I. INTRODUCTION

Two current trends in the complex and highly diverse field of analytical chemistry are miniaturization of instruments and techniques, and the development of techniques that will accommodate biologically relevant samples. These trends are manifested in the rapidly developing field of microfluidics. Microfluidic devices are in many ways the chemical equivalent of the printed circuit board. That is, after the boom in microfabrication techniques developed for the electronics industry, the same methods were adapted to moving liquids, rather than electrons, around a 'chip'. What began as a way to carry out microscale separations[1] now has applications including drug screening, microreactors for PCR (polymerase chain reaction)[2,3] and enzymatic reactions,[4] and biochemical assays.[5] All of these applications have proven to benefit from the reduced size of the device components, because with reduced size comes the use of less reagents and faster analysis times.

The broad goal of my project is to fabricate a polymer-based fluorescence biosensor within a microfluidic device that will act as a high throughput screening (HTS) device for genetically modified cells. Currently, there are large scale devices available commercially to do HTS with whole cells, primarily targeting the drug screening market. Two companies that are very active in this area are Cellomics and Molecular Devices. Each company uses a detection scheme based on a different sensing principle. Molecular Devices uses a light addressable potentiometric sensor (LAPS).[6,7] In the Cytosensor®, mammalian or bacterial cells are cultured within a specially designed porous cell capsule. The capsule is enclosed inside a sensor chamber that is made of silicon nitride (Si_3N_4). This system monitors the cells respiration rate by determining the extracellular acidification rate via an electrochemical route. The change in pH causes a change in the surface charge of the Si_3N_4. A voltage is applied across the Si_3N_4 and it is addressed with a light pulse. The light pulse creates a photocurrent that is dependent upon the charge on the surface of the Si_3N_4 and hence the pH. Cellomics is the other company using cells as a sensor in their Array Scan™.[8] Cellomics, uses a fluorescence based detection scheme in which dyes are placed within the cells and the cells are cultured in microwell plates. The cells responses to stimuli are monitored optically via an objective lens and a CCD camera. The image is then processed digitally to correlate the fluorescence data with the cellular response in each of the microwells. Cellomics is also working in collaboration with ACLARA Biosciences on producing CellChip™, a microfluidics-based whole cell biosensor.[9]

In comparison to the systems described above there are two distinct advantages of the system I am proposing to construct. First, the previously described systems rely on the average response of a large number of cells, but because the proposed biosensor confines cells within micron-scale corrals it will be possible to interrogate individual cells. This is an advantage because we will be using a combinatorial library composed of genetically modified cells and each cell's response to organophosphate compounds will be different and need to be independently monitored. In the systems described above a library of compounds, usually drugs, are screened against a single cell line, so collecting an average signal is not a problem. Second, we will not have to label the cells with fluorescent dyes that can interfere with normal cell function; we will instead label the polymeric support.

This project involves two very distinct individual components. The first concerns preparation and handling of biological materials and the second involves the analytical aspects of the microfluidic system and detection within these systems. My dissertation

studies focus on the latter aspect of this project, and my colleague Brooke Kocsis will handle the former. Accordingly, the primary focuses of my research proposal are as follows:

1) Fluorescence detection of chemical processes, such as pH changes, in polymer films.
2) Design and fabrication of microfluidic devices.
3) Incorporation of hyperbranched polymer technology into these microfluidic devices.

II. BACKGROUND

Fabrication of Microfluidic Devices

Many different approaches have been used for the fabrication of microfluidic devices. The traditional method of fabricating microfluidic devices involves the preparation of a mask using standard photolithographic methods followed by selective etching of the substrate.[10,11] This approach has been used for fabricating silicon, quartz and glass devices.[11] For example, Figure 1 shows the steps required to prepare a microfluidic device based on a silicon substrate. First, the substrate is spin-coated with layer of positive photoresist and the photoresist is then exposed to UV light through a photomask. The resist is then developed causing the unexposed regions to be dissolved away, leaving the desired pattern. The substrate is then exposed to a wet chemical etch which will etch away the substrate in the regions without photoresist. To enclose the open channel that results from lithography, another substrate must be bonded using high temperature and voltage procedures and very planar substrates. There are many drawbacks to this methodology. Specifically, these methods are expensive and it is time consuming to change channel designs. In addition, the channels formed have low aspect ratios and the channel walls are not perpendicular to the surface limiting the size range of possible channels and the characterizability of flow through the channels, respectively.

