pH-Sensitive Resist Materials for Combined Photolithographic Patterning of Proteins and Fluid Lipid Bilayers
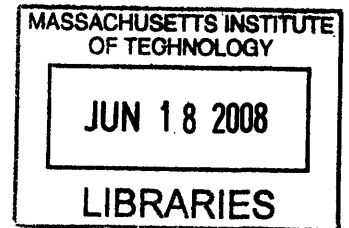
by

Mirat Shah

Submitted to the Department of Materials Science and Engineering in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science

At the Massachusetts Institute of Technology

June 2008

ARCHIVE

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Department of Materials Science and Engineering
June 2008

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ABSTRACT

Photolithography of a pH-sensitive photoresist polymer was performed to pattern both lipid bilayers and proteins onto the same surface. The motivation behind this was to create a substrate mimicking an array of antigen- presenting cells. The substrate would consist of signaling ligand, biotin anti- CD3, bound to a lipid bilayer in a regular array of patches. The fluidity of the lipid bilayer would impart mobility to the signaling ligand.

It was found that under appropriate substrate fabrication conditions, lipid bilayers and their associated ligand do segregate to the desired signaling patches. Additionally, the bilayer in these regions is fluid, and is potentially bioactive. This bodes well for our system as a future platform to study the actions of the helper T cell and antigen- presenting cell at the immunological synapse.

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Introduction

The immunological synapse is the junction which forms between an antigen-presenting cell and a helper T cell. When a foreign species invades the body, it is chewed up and its peptide fragments are presented on the surface of an antigen-presenting cell (APC). The APC uses molecules of the major histocompatibility complex (MHC) for this display. To actually generate the intracellular and extracellular signaling associated with the immune response, a helper T cell not only has to "see" the foreign fragments. It must then physically interact with the peptide-MHC complex on the surface of an APC. When this physical interaction takes place, the immunological synapse (IS) is formed (Figure 1) [1].
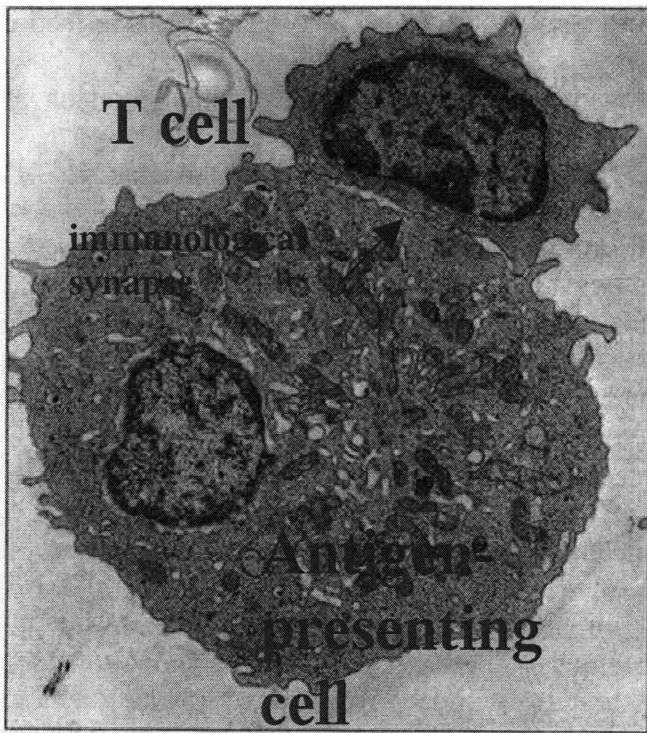

[2]

Figure 1: The physical interaction between a T cell and an antigen-presenting cell. The region of contact between the two is the immunological synapse.

Two different types of interactions take place between the T cell and the APC in the immunological synapse- adhesion and signaling. Adhesion allows the cells to stick together so that signaling can occur, and signaling actually stimulates the immune response. The APC presents ICAM-1 as its main adhesion ligand and peptide-MHC as its main signaling ligand. The T cell uses LFA-1 receptors to interact with ICAM-1 for adhesion, and T cell receptors (TCRs) and associated complexes to interact with peptide-MHC for signaling [3].

