actuation can then be compared to a calibration curve created using turbidity measurements of various known concentrations of the suspension. A possible experimental apparatus is shown in Figure 68.
CHAPTER 6

Design of a Novel Point-of-Care Diagnostic Device
6.1 The Immunodiagnostic Market

The diagnostic market including reagents creates revenues exceeding $20 billion annually and has grown at better than 4% a year in spite of negative effects resulting from managed care and hospital consolidation (76). In some cases immunodiagnostic technologies have been sold or licensed for several billions of dollars (77). The expansion in this market stems from scientific and technological advances that have brought forth an increasing supply of new equipment offering greater variety for in-vitro diagnostics as well as an increase in the accuracy for point-of-care testing (78). Additionally, fluid control systems have adapted to continuing changes in diagnostic and monitoring equipment by becoming more compact, using smaller fluid volumes, and integrating system components (76). Indeed, point-of-care diagnostic tests rival the accuracy found in testing performed in central laboratories, offering additional benefits of immediate bedside assays resulting in better and faster patient care (78).

The design and manufacture of a fully integrated point-of-care diagnostic device offers many unique challenges. A schematic of a typical immunochemistry system is shown in Figure 69. The full system cycle usually incorporates the following actions: sample pickup, reagent dispense, liquid aspiration, reagent dispense, analysis, cuvette waste (76).

The investigation of a novel assay method has been evaluated and discussed within this work, as well as the design and modeling of a unique method for sample processing and purification, offering an alternative to laboratory instruments such as a centrifuge. In this chapter, equipment available for use in a prototype design is discussed.
6.2 An Appropriate Laser

As demonstrated in Chapter 4, for the photoexcited molecule, $[\text{Ru}(\text{bpy})_3]^{2+}$, used in the proposed assay method MLCT occurs in the range of 400-500 nm, with a peak occurring at approximately 440-460 nm. A high power laser was used for laboratory experimentation in this work, but an appropriate laser providing the necessary excitation wavelength with a compact design may be purchased for use in a prototype. The LDM-Serie UV/ blue and the Highpower LDM-Serie UV/blue provided by Micron Laserage are shown in Figure 70 and Figure 71, respectively. These lasers are rated for 40 mW of beam energy.

Figure 69. A typical automated immunochemistry system (75).

Figure 70. LDM-Serie UV/blue - CW Operation.
power at 440 nm, matching well with preliminary data for the proposed PEC assay discussed in the presented work.

**Figure 71.** Highpower LDM-Serie UV/blue - CW Operation.

### 6.3 Available Micropumps and Assemblies

As discussed previously in this work, fluid handling bears significant importance in diagnostic devices, particularly in a fully integrated design. Compact self-priming

**Figure 72.** Self-Priming Micro-Pump Bio-Chem Valve, Inc.

**Figure 73.** Solenoid Valve Assembly Bio-Chem Valve, Inc.
micropumps and solenoid valve assemblies available through Bio-Chem Valve, Inc. are shown in Figure 72 and Figure 73, respectively. These devices offer a compact design using inert, non-reactive materials and provide precision pipetting of 5 μL to 250 μL per solenoid actuation.

6.4 An Optimized Biotinylated Anti-Estradiol Antibody

In the pursuit of an assay for estradiol using PEC technology with a human saliva sample an appropriate antibody must be found that could be used as a key component in the developed assay. Several papers have been published by Lamminmaki, et al. describing their development of an anti-estradiol antibody with improved specificity. This antibody is available for purchase in biotinylated form through Antibody Shop. This antibody seems to bind estradiol at the D-ring, or the C17 hydroxyl group (see Figure 1), leaving the C3 hydroxyl group open. A competitor to the estradiol naturally present in a patient sample could then be prepared with a photoresponsive label, such as \( [\text{Ru}(\text{bpy})_3]^{2+} \).

6.5 Available Actuators

The simulated performance of a novel means of sample processing and purification has been evaluated and was discussed in Chapter 5. In order to experimentally validate this work and understand the true performance of such an actuation process an apparatus such as the one shown in Figure 75 must be constructed. Piezoelectric actuators capable of 10,000 g’s and continuous operation at up to 1KHz are available through PI, or Physik Instrumente.
headquartered in Germany. A example of one possible actuator appears in Figure 74, the P244.10. This model is a compact actuator with a high preload enabling it to handle the

**Figure 74.** P244.10 Piezoactuator provided by Physik Instrumente.

accelerating and decelerating loads. It also comes ready with a tapped hole on top for easy mounting of a fluid sample. The actuator may be moved in an arbitrary wave form, such as the one defined and examined in the presented work, by using a Data Acquisition Card (DAC) to output the waveform from a PC via a 0-10 V signal from the DAC to a piezo amplifier.