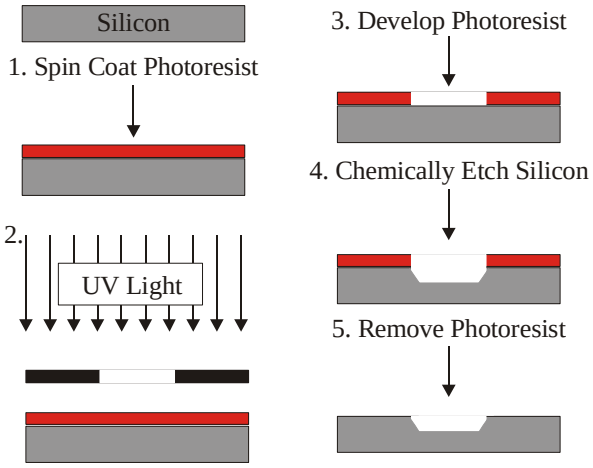


Figure 1: Fabrication of channels in silicon

Recently, there has been a move in the field of microfluidics to polymer-based devices, because they are cheaper and usually easier to produce.[12,13] Polymers that are commonly used include polyamide, polycarbonate, polyethylene, poly(methyl methacrylate) and polystyrene.[14] Methods to produce polymer-based microfluidic devices often involves the use of photolithography at some point in the process to form a master (that is, a durable template of the desired pattern) but then multiple replicas can be made from that one master. Techniques for fabricating the polymer replicas include laser ablation, casting, hot embossing, and injection molding.[13,14]

Hyperbranched Polymers

Hyperbranched polymers are a class of materials that have the unique property of a highly branched structure. This gives rise to a large number of end or reactive groups per chain and makes them useful for a variety of applications including coatings, catalysis and as platforms for chemical sensors.[15] Our group, along with Dr. Bergbreiter's research group, have developed a method of fabricating these hyperbranched polymers on surfaces by first forming a monolayer of mercaptoundecanoic acid (MUA) followed by sequential grafting of amine-terminated poly (*tert*-butyl acrylate) (PTBA) and its subsequent hydrolysis to poly(acrylic acid) (PAA). PAA has multiple carboxylic acid groups on each polymer chain, which have the potential to undergo further grafting by PTBA in the subsequent cycles. The films that are formed in this way grow in a non-linear fashion leading to thick polymer films (50-100 nm) in just a few steps. The biggest advantage is that the polymers films are bound to the substrate, in this case a gold wafer, by gold-thiol interaction. This anchors them to the surface and delamination is avoided. In addition, the hyberbranched polymer films can be easily modified covalently[16,17] or electrostatically.[17,18]

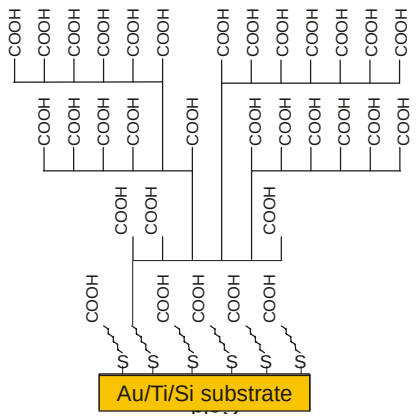


Figure 2: 3PAA hyperbranched polymer film

Polymer Patterning

Microcontact printing (μ-CP) uses an elastomeric stamp made from polydimethylsiloxane (PDMS) to transfer chemical ink onto a surface. The ink is typically a thiol that can be stamped onto a gold surface forming a monolayer of the thiol in the stamped regions. This creates a pattern of the stamped thiol and bare gold. The gold region can then be further modified with another monolayer or multilayer to create a surface pattern of two chemical species.

Our group, along with Dr. Bergreiter's research group, has recently combined the ideas of μ-CP and hyperbranched polymers to prepare patterned polymer films. By stamping a gold substrate with hexadecane thiol (C16SH) and then soaking the stamped substrate in MUA we create a pattern that has chemically reactive end groups (MUA) in predefined areas and non-reactive groups (C16SH) passivating the rest of the surface. The stamped region containing carboxylic acid end groups can then be activated and chemically modified with PTBA. This yields robust patterns of PTBA or PAA and C16SH. These patterns have been used as a template for controlling cell growth on gold substrates.[19] In comparison to other stamping techniques, using hyperbranched polymers is unique since we can build up a thick polymer layers in the pattern.

