Introduction

Biosensors employ biological entities, such as proteins and cells, to detect a specific analyte or group of analytes. At present, efforts are underway to incorporate the active sensor components of biosensors onto microfluidic chips. The main advantage of integrating chemically sensitive components onto microfluidic devices is that additional functions, such as sample preconcentration, separation, and transduction, can be combined to yield a complete, integrated detection system. These three functions are highly desirable for biological analytes because sample volumes are often small, they may require preprocessing prior to analysis, and it is frequently convenient to screen for multiple analytes in a single sample. Finally, the small size of microfluidic devices is compatible with high throughput screening (HTS) of analytes. [1] The CellChip™ System is an excellent representation of the current state of the art in microfluidics and HTS analysis. Cellomics, Inc. (Pittsburgh, PA) and ACLARA Biosciences, Inc. (Mountain View, CA) collaborated to create this microfluidic device. It consists of an array of patterned cells located in microfabricated microwells combined with a microfluidic delivery system that permits each microwell to be individually addressed. [2]

The overall objective of my dissertation research is to fabricate a biosensor using a combination of microcontact printing (μCP) and microfluidics for HTS of a combinatorial library of genetically modified enzymes. The enzyme that we are interested in is the organophosphate hydrolase (OPH) enzyme. This enzyme is a highly effective catalyst for hydrolyzing, and thus detoxifying, nerve agents and other related organophosphates. [3-7] Figure 1 is a diagram of the fabricated biosensor and how it is used in HTS. The biosensor consists of a microfluidic device, which is useful for handling reagents, and an array of bacterial cells that will host the mutant OPH enzymes. Step A consists of the fabrication of the microfluidics device. A microfluidics channel is formed from a channel molded from poly(dimethylsiloxane) (PDMS) that is bonded to a silicon wafer. An array of cell corrals is formed within the channel using photoacid-based patterning of a hyperbranched polymer film (HPF). A pH-sensitive dye is then incorporated into the polymer at the bottom of the corrals. In step B, a combinatorial library of genetically modified bacteria is seeded onto the wafer. In step C, organophosphates are flowed through the channel to be hydrolyzed by the bacteria. The hydrolysis of the organophosphate results in the release of two protons, which changes the local pH of the cell. This pH change is then observed via fluorescence of the pH-sensitive dye located in the bottom of the corral in step D. Corrals that fluoresce the most contain the most active enzymes. These bacterial cells can then be collected and their DNA sequenced to determine what changes occurred to improve the catalytic activity of the enzyme.

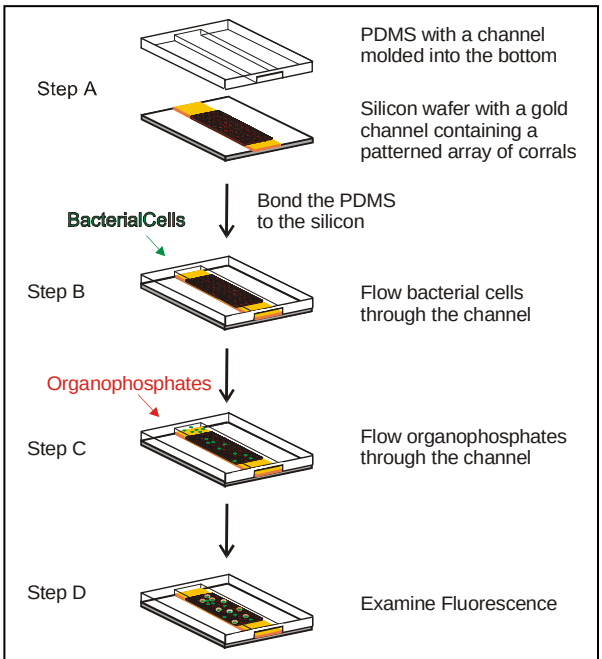


Figure 1: Microfluidic System

Background

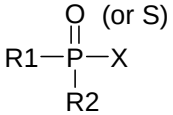
Cell Patterning

It is often necessary to pattern biomolecules, such as proteins and cells, for use in biosensors. Due to their ease of use, the most common methods for patterning cells and proteins are microcontact printing [8,9] and membrane-based patterning. [10] Biomolecules have also been patterned using the laminar flow conditions in the microchannels of microfluidic devices. [8,11-13] Another common way to pattern cells is by employing proteins that specifically bind certain types of cells. [13-15] A few commonly used protein sequences are the RGD (arginine, glycine, aspartate) sequence [14-16] and the YIGSRG (tyrosine, isoleucine, glycine, serine, arginine, and glycine) sequence. [15,17]