**Figure 75.** Apparatus for Piezo Actuator Experimentation.
6.6 The Internal Review Board at DUMC and Collection of Human Saliva Samples

The collection of samples from humans is essential for testing of a new assay method for estradiol in saliva. With this in mind, a proposal for collection of human samples was submitted to and approved by the Internal Review Board (IRB) at DUMC. This process exists to ensure the protection of humans involved in research and is guided by the ethical principles set forth in the report of the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research entitled: Ethical Principles and Guidelines for the Protection of Human Subjects of Research. The following factors are of significant importance and must be addressed for review by the IRB:

1. The risks to the subjects.
2. The anticipated benefits to the subjects and others.
3. The importance of the knowledge that may reasonably be expected to result.
4. The informed consent process to be employed.

The approval of the IRB at DUMC for collection of human saliva samples was a great accomplishment to ensure the success of the overall scope of the research at hand. The patients targeted for sample collection would be those in the care of Dr. Grace Couchman in her reproductive therapy practice. As these patients provide serum samples for measurement of estradiol levels, they may also, upon their consent, provide a saliva sample to be tested in parallel using the developed novel assay planned through the research described here. The resulting levels of estradiol in the sample donor may then be compared and the data evaluated. While this data would provide an excellent means for qualification of the
proposed assay technique it should be noted that is not a clinical trial. The complete IRB submission for this research project may be viewed in Appendix VII.
CHAPTER 7

Conclusions & Recommendations
In this work, the design of a point-of-care diagnostic device has been examined in careful detail. The following objectives have been achieved:

- Saliva may be used as a diagnostic fluid for quantifying estradiol.
- Experimentation with a novel photoelectrochemical procedure for estradiol assays shows promise, yet more data is needed for a conclusive result.
- A novel method for purifying a fluid sample has been identified and demonstrated to be an effective alternative to centrifugation.

The validity of using human saliva as a diagnostic medium has been well studied and validated experimentally in this work. Estradiol has been shown to exhibit native fluorescence capable of detection in concentrations of less than 1 μg/mL through absorption measurements. Additionally, a qualified state-of-the-art technique for quantifying estradiol levels in human serum has been shown to accurately measure estradiol concentrations of a few pg/mL present in a saliva-like matrix. A unique approach to an estradiol immunoassay through a photoelectrochemical process has been defined and investigated. Initial experimental results using a TiO$_2$ coated ITO electrode demonstrate that a photocurrent of 0.50 μA relative to control experiments exists for an immunocomplex containing E2-[Ru(bpy)$_3$]$^{2+}$ in the presence of EDTA as a sacrificial donor. It should be noted that, while encouraging, these results are but a first step in development of a novel assay. These results should be confirmed through further experimentation and expanded upon in great detail. Further work is necessary in verifying a correlation between an increased photocurrent response relative to an increased concentration of E2-[Ru(bpy)$_3$]$^{2+}$. Additionally, the
proposed assay method should be verified for performance with known concentrations of estradiol in solution, possibly distilled water in order to mimic human saliva. Finally, assay development should be verified experimentally using human saliva samples in comparison with assay results from serum samples tested at Duke. Collection and testing of human saliva samples has previously been approved by Duke University Medical Center through the Internal Review Board.

A novel process for sample processing and purification as an alternative to centrifugation has been validated through CFD analysis. The proposed method has been shown to be approximately half as effective in accelerating sedimentation when compared to centrifugation (using operation conditions of 1000 g’s applied constantly under centrifugation and 1000 g’s applied at 100 Hz with the proposed actuation method). In addition the proposed method has potential for being small and compact, remaining more suitable for small volumes of fluid needing processing, providing an alternative which could become part of a fully-integrated immunodiagnostic system or device. This would eliminate the need for a separate apparatus for centrifugation of the fluid sample.

Finally, equipment and instrumentation for prototype design along with future work and investigation has been identified and discussed.