Photoacid patterning is another technique that we have developed to pattern hyperbranched polymers. Photoacids are compounds that release protons upon irradiation. These compounds have found uses in industry as photoresist amplifiers and as photoinitiators for cationic polymerization.[20] In the context of patterning polymers, we use the photoacid to hydrolyze the PTBA in regions which have been exposed to a UV light source through a photomask. The pattern created from this technique is a combination of two hyperbranched polymers, PTBA and PAA. By using the chemical functionality of the hyperbranched

polymers we have been able to covalently graft multiple dyes to the patterned surface.[21] Husemann et al. have done similar work using a photoacid with a spin-coated layer of PTBA. The purpose of their work was to show a modification of surface properties such as hydrophobicity.[22]

III. TECHNIQUES

Fluorescence Microscopy

Fluorescence microscopy is a technique that allows non-destructive visualization objects down to a lateral resolution of 0.2 μm. It uses conventional optical microscopy instrumentation to image fluorescence emission from labeled objects. Fluorescence microscopy has become a valuable technique in biological imaging because it allows a researcher to obtain visual information about location and chemical concentrations using ion-specific fluorescent dyes.

Fluorescence Microscope/Spectrometer Apparatus

The detection system for the proposed studies will be composed of an inverted epi-fluorescence microscope and a spectrometer. The setup is illustrated in Figure 3. In an epi-fluorescence microscope the objective delivers and collects light from the sample. After passing through an excitation filter, the sample is illuminated by light from the objective. The resulting fluorescence is collected by the objective and passed through an emission filter that eliminates any stray source of light. One of two detectors, the imaging CCD or the spectrograph, can then collect the fluorescence. This allows for the imaging of the surface of the wafer as well as sampling of its fluorescence emission spectrum. An image of the entire surface can be obtained by using a low magnification objective, or by focusing in on a small region of interest with a higher magnification objective, it is possible to obtain the emission spectra of the dye in that region.

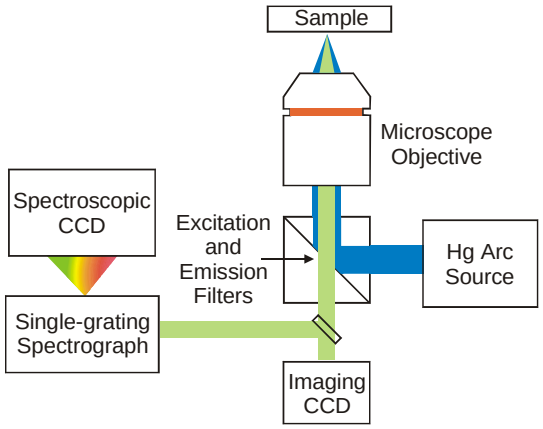


Figure 3: Fluorescence miroscope/spectrometer

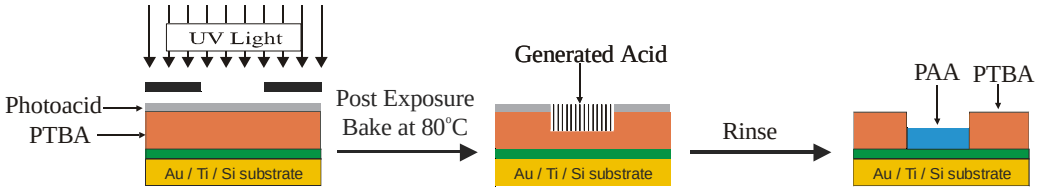
Atomic Force Microscopy

Atomic Force Microscopy (AFM) is a scanning probe technique that can be used to obtain topographical maps of conducting or non-conducting samples. The tip is brought into contact or close proximity to the surface and may be scanned across the surface with the use of a piezoelectric scanner coupled to feedback electronics. An AFM can be operated in two different modes: contact and tapping. In contact mode the height information is gathered by

measuring the force of the tip-sample interaction. In tapping mode the height information is obtained by measuring the changes in oscillation frequency of the tip.