The immunological synapse contains two distinct regions which together resemble a bulls-eye. These regions are the central supramolecular activating cluster (cSMAC) and the peripheral supramolecular activating cluster (pSMAC). In the early stages of its formation, the adhesion region of the synapse is contained in the cSMAC and the signaling region is contained in the pSMAC. However, this entire structure is dynamic with the ligand-receptor complexes migrating from their starting locations. The adhesion and signaling regions swap places and once in its mature stage, the immunological synapse resembles an even tighter bulls-eye, with the signaling region now in the middle, and an adhesion field all around (Figures 2a, 2b). Even in these later stages, with the characteristic signal in the center and adhesion region on the outside, the synapse is not static [4].

a.

[4]

b.

	T cell	APC
cSMAC	TCR, CD28, PKCθ	pMHC, CD80
pSMAC	LFA-1, talin	ICAM
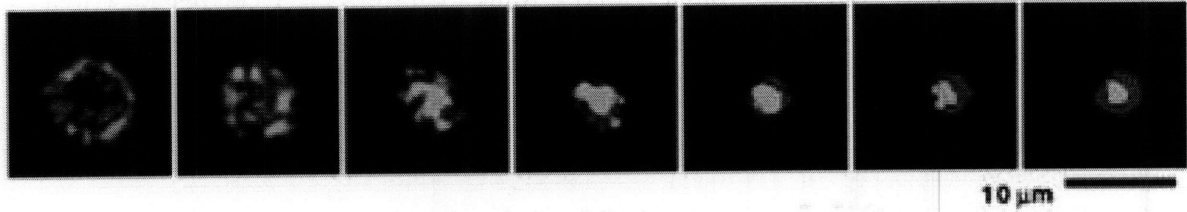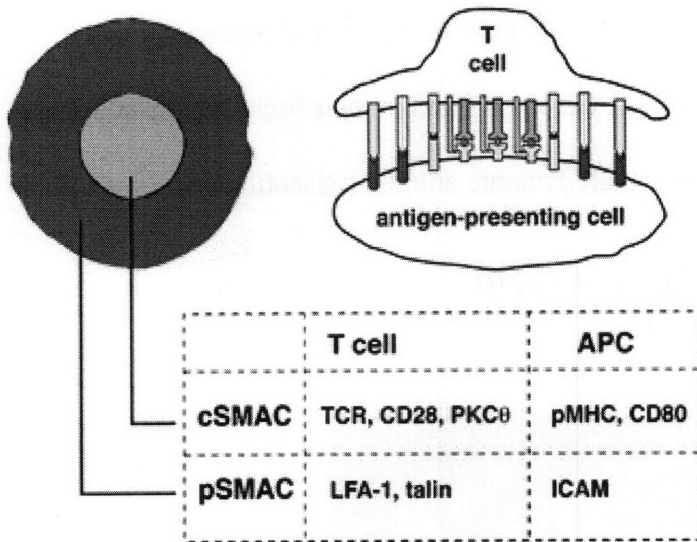
[5]

Figure 2: a. *Inversion of signaling and adhesion receptor-ligand complexes between the early and mature immunological synapse. In the early stages of synapse formation (panels 1 and 2), adhesion receptor-ligand(red) is in the cSMAC and signaling receptor-ligand (green) is in the pSMAC. At later stages (panels 6 and 7), the cSMAC becomes the signaling region and the pSMAC becomes the adhesion field.* ***b.*** *Mature immunological synapse "bulls-eye" and T cell receptors and APC ligands in each region*

To better visualize what actually occurs between the T cell and APC at the immunological synapse, there is a precedent of fabricating a substrate to mimic an array of antigen-presenting cells and letting live T cells interact with it [3]. Synthetically creating an APC array is advantageous for several reasons. The type(s) of signaling ligand presented, their

surface densities, and their total amounts may be controlled. Additionally, it is much easier

to acquire and interpret images from a flat APC array than from the T cell response to a

live APC cell.