This study demonstrates the merit in the proposed approaches to a novel salivary assay technique as well as a novel sample processing and purification procedure. Future work and experimentation has been specifically defined for each approach. Continued work guided by
the framework herein may well result in a fully-integrated point-of-care diagnostic device, capable of not only hormonal tests, but an expanded set of tests limited only by the diagnostic medium. Successful completion of this endeavor will result in enhanced patient care for infertile patients undergoing treatment, as well as anyone benefiting from rapidly responsive diagnostics.
References:


(23) Radioimmunoassay, Serology. Texas A&M University College of Veterinary Medicine, Copyright 2004, College Station, TX. http://vtpb-www.cvm.tamu.edu/vtpb/vet_micro/serology/ria/default.html


(40) Franzen, S. Photoelectrochemistry, Personal Communication, 2003: Raleigh, NC.


(60) J.C. Ritchie. *Human Saliva as used in Estradiol Measurement-Personal Communication*. 2004: Emory University, Atlanta, GA.


(72) Introduction to CFX-5, ANSYS Canada Ltd., 2004.


(77) A. Bouchie. Roche and Igen in Shotgun Wedding. Nature Biotechnology. 2003 Sep;21(9).


Appendix A. Request to Collect Human Saliva Samples through Duke University Medical Center, as submitted to the Internal Review Board.

1. Purpose of the Study

   The purpose of the study is to identify a correlation between estradiol levels measured in saliva and serum samples collected from a particular sample donor. The hope is that the results will demonstrate that using saliva samples for estradiol assays will produce the same results, relative to estradiol levels in a particular sample donor, as would be shown using a serum sample. This will be a significant step in laying the groundwork for the development of a compact, rapid estradiol device that uses a saliva sample as opposed to a serum sample.

2. Background and Significance

   Over the past 20-30 years numerous research studies have validated the use of saliva as a diagnostic medium to measure the biologically active fraction of steroid hormones in the bloodstream [1]. The use of saliva offers many advantages to the use of blood serum. The saliva represents the biologically active (free) fraction of steroids in the bloodstream as opposed to blood or urine which measures total levels. In addition, the saliva sample is a non-invasive procedure that can be performed by patients themselves if necessary. While many patients may have an aversion to invasive blood serum sampling, the use of a saliva sample may aid in reducing stress and eliminating pain, as well as the possibility of blood contamination.
Monitoring blood levels of estradiol is critical to reproductive therapy via IVF. Work to provide a rapid estradiol assay instrument would significantly improve the current process. “Rapid estradiol assays would revolutionize the way in which infertility clinics are run”, says Dr. Couchman [1], “and would greatly improve the ease of decision making in ovarian stimulation cycles, as well as the communication between patient and doctor.” Using a device that is available in the doctors office, obtaining results almost instantaneously, and then engaging in a face-to-face dialog with the patient about their current status in the treatment would be a great step in improving fertility treatment.

The research that this proposal is related to seeks to provide a rapid estradiol assay device which uses saliva samples for measurement of estradiol levels in the patient.

3. **Design & procedures**

Saliva samples will be collected by passive drool using the following:

*Guidelines for sample collection:*

- Prescription and over-the-counter medications taken by the subjects should be documented at the time of sample collection.
- Ordinary plastic drinking straws, typically found in grocery stores, should be cut into ~2-inch segments.
- Subjects should be told to visualize their favorite food and gently chew on the straw.
- Subject will passively drool down the straw into a 2 ml cryovial (Salimetrics P/N 5002-1) with their heads tilted forward until adequate sample is collected.
Subjects should be instructed not to attempt to get every last drop of saliva out of their mouths, as a high amount of foam may be produced.

Absolutely no saliva stimulant will be used.

The sample must be placed in a freezer unit immediately (within 30 minutes of sample collection) that supplies at least –20 degrees Celsius. Samples not placed in a freezer unit within this time period may be compromised, and should not be used.

If a slight reddish color is noted in the saliva sample it should be discarded and another sample collected at a time when oral bleeding or trauma is not evident.

Guidelines for patients providing samples:

Subjects should not have eaten anything at all within 60 minutes prior to sample collection.

Subjects should avoid alcohol for 24 hours prior to sample collection, as it is resorbed into saliva and interferes with antibody binding to immunoassays.

Subjects should rinse their mouths thoroughly with water 10 minutes before giving a sample in order to minimize the potential for saliva contamination.