IV. PRELIMINARY RESULTS

My preliminary work has focused on the photoacid patterning of hyperbranched polymer surfaces. The process of photoacid patterning is outlined in Scheme 1. First, a three layer PTBA film is prepared. The polymer film is then coated with a layer of photoacid (triarylsulfonium hexafluoroantimonate) and irradiated with UV light through a photomask. The photoacid generates protons upon irradiation. The wafer is then heated in a post exposure bake (PEB) step allowing protons that were generated to diffuse through the polymer film. These protons hydrolyze the *t*-butyl ester groups of PTBA, converting it to PAA.



Scheme 1. Photoacid patterning procedure

Photoacid patterning is an important aspect of my research because it affords us a chemically flexible patterned surface (Figure 4). Although the patterned surfaces prepared by μ-CP have many valuable characteristics, the parts of the pattern that are C16SH are dead ends chemically. That is, no further chemical modification can be carried out in these regions. Using photoacid patterning we can create a situation where the regions that were previously C16SH are now PAA. PAA is a very robust and versatile scaffold, for expamle, it can be functionalized with dyes[16,21] and by electrostatic immobilization of enzymes.[18]

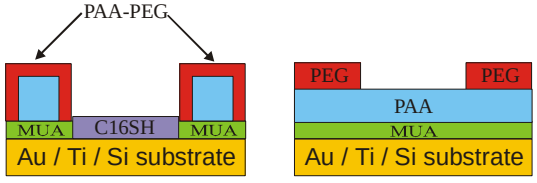


Figure 4: μ-CP surface vs photoacid patterned surface

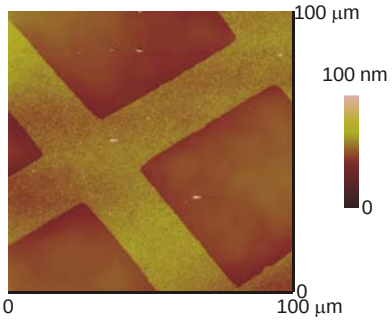


Figure 5: AFM of photoacid pattern (PAA boxes and PTBA lines)

I have characterized the photoacid patterned film surfaces using AFM to assess the ability of a photoacid to pattern hyperbranched polymers. The results of this study shows that uniform hydrolysis of PTBA to PAA occurs and that photoacid pattering is a viable route (Figure 5). In addidion, the difference between the heights of the boxes and lines corresponds to the expected thickness changes for bulk PTBA films.[23] AFM has also been performed on photoacid patterns that have been further modified with poly(ethylene glycol) (PEG), a polymer known to resist cell adhesion and growth.[24]

We have previously shown that μ-CP of hyperbranched polymer patterns is a viable method for controlling and directing the growth of mammalian cells.[19] Preliminary results indicate that photoacid patterning should also be a suitable method for controlling the growth

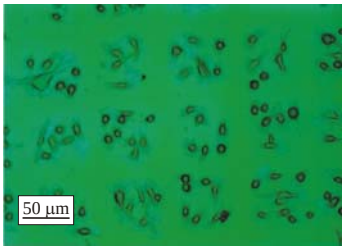


Figure 6: Murine macrophage cells on photoacid patterned surface.
(PAA corrals/PAA-PEG walls)

of cells as well. Figure 6 shows macrophage cells grown on a photoacid pattern consisting of PAA corrals and PAA-PEG walls. The cells in the corrals are extended and the lamellapodia are also in extended positions suggesting that the cells are viable and growing on the surface.

In addition to showing that cells will grow on these photoacid patterns, I have also shown that a pH-sensitive dye incorporated into a photoacid-patterned hyperbranched polymer can be used as a pH-sensitive detector. This provides the possibility of having an individual sensor in each corral. This experiment was carried out by labeling generation four poly(amidoamine) dendrimers (G4) with seminapthfluorescein (SNAFL), and selectively binding it within the PAA regions of the polymer film pattern (Figure 7). The G4-SNAFL remains entrapped within the polymer film at pH > 4.5. This is supported by previous work which shows that G4 dendrimers sorb into PAA at any pH > 4.3 and will be released only upon soaking in a solution of pH < 2.[17] This is well within the working range that will be expected when using biologically relevant samples.