The first synthetic APC arrays consisted of fixed signaling ligand against a blank glass or

substrate background. An improvement to these arrays was two protein patterning, with the

signal protein in patches, and an adhesion protein in the background. This created

essentially an array of bulls-eyes, all imitating the form of the mature immunological

synapse (Figure 3). However, both proteins were still fixed to their backgrounds and static,

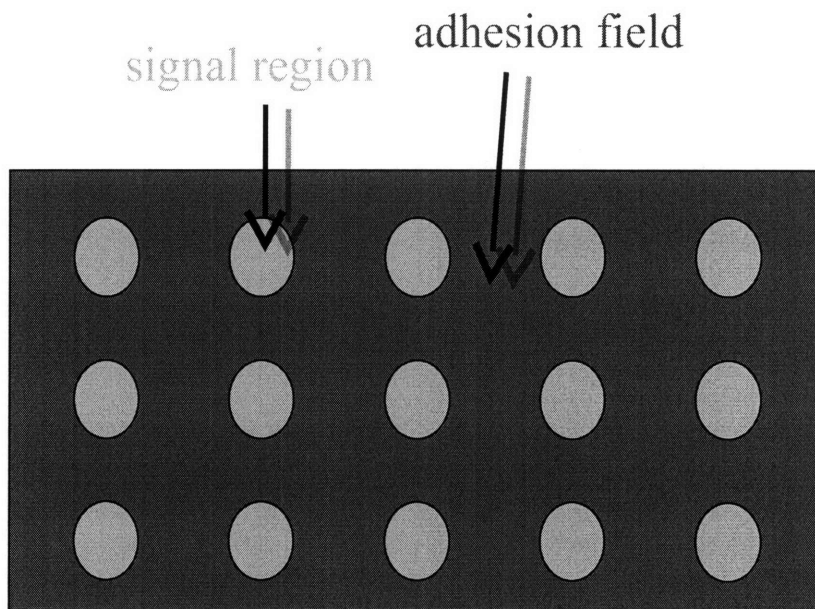making these arrays far removed from actual, dynamic antigen-presenting cells.

Figure 3: *Schematic of a synthetic antigen-presenting cell array. Signal regions are green and adhesion field is in red.*

A significant advance in the making of a synthetic APC substrate was using lipid bilayers

for parts of the array. Lipid bilayers are fluid in two dimensions, and so mimic mobile

signaling ligand much better than static arrays. Proteins may be patterned on top of the

bilayer in many different ways: via antibodies, via GPI tethers, via His tags, and via streptavidin-biotin linkages. All of these strategies bestow the protein with the fluidity of the underlying lipid bilayer [6, 7, 8, 9].

Doh and Irvine developed a unique photolithographic approach to create a synthetic antigen-presenting cell substrate. As in the other arrays, signaling ligand was in spots on the foreground and adhesion ligand was in the background. The end result resembled Figure 3. Their method is based on the novel terpolymer PNMP, from o-nitrobenzyl methacrylate, methyl methacrylate, and poly(ethylene glycol) methacrylate monomers. This polymer is exceptional in several ways; it is photo-sensitive, pH-sensitive, and may be biotinylated. When it is UV exposed, its photo-sensitive o-nitrobenzyl group cleaves to a carboxylate group (Figure 4a). At this point, the polymer becomes pH sensitive. If pH is increased past 6.6, the carboxylate becomes deprotonated, and the polymer film irreversibly dissolves off of the substrate (Figure 4b). Removal of polymer leaves behind a thin polymer film with exposed biotin groups. Proteins can be patterned onto this film using a streptavidin molecule bridge. Streptavidin is capable of binding four biotin groups via its four binding pockets. In forming a bridge, some of these pockets are occupied by the biotin groups on polymer, and some are occupied by the biotin groups attached to the protein ligand. By using a photomask during UV exposure, signaling and adhesion regions may be defined on this substrate, and through sequential UV exposure and increased pH wash, two different proteins may be patterned into these. [10].
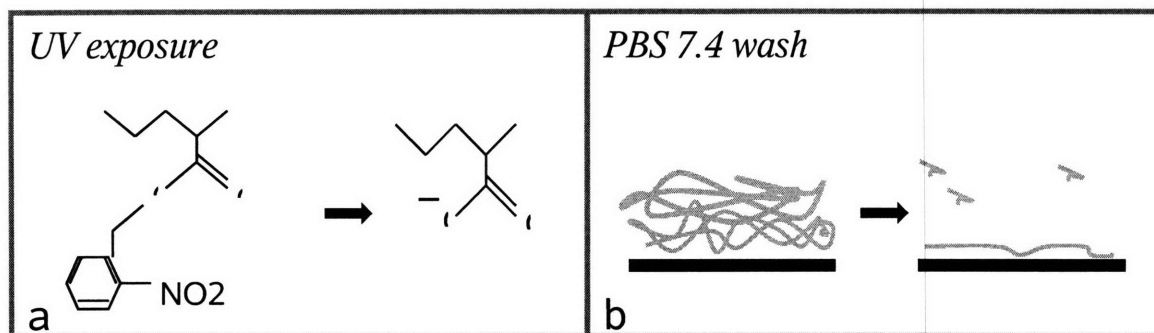
Figure 4: a. *Cleaving of o-nitrobenzyl group upon UV exposure.* **b.** *Dissolution of polymer film upon PBS pH 7.4 wash.*