Subjects should brush their teeth the day of sample collection, but not within 3 hours prior to sample collection, nor should any dental work be performed within 24 hours prior to sample collection.
4. **Risk/benefit assessment**

There is virtually no risk to the sample donor associated with the collection of a saliva sample. The benefit to this sampling could be tremendous as it will aid in laying the groundwork for development of a compact, rapid estradiol assay device.

5. **Subject identification, recruitment, and compensation**

Samples of serum and saliva will be collected at DUMC in the care of Dr. Grace Couchman. Both the serum and saliva samples will be labeled with an identification number that provides capability of matching test results with serum to test results with saliva. Once the identification numbers have been assigned, all records relating the samples to the patient donors may be destroyed. The ability to match serum and saliva samples from a common donor is required for meaningful results; however, the identity of the donor need not be known.

Patients will be recruited through the care of Dr. Grace Couchman. Patients will be providing serum samples for testing as IVF patients. These patients will be asked to give permission for their serum samples to be used in this testing, and to also provide a saliva sample for testing.

At this time there is no plan for compensation of the patients.
6. **Subject competency**

Subjects not competent to give consent to this proposal will not be included.

7. **Costs to the subject**

There will be no costs to the subjects that take part in the proposed testing.

8. **Data analysis & monitoring**

Serum and saliva samples will run in the XXXXX laboratory at DUMC on the Elecsys 2010 automated assay device. Several assays specific to estradiol will be run on both serum and saliva samples. Results will be examined in hopes of observing that estradiol levels observed in saliva samples can be readily related to those observed in serum samples, thus accurately reflecting the estradiol levels in a certain patient at a given point in time.

9. **Data storage & confidentiality**

Identification of the serum and saliva samples will be done by a number which relates one sample to the other. It is imperative that the knowledge of which serum sample matches with a respective saliva sample be available. Aside from this requirement, no further information is necessary in identifying the samples. Subject anonymity is ensured, as nothing relating the samples to the subjects identity will be collected for this project.
Use of a numbering identification method will be ensured through a joint effort between Dr. Couchman and the research staff at NCSU. As the samples are collected they will be numbered. The id number is all that will be available for the NCSU personnel. Dr. Couchman may collect personal information from the patients, as may be typical with her practice; however, none of this data would be available for use on this project.

The following institutions and businesses either directly or indirectly offered valuable information used in this request.


Appendix B. Linear and Quadratic Curve Fits of Mucus Accumulation as a Function of Time.

<table>
<thead>
<tr>
<th>g’s</th>
<th>Freq [Hz]</th>
<th># of Cycles</th>
<th>Linear $R^2$</th>
<th>Quadratic $R^2$</th>
<th>Linear Fit</th>
<th>Quadratic Fit</th>
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**Centrifuge**

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Appendix C. Literature for Proposed Lasers.

### High stabilized Laserdiode-Modules

**LDM-Serie UV / blue - CW operation**

### Product Specifications

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<tr>
<th>Parameter</th>
<th>Specification</th>
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<tbody>
<tr>
<td>Wavelength, Power</td>
<td>375 nm / 10mW 405 nm, 5mW / 65mW 440 nm / 20mW 473 nm / 5mW</td>
</tr>
<tr>
<td>Operation Mode</td>
<td>CW</td>
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<tr>
<td>Noise RMS (20 Hz - 500Mhz)</td>
<td>&lt; 0.5 % (typ. 0.2 %)</td>
</tr>
<tr>
<td>Pointing stability</td>
<td>&lt;5μrad/° C</td>
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<tr>
<td>Power stability</td>
<td>&lt;0.5% over 1h, &lt;2% over 8h</td>
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### Beam Parameters

<table>
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<th>Parameter</th>
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<tbody>
<tr>
<td>Beam Waist Diameter at 1/e²</td>
<td>1.1mm +/- 10% up to 15mm on Request</td>
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<tr>
<td>Spatial Mode (Far Field)</td>
<td>TEM0</td>
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<tr>
<td>Ellipticity</td>
<td>&lt;1,1:1</td>
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<tr>
<td>Astigmatism</td>
<td>&lt;20% (adjustable)</td>
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<td>Beam Divergence (Full Angle)</td>
<td>&lt;0,7mrad @ 1.1mm</td>
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<tr>
<td>Static Alignment Tolerances</td>
<td>+/- 250μm (xy) +/- 2 mrad (angle)</td>
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<tr>
<td>Polarisation</td>
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### Laser Head Specifications