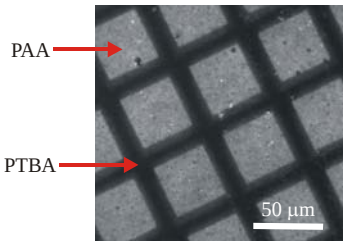


Figure 7: Selective fluorescent labeling of PTBA/PAA pattern

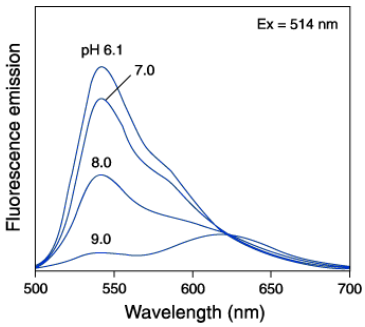


Figure 8: Fluorescence spectra of SNAFL. (Molecular Probes, Inc.)

SNAFL was chosen for this work because it is sensitive in the pH range from 6.8-8.3 and is a ratiometric dye. As seen in Figure 8, it experiences a significant decrease in emission intensity at 545 nm while the emission at 635 nm remains constant. The emission at 635 nm behaves as an internal standard that eliminates some of the problems associated with fluorescence such as photobleaching and variable dye concentrations.

Finally, I have shown that it is possible to dose individual corrals with a reagent. This technique could be used to address individual or small groups of cells within individual boxes with nutrients, enzymes, or toxins. This could be important in monitoring the reponses of similar cells to different stimuli. Dosing of individual boxes was accomplished by using a PicoPump™ (WPI, Inc. FL). Figure 9 shows an individual corral (62 µm x 62 µm) that has been dosed with G4-SNAFL. The pattern shows no indication of cross talk between corrals in this experiment. This effect is driven by the differences in hydrophobicity of different parts of the pattern. That is, the walls are PTBA, which is hydrophobic, while the bottoms of the boxes are PAA and are hydrophilic.

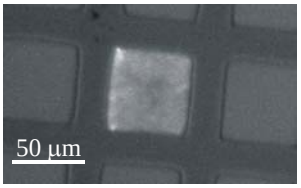


Figure 9: Individual corral dosed with G4-SNAFL

V. PROPOSED RESEARCH

I propose that hyperbranched polymer films with electrostatically bound dyes can be used as fluorescent sensors to monitor the biological responses of whole cells within

microfluidic devices. Specifically, I plan to investigate the sensing capability of dye-labeled hyperbranched polymer films confined to a surface. I also plan to fabricate microfluidic devices using different design rules to accommodate various experiments. Finally, I plan to merge the hyperbranched polymer fluorescence sensors and the microfluidics devices to create a organophosphate biosensor.

To investigate the pH sensing properties of hyperbranched polymer films labeled with G4-SNAFL, I plan to start with homogeneous PAA films that have been soaked in a solution of G4-SNAFL. I have shown in preliminary results that fluorescent dyes attached to dendrimers, such as G4-SNAFL, will electrostatically bind within PAA and remain entrapped there until very acidic conditions (<pH 2) are reached. The goal is to be able to detect pH changes in the bulk solution via the fluorescence response of SNAFL incorporated within the polymer film. A benefit to using PAA is that it is sensitive to its surroundings; for example it triples in thickness when put into acidic media.[17] The polymer film acts as a proton sponge that brings the protons into close proximity with the dye, hopefully allowing an increased ability to detect changes in the fluorescence emission of G4-SNAFL. To carry out the bulk PAA film experiments in *situ*, a PDMS flow cell (Figure 10) has been designed. Using a series of solutions of known pH, the ratio of the two peaks at 545 nm and 635 nm can be used to make a calibration curve of emission as a function of pH. The calibration curve can then be used to determine the pH of a sample of unknown pH. This will determine if pH changes can be detected via the polymer film.

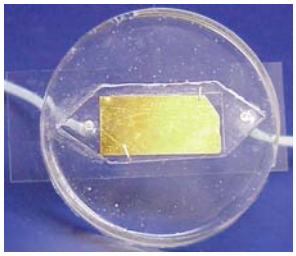
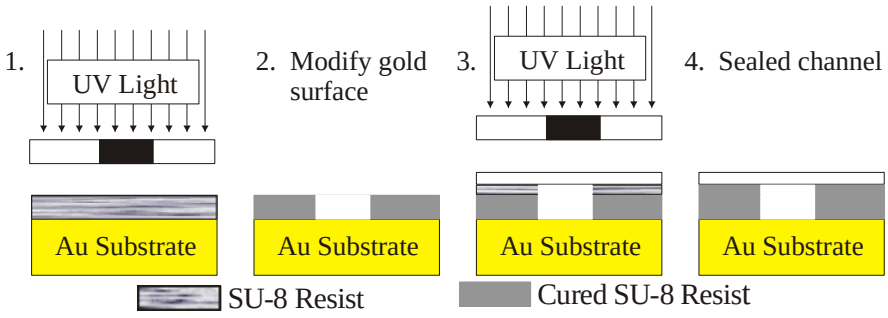