When using cells in biosensors, it is essential that cell growth be controlled. One way to control cell growth is to limit the area where cells can adhere to on the surface. Grafting a polymer onto the surface that prevents cell growth is the most common method, and poly(ethylene glycol) (PEG) is often used for this purpose. Our research group has shown that grafting PEG onto the sides of the poly(acrylic acid) (PAA) corrals can spatially segregate the growth of mammalian cells within corrals. [18,19] Currently, there are two theories that attempt to explain why PEG is able to resist bioadhesion. The first involves the conformation of PEG on the substrate. PEG assumes a helical conformation when bonded to a gold wafer. This helical conformation gives PEG the ability to interact strongly with water molecules via hydrogen bonding resulting in the formation of a water layer above the polymer surface. This water layer acts as a barrier preventing the proteins from reaching the polymer surface. [20,21] The second theory is that proteins are unable to bind to the surface because of steric repulsion. [21-23] Although the two current theories do not agree on the precise reason, they both recognize that a dense layer of PEG is needed on the surface for complete resistance to bioadhesion. Self-assembled monolayers (SAMs) have also been shown to have the ability to resist bioadhesion. SAMs that best exhibit this ability have the following characteristics: they have polar head groups, are uncharged, and are hydrogen bond donors but not hydrogen bond acceptors. [24]

Organophosphates

The principle commercial use of organophosphates is as pesticides, and thus it is not surprising that they are among the most toxic compounds known. [25,26] Unfortunately, some of the most toxic organophosphates, such as sarin, soman and tabun, are also used as chemical warfare agents. Organophosphates used as chemical warfare agents are three to four orders of magnitude more toxic than those used as pesticides. [27] The general structure of an organophosphate molecule is as follows. [26,28]



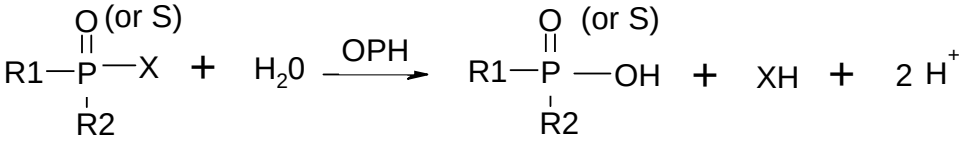
R1, R2 = alkoxy, phenyl, thiol, etc...
X = phenoxy, thiol, fluorine, etc...

The atoms that are directly connected to the central phosphorous atom are used to classify organophosphates. [28] Chemical warfare agents differ from the organophosphates used in pesticides in that they contain either fluorine or cyanide groups in their structure. Nerve agents can be further categorized into two groups: G agents, which are derived from either

phosphoramidocyanidic acid or methyl phosphonofluoric acid, and V agents, which are derived from methyl phosphonothioic acid. [27]

Organophosphates act as a neurotoxin to the body. They enter through the respiratory and digestive tracts and can even be absorbed directly through the skin. Organophosphates bind to the enzyme acetylcholinesterase (AChE) and prevent it from breaking down the neurotransmitter acetylcholine. The degree of binding depends on the structure of the organophosphate in question. Organophosphates that contain electron withdrawing groups and long alkyl groups in the side chains tend to bind more strongly. [28] Chemical warfare agents are capable of binding irreversibly to the enzyme. [27] Since acetylcholine is no longer degraded by AChE, it collects in the nerve synapses in both the central and the peripheral nervous systems resulting in overstimulation of the nerve receptors. [28,29] This build-up of acetylcholine is known as organophosphate poisoning. Some of the symptoms of organophosphate poisoning include hypersalivation, intestinal and muscle cramping, and flu-like symptoms such as vomiting and diarrhea. Restlessness and confusion may also occur along with both respiratory and circulatory failure. The symptoms experienced can vary widely and are dependant upon the specific organophosphate the subject is exposed to, as well as the amount of exposure. [28,30] Organophosphate poisoning required immediate treatment once it is diagnosed.