This work seeks to improve upon this antigen-presenting cell array by adding signaling ligand mobility. This will infuse the substrate with some of the dynamism of its live APC counterparts. To do this, the photolithographic approach will be combined with the patterning of both protein *and* lipids. Lipid bilayers will be formed in the signaling regions (regularly arranged, micron-scale patches) defined by UV exposure. Biotinylated lipid vesicles will be synthesized and induced to burst into bilayers on the substrate. Streptavidin will again act as a bridge between the underlying bilayer and biotinylated signaling ligand. However, in this case, the underlying layer is a lipid bilayer which will allow the signaling ligand to be mobile, and. The signaling ligand used will not be the physiological peptide-MHC, but instead an antibody to the CD3ε part of the T cell receptor (biotin-anti-CD3ε). This antibody induces a response from TCRs by cross-linking them. A fusion protein with the adhesion ligand, ICAM-1, can still be patterned onto the background using the original approach outlined by Doh and Irvine. The desired completed APC array substrate will have a top view like that of Figure 3. However, the signal spots will not be fixed, but will be mobile. A side view of the desired substrate is shown in Figure 5.The

most salient aspects of this model are the biotin-anti- CD3ε patterned onto the lipid

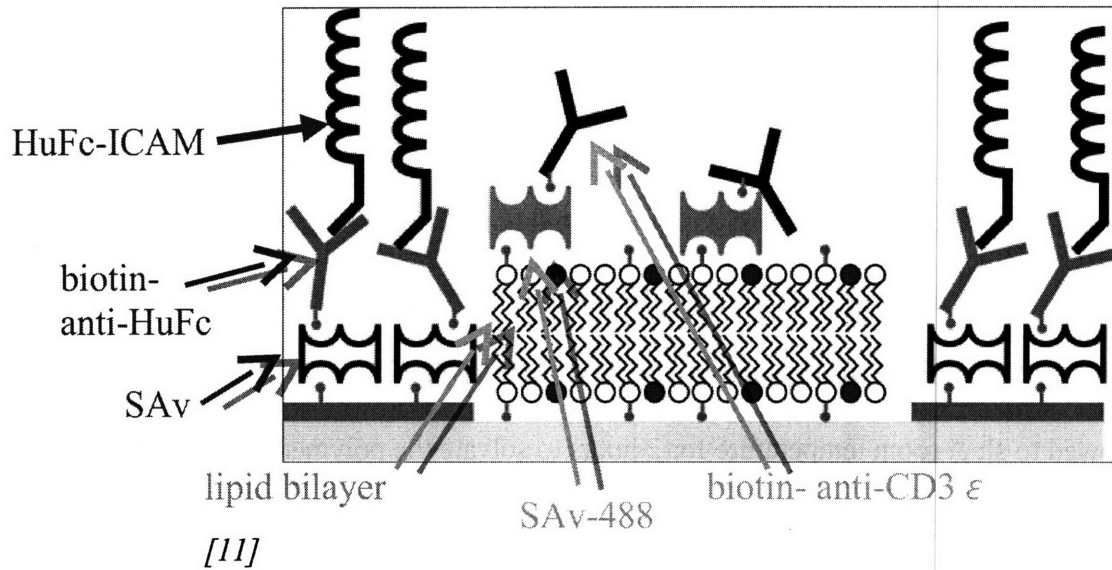bilayer in the signal region and the anti-HuFc-ICAM-1 patterned in the adhesion region.

Figure 5 *The desired completed antigen-presenting cell substrate. The signaling region is the middle, with biotin-anti-CD3ε patterned on top of a biotinylated lipid bilayer via a streptavidin bridge. The background adhesion regions are on the left and right of this signal region and contain ICAM-1 patterned on top of biotinylated polymer film via a streptavidin bridge to biotin-anti human Fc and an anti-HuFc-HuFc interaction.*

The answers this work sought are three-fold. First, can the lipid bilayers and their

associated signal proteins be segregated to desired locations in order to create a substrate

mimicking an array of antigen-presenting cells? Second, is the lipid bilayer formed in these

signaling regions mobile? Finally, is the resultant synthetic APC array bioactive?