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<th>Parameter</th>
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<td>Dimensions max. (L x W x H)</td>
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<td>Beam Height</td>
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<td>Weight</td>
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<tr>
<td>Operating Temperature</td>
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<td>Max. Heat Load from Laser Head</td>
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### Controller Specifications

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### AC Input Requirements

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<tr>
<td>Input Power</td>
<td>&lt; 20 Watts</td>
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</table>
# High stabilized Laserdiode-Modules

**Highpower LDM-Serie**

**UV / blue - CW operation**

## Product Specifications

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
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<tbody>
<tr>
<td>Wavelength / Output Power</td>
<td>375 nm / 20 mW, 440 nm / 40 mW, 473 nm / 10 mW, 405 nm (-5nm +15nm selectable) / &gt;100mW (typically 110mW) /</td>
</tr>
<tr>
<td>Operation Mode</td>
<td>CW</td>
</tr>
<tr>
<td>Noise RMS (20 Hz - 500Mhz)</td>
<td>&lt; 0.5 % (typ. 0.2 %)</td>
</tr>
<tr>
<td>Pointing stability</td>
<td>&lt;5μrad/°C</td>
</tr>
<tr>
<td>Power stability</td>
<td>&lt;0.5% over 1h, &lt;2% over 8h</td>
</tr>
</tbody>
</table>

## Beam Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beam Waist Diameter at 1/e²</td>
<td>1.1mm +/- 10%, up to 15mm on Request</td>
</tr>
<tr>
<td>Spatial Mode (Far Field)</td>
<td>TEMo0</td>
</tr>
<tr>
<td>Ellipticity</td>
<td>&lt;1:1.1</td>
</tr>
<tr>
<td>Astigmatism</td>
<td>&lt;20% (adjustable)</td>
</tr>
<tr>
<td>Beam Divergence (Full Angle)</td>
<td>&lt;0.7mrad @ 1.1mm</td>
</tr>
<tr>
<td>Static Alignment Tolerances</td>
<td>+/- 250μm (x/y) +/- 2mrad (angle)</td>
</tr>
</tbody>
</table>

## Laser Head Specifications

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESD Protection</td>
<td>Level 4 (air and contact)</td>
</tr>
<tr>
<td>Dimensions max. (L x W x H)</td>
<td>214 mm x 50mm x 87mm (93.5mm) (length depends on used optics)</td>
</tr>
<tr>
<td>Beam Height</td>
<td>23.5mm or 40 mm (with Baseplate)</td>
</tr>
<tr>
<td>Weight</td>
<td>1500g</td>
</tr>
<tr>
<td>Operating Temperature</td>
<td>15°C - 40°C</td>
</tr>
<tr>
<td>Storage Temperature</td>
<td>-20°C - 60°C</td>
</tr>
<tr>
<td>Max. Heat Load from Laser Head</td>
<td>6W</td>
</tr>
</tbody>
</table>

## Controller Specifications

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESD Protection</td>
<td>Level 4 air / Level 3 contact</td>
</tr>
<tr>
<td>Weight</td>
<td>ca. 2 x 350g</td>
</tr>
<tr>
<td>Dimensions (L x W x H)</td>
<td>2 x 200 x 130 x 70 (19° cassette)</td>
</tr>
</tbody>
</table>

## AC Input Requirements

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input Voltage</td>
<td>85 - 245 VAC 50/60 Hz optional 18-36 VDC nominal 24VDC</td>
</tr>
<tr>
<td>Input Power</td>
<td>&lt; 20 Watts</td>
</tr>
</tbody>
</table>

**Main features of the LDM-Series**

- Extremely compact design on european and US-american guidelines
- Separable laserhead and controlling unit
- Active temperature stabilized
- Flexible to customized specifications
- High efficiency of optical design, > 95%
- Astigmatism adjustable
- Optional mode-hopping-free operation
Appendix D. Literature for Proposed Micropumps.

**Self-Priming Micro Pumps**

Bio-Chem Valve micro pumps provide an inert path for precise, repeatable dispensing with discreet, fixed output flows for high purity or aggressive fluids. The reliable, low maintenance Bio-Chem Valve Self-Priming Micro Pump replaces pump and valve combinations, or peristatic pumps.