Over 40 thousand tons of pesticides are used annually by the US alone. The US also produces 20 tons of pesticides for export to other countries. A chemical weapons treaty has also been recently signed agreeing that the participating countries will destroy the arsenals of chemical warfare agents by the year 2007. [26,31] Thus, it is essential that effective means for organophosphate detection and detoxification be developed. There are several ways that organophosphates can be detoxified. One of the most common is through the use of the enzyme OPH. OPH is able to hydrolyze toxic organophosphate compounds to form products that are approximately two orders of magnitude less toxic than the original organophosphates. [32] The overall hydrolysis of organophosphates can be described by the following reaction. [25,33]



Two examples of the OPH enzymes are the *Flavobacterium* phosophtriesterase (PTE) and human paraoxonase (HuPON). [34] The most common OPH enzyme used is found in *E. coli* BL21, which expresses a cloned form of PTE from either *Pseudomonas diminuta* or *Flavobacterium*. [25,34] It has also been shown that sodium perborate ($NaBO_3$) [35] as well as organometallic complexes such as molybdenocene dichloride (Cp_2MoCl_2) [36] are capable of organophosphate hydrolysis.

For health, environmental, and national defense reasons it is essential that sensitive detection methods be developed for organophosphates. Organophosphates can be easily analyzed through the use of chromatographic techniques such as gas chromatography and high-performance liquid chromatography. The problem with these methods is that they are expensive, have to be done in a laboratory environment, require highly trained personnel, and often require extensive sample preparation. [29,37] Biosensors offer another form of organophosphate detection. The first organophosphate biosensors employed the enzyme AChE. Another common enzyme used in organophosphate detection is OPH. These biosensors have detected organophosphates both electrochemically [25,30,38-41] and spectroscopically. [4,37,42] Whole bacterial cells capable of

expressing OPH have also been used in organophosphate biosensors. The main advantages for using whole cells in biosensors is that the cell helps protect the enzyme and eliminates the need for enzyme purification. [25] When using whole cells in biosensors, the rate-limiting step is the diffusion of the organophosphate into the cell. This can be avoided either by permeablizing the cell membrane [32] to allow easier entrance of the organophosphate or by immobilizing the enzyme to the outside of the cell. [6,7]

Methods and Materials

Fourier Transform Infrared External Reflection Spectroscopy

In this project we use Fourier transform infrared external reflection spectroscopy (FTIR-ERS) to characterize HPFs on gold wafers. In FTIR the absorption of infrared radiation (IR) (2.5 to 15 μm or 4000 to 600 cm^{-1}) by molecules is monitored. The result of this absorption is that the vibrational modes of the molecule become excited. The two basic vibrational modes are the stretching mode, which involves a change in the bond length in a molecule, and the bending mode, which involves a change in a molecule's bond angle. IR absorption is restricted to vibrational modes resulting in a net change in dipole within the molecule. Functional groups of the molecules are identified based on the frequencies absorbed, the intensity of the absorption, and the shape of the peak on the IR spectrum. The infrared spectrometer that we will be using in for this project requires the use of a grazing angle reflection accessory. This accessory permits IR spectra to be obtained for samples located on reflective surfaces.

Ellipsometry

In this project we use reflection ellipsometry to measure the thicknesses of HPFs. In reflection ellipsometry a beam of polarized light is reflected at an oblique angle from a reflective surface containing the sample. The sample interacts with the polarized light resulting in a change in its polarization. The change in polarization is measured and a computer is used to analyze the data to determine information about the sample. [43]

Fluorescence Microscopy

Fluorescence microscopy is an important tool used in biological research for imaging purposes. In fluorescence microscopy biomolecules, such as proteins, antibodies, and even cells, are labeled with a fluorophore. When the fluorophore is illuminated with light, it absorbs the energy and becomes excited. The fluorophore is designed so that the excess energy is lost through the release of a photon with a longer wavelength. Molecular Probes (Eugene, Oregon) specializes in designing fluorophores that can be used for a wide range of biological applications. There are many advantages to using fluorescence labeling to image biological processes. Fluorophores can be easily conjugated to many molecules and can be used to label materials that cannot be viewed using brightfield microscopy. They are also very specific in the types of molecules that are labeled. Most fluorophores are not detrimental to cells, which permits live cells and cellular processes to be imaged.