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Methods

Preparation of glass/polymer film substrate.

The substrate was built on an aminosilane coated glass slide (24x50 mm, Xenopore) which has a positive surface charge. To form the polymer film, biotinylated PNMP photoresist was first dissolved in 1,4-dioxane at a concentration of 3 wt. % PNMP. The solution was allowed to sit at room temperature for 2 hours to solvate the polymer. This solution was then spincoated onto the aminosilane glass slides, and the slides were dried in a vacuum oven overnight at 60 ° C. The resultant polymer film layer was 120-130 nm thick (as characterized by ellipsometry).

Two different chrome photomasks were used for UV patterning: one with 100 μm squares, 100 μm apart, and another with 10 μm diameter circles, with a 35 μm center to center distance. In preparation for UV patterning, these chrome patterns were washed with acetone and dried in the vacuum oven for 20 minutes for cleaning. The spincoated slides were then UV exposed in the presence of a photomask for 20 minutes (Figure 6i). The photomasks were used to define two regions on the polymer film- the signaling patches, and the adhesion background. The photomasks protected the background adhesion region, and UV exposure of the selected (signaling) areas converted the o-nitrobenzyl groups to carboxylate groups. The slide was then incubated in PBS pH 7.4 for 2 minutes which dissolved the UV exposed regions of the polymer film (Figure 6ii). A thin, negatively-charged film containing carboxylate groups remained because this film was

electrostatically attracted to the underlying cationically functionalized glass. The slide was then washed with distilled water, and dried with air. Finally, the slide was UV exposed again, this time without the presence of a photomask (Figure 7ii). This prepared the background for dissolution (Figure 6iii).

A lipid bilayer will only form on a clean, extremely smooth glass surface. In order to prepare the slide for lipid bilayer formation, it was plasma etched with oxygen for 60 seconds at a pressure of 100 mTorr using a Harrick Plasma Plasma Cleaner PDC-32G (Figure 6iv). This etching removed the remaining photoresist polymer from inside the UV patterned wells, while only minimally affecting the background.
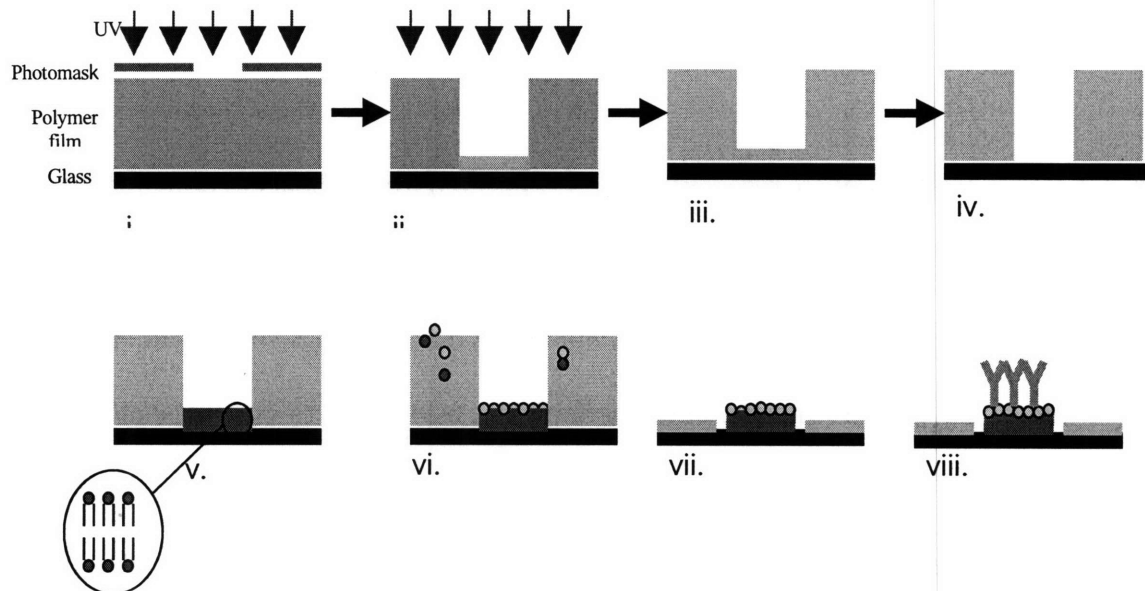

Figure 6: i.UV exposure with a photomask. ii. PBS pH 7.4 wash, followed by UV exposure with out a photomask. Note that the UV exposed regions turn from gold to violet. iii. Background is ready for liftoff. iv. Plasma etch with oxygen. v. Incubation with lipid vesicles and wash with PBS pH 6.0. vi. Incubation with streptavidin-488. vii. Liftoff wash with PBS pH 7.4. viii.Incubation with biotin-anti-CD3ε and wash with PBS pH 7.4.