Figure 10: PDMS Flow cell

Once these experiments are working in the bulk form, I will incorporate the polymeric sensor films into microfluidic devices. There are three main reasons one would choose to use microfluidic systems in this case. The first reason is the reduced consumption of solvents and analytes. For example, in the final stages of this project we plan to use nerve agent simulants as analytes. These compounds can be harmful to humans and therefore limiting the amount used will limit the potential exposure. Additionally, the analytes will be contained within the microfluidic system. The second reason is with the reduced size of the apparatus, imaging and detection should be easier and faster. The distance between the detector (the microscope objective) and the sample will be minimized giving greater working ranges to the microscope and enhancing our ability to use higher magnification objectives. Finally, in the future it may be desirable to integrate our HTS approach with other analytical techniques. The microfluidic platform is ideal for this integration.

There are two key issues in the development and fabrication of the microfluidic devices. First, we must be able to visualize the polymer surface through the microchannel. Accordingly, the top or cover of the channel must be transparent at the wavelength of interest. Second, the chemistry of hyperbranched polymers has been developed using gold as the substrate. This means that we will need to fabricate our devices out of a materials that will be compatible with gold. If using gold as the substrate presents problems, self-assembled monolayer chemistry of silanes provides a route to using glass and silicon as substrates.[25] Silanes have also been used in conjunction with μ-CP.[26]

As discussed above the choice of materials to fabricate our microfluidic devices is very important. I have chosen to use NANO™ XP SU-8 as the material to use in the fabrication of our microfluidic channels. SU-8 is a low-cost epoxy-based negative photoresist that allows high aspect ratio patterns to be made. SU-8 is a chemically robust epoxy that is inert to most solvents and chemical etches.[27] It is typically used as a photoresist in photolithography, but it has also been used for the fabrication of microreactors,[4] MEMS,[28] and microfluidic channels.[29,30] Because SU-8 is a negative photoresist the unexposed regions are rinsed away during development. This allows the build-up of several layers upon one



Scheme 2. Fabrication of microfluidic device

another to create complex patterns. Scheme 2 shows how SU-8 will be used for the fabrication of microchannels. A gold wafer can be spin-coated with SU-8 having a thickness ranging from 5 μm to 500 μm. The SU-8 crosslinks upon exposure to UV radiation and forms a rigid photopolymer in the exposed regions (Step 1). After development the gold substrate will be exposed and ready for the hyperbranched polymer chemistry (Step 2). Preliminary results show that the SU-8 will be able withstand the conditions necessary for the synthesis of hyperbranched polymers. Following polymer modification of the gold substrate the channel can then be sealed using SU-8 as a sealant.[29] SU-8 will be applied to the already cured SU-8 and a coverslip put in place forming a channel (Step 3). Exposing the entire device to UV light will photopolymerize the sealant SU-8 forming a sealed channel (Step 4).

To test the imaging and spectroscopic capabilities of our system within the microfluidic devices, the initial devices will have a simple Y-shaped channel with dimensions of 20 μm x 50 μm. In channels of this dimension, laminar flow will occur within the channel. This means that multiple fluids can be flowed through the channel and they will only mix at the interface due to diffusion. Therefore, two solutions of different pH should remain distinct from one another within the channel. Being able to detect this will conceptually show that we can distinguish different pHs within a confined space. In addition, due to the diffusional mixing at the interface, it should be possible to detect a third region as well. Being able to detect this third region should give us an idea of the sensitivity we will be able to achieve by incrementally changing the incoming pHs to narrower and narrower ranges.