Cassette Mutagenesis

The *E. coli* that we plan to seed onto the patterned arrays will have undergone cassette mutagenesis. Cassette mutagenesis is a process whereby a sequence, or cassette, of DNA is synthesized with one or more positions randomly mutated. These mutations produce genes with

one or more varying codons encoding for various amino acids in those positions. [44] The DNA is then reinserted into the cell and the cells are screened to see if the protein retains its activity. [45,46] This is a good technique for determining which portions of the genome are essential. Regions that undergo many substitutions while the proteins retain their activity are usually not essential. Regions that undergo only a few substitutions but yield proteins that are not active are usually essential. [44,45] The gene sequence targeted for mutation in these studies will be the gene encoding for the OPH enzyme. Professor Frank Raushel, at Texas A&M University, has kindly agreed to supply the *E. coli* used in this project.

Preliminary Results

The first step in this project is to prepare patterned arrays of corrals for cell growth. Hyperbranched polymer films (HPFs), which our research group has worked extensively with, will be used to prepare these corrals. [47-50] The procedure involves the formation of a self-assembled monolayer (SAM) of mercaptoundecanoic acid. Poly(*tert*-butyl acrylate) (PTBA) is then grafted onto the monolayer followed by the hydrolysis of the PTBA to form poly(acrylic acid) (PAA). Hyperbranched polymer films are formed through grafting on multiple layers of PAA. [48] The main advantage to using HPFs is that any mistakes in the base monolayer are subsequently corrected by grafting additional layers of polymer. The use of multiple layers of polymer results in a high density of acid endgroups on the HPF. This enables us to subsequently graft PEG onto the polymer in a high enough density to prevent bioadhesion. [18,19]

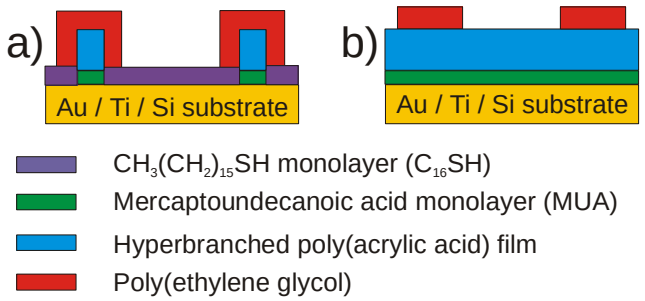


Figure 2: Comparison of Patterns made by a) μCP and b) photoacid-based patterning

We have demonstrated that hyperbranched polymers can be patterned using microcontact printing (μCP)[48,51] and photoacid patterning. [52] The patterns formed contain up to 1000 corrals in the space of only a few millimeter squared. In μCP, an alkanethiol pattern is stamped onto a clean gold wafer. The wafer is then soaked in mercaptoundecanoic acid (MUA), which forms a monolayer in the regions of the wafer that are not occupied by the alkanethiol. Successive layers of PAA followed by PEG can then be grafted onto the MUA resulting in the formation of an array of corrals having hexadecanethiol ($C_{16}SH$) bottoms and sides of PAA capped with PEG (Figure 2a). [51] In photoacid patterning, a thick PTBA polymer film is prepared. Photoacid is then used to hydrolyze selected regions of the PTBA to form PAA. PEG is then grafted onto the PAA and the rest of the PTBA film is hydrolyzed. This results in the formation of an array of corrals with PAA bottoms and sides of PAA capped with PEG (Figure2b). [52] This type of corral has the advantage of having a polymer bottom, which can be further functionalized.