Preparation of lipid vesicles.

The following lipids: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, 786.15 g/mol), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rho-DOPE, 1,301.73 g/mol), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)2000] (biotin-PEO-DSPE, 3,016.81 g/mol), were purchased from Avanti Polar Lipids. The structures of these lipids are shown in Figure 7. All three of these lipids were stored in chloroform at -20 °C. The major component of the vesicles was DOPC, a neutral phospholipid. Rho-DOPE was present because of its fluorescence, and biotin-PEO-DSPE was present because of its biotinylation. These lipids were vortexed for one minute to mix. The lipid mix was then formed into lipid cakes by drying with N2 gas, and then drying in a vacuum oven for 1-2 hours.

After drying, the lipids were reconstituted in PBS pH 6.0 for 1-2 hours to bring the final concentration of DOPC to 1 mg/mL. Rho-DOPE was present at a concentration of 0.5 mol %, and biotin-PEO-DSPE was at 1 mol %. Following this, the lipids were again vortexed for 1 minute and agitated via sonication for 5 minutes using a Microson Ultrasonic Cell Disrupter XL to induce vesicle formation. Finally, the vesicle solution was centrifuged in an Eppendorf Centrifuge 5424 for 5 minutes at 5,000 rpm. The pellet was discarded, and the supernatant saved.
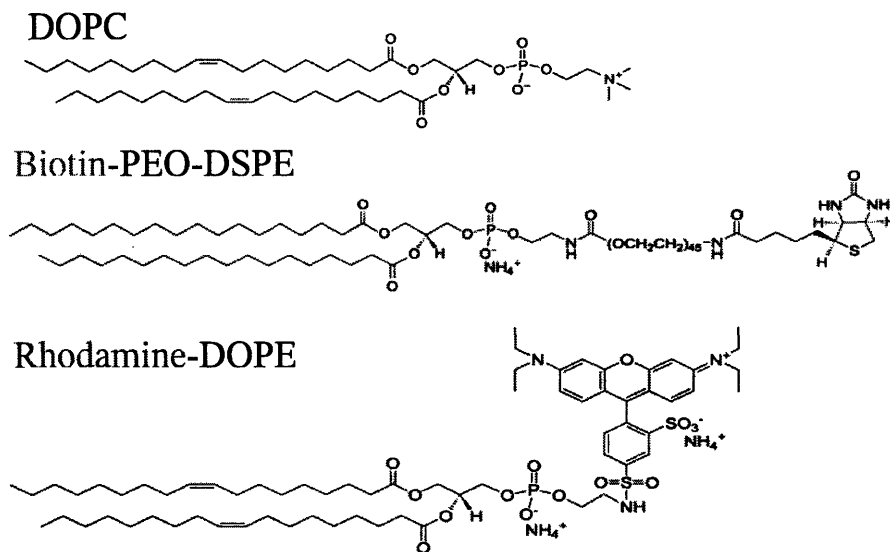
DOPC

Biotin-PEO-DSPE

Rhodamine-DOPE

Figure 7: *Structures of lipids used for vesicle formation from Avanti Polar Lipids, Inc.*

Formation of lipid bilayer and subsequent protein patterning.

To create wells for patterning on the flat glass slide, adhesive silicone isolators (JTR8R-

A2-2.5 , Grace Biolabs) were affixed to the already UV exposed and plasma etched slides.

In these regions, lipid vesicle solution along with additional PBS pH 6.0 solution was

incubated (ratio of 1:3) for 1 hour. After this time, the wells were washed with PBS pH 6.0

to remove any lipid vesicles that had not fused into bilayers (Figure 6v). This low pH

buffer also protected the background from dissolution. The slide was then incubated with

streptavidin-Alexa 488 (SAv-488) at a concentration of 2 wt % (to DOPC) (Figure 6vi).