- Precision controlled dispensing in volumes from 5 µL to 250 µL per solenoid actuation
- Compact, economical and reliable
- Dispensing precision as tight as ±2% of set volume

**Operating Instructions:**

The diaphragm of the pump is held closed with an internal spring mechanism. When voltage is applied, the solenoid coil becomes energized and pulls the diaphragm open. This opening action causes fluid to be drawn into the pump chamber. The fluid is dispensed from the pump when the voltage is dropped enough to de-energize the coil and allow the internal spring to force the diaphragm back to the closed position. To operate the pumps:

- The pumps require a square wave signal. For ideal dispense properties, provide a 12 volt signal (or 24 volt for 24 volt rated pumps) for 150 milliseconds (250 milliseconds for the 150SP).
- Cut the voltage for a minimum of 150 milliseconds (250 milliseconds for the 150SP). This will provide a maximum cycle rate of 3.33 hertz (2 hertz for the 150SP).

To control the flow or dispense timing, modify the off-time. Lengthening the off-time will cycle the pump slower. The pump can cycle faster, but accuracy and dispense volume may be affected, depending upon the application setup.

**Manifold Assembly: Series 089M & 079NC**

- Convenient Servicing
- Low Power Consumption
- Isolated Solenoid
- Continuous Duty
- High Cycle Life
- Zero Dead Volume Ports
- Fast Response Time
- Vacuum or Pressure Service
- Inert Wetted Parts
- All solenoid operators are adjusted for equal flows

One of the attractions of the 079NC 2-way isolation valve is its ease of self-sealing. The convenience and versatility of a quick exchange of the 079NC valve assists in changing or upgrading the manifold block or valve without having to replace the entire unit.

These manifold valves are excellent for multi-liquid or gas control systems, employing aggressive or high purity fluids.

Combine the 079NC with a manifold mountable version of any other Bio-Chem valve or pump and you can create your own custom fluid system.
Appendix E. Literature for Proposed Antibody.

Anti Estradiol-17-beta

mouse monoclonal antibody

**PRODUCT NO.**

**HYB 057-02**

**PRESENTATION**

Preparation: Protein G purified
Content: 1 ml, 1 mg/ml.
Solvent: 0.01 M phosphate buffer, pH 7.4, with 0.5 M NaCl and 10 mM sodium azide
Storage: In the dark at 4°C

**ANTIGEN**

Biologically active oestrogen, estradiol-17β, is an important sex hormone, but also important contribution to the high concentrations of oestrogens which are present in breast tissues.

**IMMUNOGEN**

Estradiol-17β-6-CMO coupled to BSA

**SPECIFICITY**

HYB 057-02 reacts specifically with estradiol-17β. No crossreactivity is seen with estrone sulphate, 16-ketoestradiol, 2-hydroxyestradiol, progesterone, androstenedione, or cortisol when measuring on tritium-labeled steroids in radioimmunoassay.

**EPITOPE SPECIFICITY**

Epitope specific for the hormone, no reactivity to the CMD linker

**REACTIVITY**

HYB 057-02 reacts specifically with estradiol-17β with a measured Ka of 3; radioimmunoassay. HYB 057-02 can be cleaved with papain and reduced to generate F; with a Ka of 13.4x10^9 (1).

**CULTURE MEDIUM**

RPMI 1640 with 2-10% fetal calf serum

**FUSION PARTNER**

X63-Ag8.653.

**IMMUNIZATION**

Female C57BL/6 or BALB/c mice immunized i.p. with immunogen adsorbed onto Al(OH)3
Appendix F. Literature for Proposed Piezoelectric Actuators.

P-245 models are equipped with thinner PZT ceramic layers, providing more displacement than P-244 models in the same package. On the other hand, their "maximum" operating voltage is limited to -1000 V with voltages in excess of -750 V recommended only for short durations.

The PZT ceramic stack is protected by a stainless steel case with internal spring preload. The standard translator tip and base have tapped holes. Select the P-244.95 ball tip option to help decouple off-axis or torque loads from the translator. For push-pull forces up to 5 N, the translator can be mounted by clamping around the case. For larger forces, the translator must be mounted by the base.

For positioning of magnetic parts the P-176.10 Magnetic Adapter can be screwed into the translator tip.

- Displacement to 120 µm
- Pushing Forces to 2000 N
- Pulling Forces to 300 N
- Sub-msec Response
- Sub-nm Resolution
- Options: Vacuum, Low- & High-Temperature

P-244, P-345 PZTs