The second step in this project is to be able to grow cells on the pattern and determine if cell growth is indeed contained within the corrals. Although it is our ultimate intent to grow bacterial cells within the corrals, we chose to first work with mammalian cell due to their large size. Mammalian cells are approximately ten times larger than bacterial cells and do not require the use of high magnification objectives that would be necessary to view bacterial cells. [18,19] Previous work from this group demonstrated that mammalian cells could be grown on μCP patterns. [18,19] My preliminary results show that bacterial cells will grow on the pattern and that their growth is confined within the corrals. Figure3a shows a 300-mesh pattern with *E. coli* seeded onto it. The width of the corrals in the pattern is approximately 63 μm and there are approximately 18 cells in each corral. In order to place fewer cells in each corral we have also worked with smaller sized corrals. These patterns were made using a 1500-mesh stamp (the stamp is made using a 1500-mesh transmission electron microscopy (TEM) grid). Figure 3b shows a 1500-mesh pattern that has been seeded with *E. coli*. The width of the corrals is approximately 12 μm and there are approximately 4 cells in each corral.

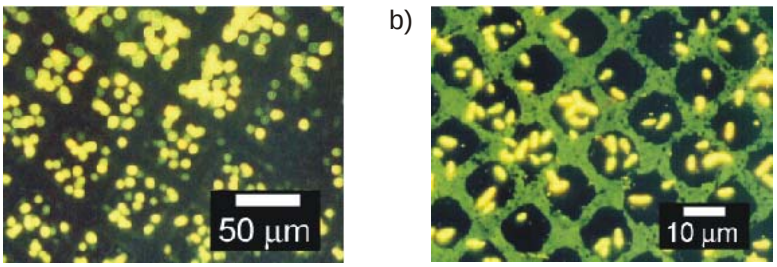


Figure 3: *E. coli* grown on a a) 300-mesh pattern and b) 1500-mesh pattern. *E. coli* imaged using Molecular Probes *Bac*Light LIVE/DEAD assay kit.

This project requires that the bottom of the corrals be functionalized through the incorporation of a pH-sensitive dye. It is not possible to do this with patterns prepared by μCP since they have an unreactive alkanethiol bottom. An alternative to μCP is photoacid-based patterning. Patterns formed by photoacid-based patterning result in corrals that have PAA bottoms and sides of PAA capped with PEG. [52] Further functionalization of the corral's polymer bottom is now possible. We have been able to grow both mammalian cells and bacterial cells on patterns generated using photoacid patterning. [53]

Proposed Research

I propose to fabricate a biosensor using a combination of microcontact printing (μCP) and microfluidics that can be used in the detection and detoxification of organophosphates. It will consist of a microfluidic channel containing a patterned array of *E. coli* that have been genetically modified using cassette mutagenesis. This organophosphate biosensor will be used for the HTS of a combinatorial library of genetically modified *E. coli*. The first step is to determine if a pH change can be observed using this design. The second step is to then determine if the organophosphate can enter the cell and produce a localized pH change, and if this pH change is observable. The third step is to perform the experiment on a patterned surface to determine if the contents from one corral effect neighboring corrals, and if the fluorescence from one corral can be distinguished from the fluorescence of neighboring corrals. Once we start working with patterned surfaces, we will also need to determine if it is possible to grow

cells on the pattern using the microfluidic device. The final step in this project involves the actual fabrication of the microfluidic system shown schematically in Figure 1.

Observation of pH Change

The first step in this research is to determine whether a pH change can be detected using the design shown in Figure 1. Before we start working in a microfluidic channel or on patterned surfaces, we are going to use an unpatterned wafer in a macroscopic flow cell. Figure 4 shows a simplified flow cell that we are going to fabricate and use for the start of this project. It consists of PDMS molded so that it contains a place for a 1.0 x 0.5 inch wafer with a small space above the wafer forming the channel. Two Teflon tubes inserted through the PDMS provide an inert path for fluid to flow through the channel. A glass cover slip is bonded to the PDMS to enclose the channel and also allows us to view the wafer.

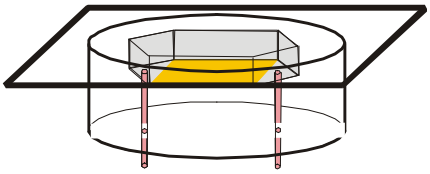


Figure 4: Flow Cell

The substrate that we will use for this experiment is an unpatterned 3 PAA coated gold wafer with SNAFL (Molecular Probes, seminaphthofluorescein) electrostatically incorporated within the polymer. The *E. coli* are going to be grown directly onto the surface of the polymer. I plan to simulate the protons that will be released from the *E. coli* by manually changing the pH using a dilute acid solution. We will be able to monitor the change in pH as a change in the SNAFL intensity using a fluorescence microscope.