Incorporating hyperbranched polymer patterns into the channels is going to require a different device design. There are two possible avenues to pursue depending upon the number of corrals needed. To screen large numbers of corrals the polymer patterns that we make are 5 mm across. A new channel design will need to be developed that will accommodate the large patterned region. A design like the one shown in Figure 11 will possess the desired requirements. The 100 μm channel will open up into a circular region to accommodate the polymer pattern. The alternative is to pattern a smaller number of corrals

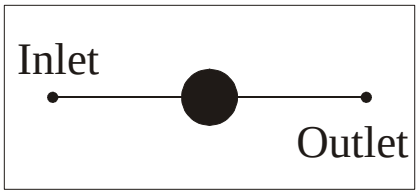


Figure 11: Design of microfluidic device with hyperbranched polymer

within a 100 μm channel. In either case the channel will have a low aspect ratio, this is one of the reasons that PDMS was not the polymer of choice for the fabrication of the microfluidic devices. Since PDMS is a soft flexible polymer the middle of the large circular region or the wider channel would cave in, thereby destroying the channel. SU-8 has a much more rigid consistency that will support a low-aspect-ratio structure.

To demonstrate that the hyperbranched polymer pattern will be able to function as an array of individual biosensors, it is necessary to demonstrate that the individuals corrals will not cross contaminate one another. By using the PicoPump, I can individually dose corrals with different ion-sensitive dyes. Flowing solutions of the ions of interest through the channel will cause the corrals specific to those ions to fluoresce. Since we have a patterned surface, the boxes that have been dosed will have an address and we can determine if the appropriate corrals are responding to the correct ions. If the boxes for the ion are the only regions to fluoresce, we will know that there has been no cross talk between corrals. If there is cross talk the corrals surrounding the dosed corral will also fluoresce. Should cross talk become a problem, we can modify the patterned regions to include wider walls, thereby separating the corrals by a further distance. In this set up, the minimum concentration of ion in the solution that can be detected can also be determined, assessing the sensitivity of the system. In addition, by performing this experiment we will be able to demonstrate that the polymer biosensor would be applicable to ions other than protons. For example, fluoride ion detection would be of interest if a chemical warfare agent were being used as the analyte.

It is important to note that each time the device geometry changes, a new mask for photolithography will be needed. The method used to fabricate the masks will be a transparency based system, where the desired pattern is designed on a computer drawing application and printed out. This printout is then photographed using high quality film and the negative is then used as the mask. This technique allows for rapid fabrication and easy changes of master designs. The same techniques can be used to design the masks for the photoacid patterning of hyperbranched polymers.

The culmination of this project is to employ the use of a patterned hyperbranched polymer film fluorescence sensor modified microfluidic devices for detection of organophosphate with live cells. The design of the microfluidic device for this task will need to take in to consideration the size of the cells; mammalian (10 μm) and bacterial (1μm) as the channel needs to have a large enough cross section so that the channels do not become clogged with cells. Once the cells are grown within the microchannel a Live/Dead assay can be performed to determine if the cells are viable under the conditions of the channel. A Live/Dead assay is a staining kit available from Molecular Probes consisting of SYTO10 and DEAD Red. DEAD Red will only enter dead cells while SYTO10 will enter live and dead cells. However, when doing the fluorescence assay the DEAD Red in the dead cells will overwhelm the signal and they will appear red. The live cells will appear green. This way we can determine if the cells are viable with in the channels.

The cells that will be used are going to be genetically modified to respond with different efficiencies to a given analyte; in this case organophosphates. The cells respond by breaking down the organophosphate, and as a result release protons. These protons are the species that will be monitored by the polymer fluorescence sensors in the individual corrals.

The fluorescence response of the corrals can be monitored via two methods of detection. The first is to use low magnification objectives and look at the entire pattern. Using this method would depend upon the eye or the CCD to determine which of the corrals are fluorescing the brightest. The other, more accurate, way of identifying the most highly fluorescent corrals is to use a high magnification objective to magnify the individual corrals and then spectroscopically monitor the differences in the pH using SNAFL's ratiometric properties. This will determine which corral contains the cell that has the most efficient enzyme. These cells can then be recovered from the pattern, as they will have a known position in the pattern, and can then be sequenced to determine the mutations that have been most effective.

VI. SUMMARY AND CONCLUSION

I have proposed the use of patterned hyperbranched polymer films modified with dyes as an array of fluorescence biosensors whose detection is performed on an epi-fluorescence inverted microscope which allows the optical visualization as well as the spectroscopic characterization within microfluidic devices. The design and fabrication of the microfluidic devices for the project has been discussed. In addition I have proposed a series of studies that will test the system for its sensitivity, selectivity and response. The incorporation of these polymer films and the growth of living cells in microfluidic devices have also been proposed.

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