Before we determine if we can see a pH change by fluorescence, we have to optimize the buffer being used. The purpose of the buffer in the system is to provide the essential nutrients to keep the bacterial cells alive on the wafer. The problem with the buffer is that it counteracts the pH changes resulting from hydrolysis. Therefore, the buffer concentration must be optimized so that the essential nutrients are provided without preventing localized pH changes. I plan to optimize the buffer by first monitoring the pH with a pH meter at the output of the channel. Once the buffer concentration is optimized, I can then determine if a pH change can be observed by fluorescence using SNAFL.

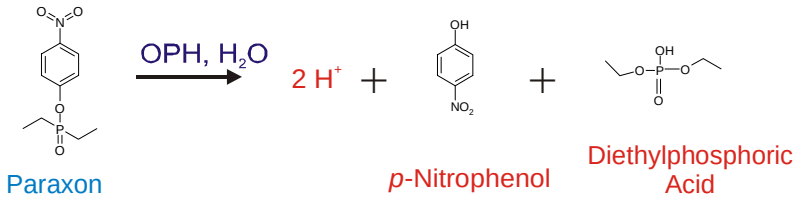
The buffer that I plan to use is a 0.1 mM phosphate buffer. The buffering capacity of this buffer requires the addition of approximately 5 μM of protons to yield a 0.01 pH change. The average bacterial cell is capable of producing 0.5 pg of OPH with an average enzyme activity of 18,000 U/mg. This results in the release of $3x10^{-7}$ μM/sec of protons from a typical bacterial cell under substrate limiting conditions. Even if only 1/3 of the protons produced reach the bottom of the corral, they will be collected in a polymer volume of 6 fL. The resulting concentration of protons in the bottom of the corral is approximately 17 mM. This concentration of protons is much larger than the concentration required to change the pH by 0.01 units.

Organophosphate Hydrolysis

If the previous experiment works, we will know that the SNAFL incorporated in the polymer is sufficient to detect pH changes on the same order of magnitude that the cells will release upon exposure to organophosphates. The next step in the experiment is to determine if the actual hydrolysis of organophosphates by *E. coli* can be observed using SNAFL. For this experiment we will be using the same setup used previously. The wafer inside the flow cell will

contain an unpatterned HPF seeded with *E. coli*. Organophosphates will then be flowed through the channel to be hydrolyzed by the *E. coli* resulting in the release of protons from the bacterial cells. The localized pH change will then be observed using the pH sensitive dye, SNAFL, located on the bottom of the corral.

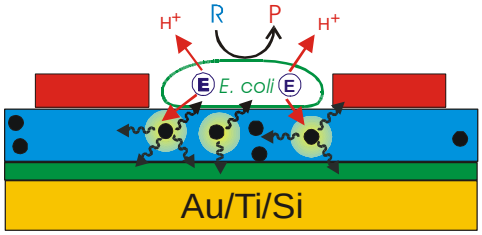
At this point it is also necessary to examine the rate that organophosphates can be hydrolyzed using whole bacterial cells. I propose to do this using the organophosphate paraoxon. Paraoxon is hydrolyzed by OPH by the following reaction.



The production of the product, *p*-nitrophenol can be directly measured by measuring its absorbance at 400 nm (E_{400} = 17 mM^{-1} cm^{-1}. [25] By measuring the production of *p*-nitrophenol we are directly measuring the hydrolysis rate of the *E. coli*.

Examination of Patterned Surfaces

Now that we know it is possible to detect pH changes on a macroscopic scale using SNAFL incorporated into the polymer, it is time to incorporate patterned surfaces onto our wafer in the flow cell. We will use the setup described in Figure 4 containing a wafer with an array of 25 corrals seeded with *E. coli*. Figure 5 shows a diagram of one of the corrals of the pattern. Photoacid-based patterning will be used to pattern the wafer resulting in the corrals having a PAA polymer bottom and PAA capped with PEG sides. A pH-sensitive fluorescent dye, SNAFL, will be electrostatically incorporated into the bottoms of the corrals. *E. coli* will then hydrolyze the organophosphates resulting in the release of two protons, which are subsequently released from the bacterial cell. These protons will change the local pH resulting in the increase in fluorescence intensity.



Escherichia coli: E. coli

●: Seminapthofluoresceins: SNAFL (Molecular Probes)

R: Paraoxon

E: Organophosphorus Hydrolase: OPH

P: Diethylphosphoric Acid, *p*-Nitrophenol

H^+: Protons

Mercaptoundecanoic acid monolayer (MUA)

Hyperbranched poly(acrylic acid) film

Poly(ethylene glycol)

Figure 5: Organophosphate Hydrolysis

First, we need to determine if the protons in one corral will leak into neighboring corrals. This can be done by killing the bacterial cells within a single corral and then flowing organophosphates through the channel. If the corral containing the dead cells fluoresces, we will know that we have leakage. Leakage may occur by the protons traveling directly through the

walls of the corrals or more likely they will travel over the top of the walls into neighboring corrals.

We also need to determine if the fluorescence in one corral can be differentiated from the fluorescence in another corral. One method for testing this is to work outside of the flow cell. Buffers of two different pHs can be micropipetted into neighboring corrals. Another way to test this is to work with a batch of *E. coli* that have actually undergone cassette mutagenesis. So far we have been working with only one type of *E. coli*. Since the OPH enzyme will be slightly different in each corral, the fluorescence intensity should also vary between the corrals.

Seeding *E. coli* onto Patterned Surfaces

The next step in this project is to determine if the flow cell, and later the microfluidic channel, can be used to seed the wafer with *E. coli*. The first step is to introduce bacterial cells into the channel by flowing through a buffer containing the bacterial cells. The buffer flow is then stopped for a period of time so that the bacterial cells have a chance to adhere to the pattern. The amount of time that the flow is stopped depends on the initial cell density of the buffer and the seeding density desired on the pattern. The channel is then rinsed with buffer to remove any cells not attached to the pattern. The microfluidic system should now be ready to use for organophosphate detection. A few experiments must be done to characterize the system. The correlation between the amount of time the flow is stopped, the initial seeding density, and how many cells are on the pattern needs to be examined. Then, depending on the initial cell density on the wafer, it needs to be determined approximately how long the cells will live on the wafer once it is seeded. This depends on the amount of room the cells have left to grow, as once the cells run out of room, they will die.

Fabrication of Microfluidic Device

The ultimate goal of this project is to fabricate a microfluidic organophosphate biosensor. This will involve first fabricating a microfluidic channel and patterning a polymer array inside the channel. Figure 1 shows a diagram of the microfluidic device we plan to fabricate. A gold channel can be chemically etched from a gold wafer and a corresponding channel can be molded into a piece of PDMS. The gold channel on the silicon wafer can then be patterned using photoacid patterning. The PDMS can then be bonded to the silicon surface of the wafer so that the gold channel aligns with the channel formed in the PDMS. It has been shown by Whitesides et al. that oxidized PDMS can bond to silicon. This bonding is the result of the covalent bridging of the silanol groups on the surfaces of the oxidized PDMS and silicon. [54] Once the patterned channel is formed, the rest of the channel needs to be passivated in order to prevent bioadhesion in areas besides the pattern. The wafer can be seeded with *E. coli* following the above procedure. The microfluidic device should now be ready for organophosphate detection.

Summary and Conclusion

The objective of my research is to fabricate a microfluidic organophosphate biosensor that can be used in the HTS of a combinatorial library of genetically modified *E. coli*. This device operates on the principle that *E. coli* are capable of expressing OPH, which is capable of hydrolyzing organophosphates. The *E. coli* have undergone genetic modification of this OPH gene resulting in a combinatorial library of enzymes being expressed. These enzymes will hydrolyze organophosphates at different rates resulting in a different localized pH around each bacterial cell. The enzyme that is the most efficient will hydrolyze the most organophosphates

resulting in a larger pH change. This large pH change is detected through the increased fluorescence of the pH-sensitive dye incorporated in the bottom of the corral. The *E. coli* producing these enzymes can then be collected to have their DNA sequenced. In this way it is possible to directly correlate OPH enzymatic activity and genetic sequence.

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