After 1 hour, the background was lifted off by washing with PBS 7.4 (Figure 6vii). This

removed the background and any SAv-488 not bound to biotinylated lipid. Finally, the

slide was functionalized by adding the signaling ligand, biotin-anti-CD3 ε (b-anti-CD3 ε,

0.5 mg/ml), at a concentration of 0.5 wt % (to DOPC). After 1hour, the unbound biotin-anti-CD3 ε was also removed by washing with PBS pH 7.4 (Figure 6viii).

Fluorescence Microscopy

The synthetic APC array was imaged using a Zeiss Epifluorescence 900 Microscope with a Xenon lamp. The purpose of this microscopy was to visualize both the lipid bilayer and the streptavidin bound on top. The lipid mix contains DOPE bound to rhodamine which fluoresces red. SAv-488 fluoresces green. An exposure time of 100 milliseconds was used.

Fluorescence Recovery After Photobleaching

To create a bilayer capable of FRAP analysis, an additional lipid needed to be added to the lipid mix- NBD-DOPE (Figure 8)
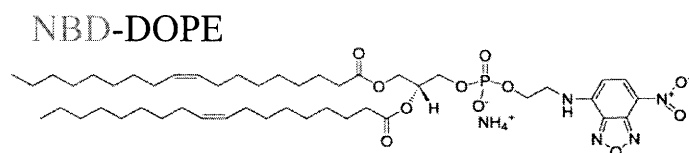
NBD-DOPE

Figure 8: *Structure of NBD-DOPE, the green fluorescing lipid needed for FRAP analysis. From Avanti Polar Lipids, Inc.*

. During vesicle formation, this lipid was added to the mix at a concentration of 1 mol % . Its addition to the lipid mix at such a low concentration should not alter the properties of the lipid bilayer formed. NBD-DOPE fluoresces green and is a weak fluorescer. This makes it extremely useful for FRAP because it photobleaches extremely rapidly when

exposed to a laser. FRAP was performed using a Zeiss LSM 510 Microscope and two lasers- a green Argon 488 laser and a red Helium-Neon (HeNe) laser. A 63x water dipping lens was employed for imaging.

Addition of Cells.

The T cells used to test the bioactivity of the substrate were harvested from a model 5C.C7 transgenic mouse. This mouse model is a knockout for the Rag gene which means it does not naturally produce any T cells or B cells except for transgenic T cells with a 5C.C7 T cell receptor which is specific for the moth cytochrome C (MCC) peptide on the major histocompatibility complex molecule IE^k. To gather the T cells, males from this mouse line who were 5 weeks to 3 months old were euthanized and their spleens isolated. The red blood cells in the spleens were lysed, and the resultant cell mix was cultured for two days with MCC peptide (to activate the T cells). On day 2, IL-2, a T cell growth factor was added to the cell culture. T cells grew extremely rapidly after this, choking out other types of cells in the culture. The T cells chosen to interact with the synthetic antigen-presenting cell substrate were from day 4 or day 5 of this culture.

Results

The parameters for antigen-presenting cell substrate preparation were varied to find the

optimal procedure for fabrication. The methods varied include the plasma etching process,

the stages of vesicle preparation, and the polymer background liftoff process.

Increasing etch time decreases the photoresist polymer film thickness.

The goal of plasma etching was to clean the signaling spots of polymer film for lipid

bilayer formation while keeping the background in tact (Figure 7iv). This way, a lipid

bilayer can be formed in the spots and functionalized, and then the background can be

erased, ensuring that only the spots are functional. The polymer film thickness may be

determined using ellipsometry, but this technique is not accurate for thicknesses less than

20 nm. Therefore, an indirect approach was used to find the idea etching time. The

thickness of the polymer background film was measured before and after etching for

different amounts of time. The assumption made was that if the background was being

etched away, so was the thin polymer film in the signal spots.

To measure thickness using ellipsometry, a reflective surface is required, and so silicon

wafers (instead of aminosilane glass slides) were spincoated with photoresist. The wafers

were UV exposed and then etched using oxygen for four different times: 30 seconds, 60

seconds, 90 seconds, and 120 seconds. The pressure inside the chamber was stabilized at

100 mTorr. The results of these different etch times on polymer film thickness are shown in

Figure 9. The thicknesses of the slides before etching are also shown, as are the thicknesses
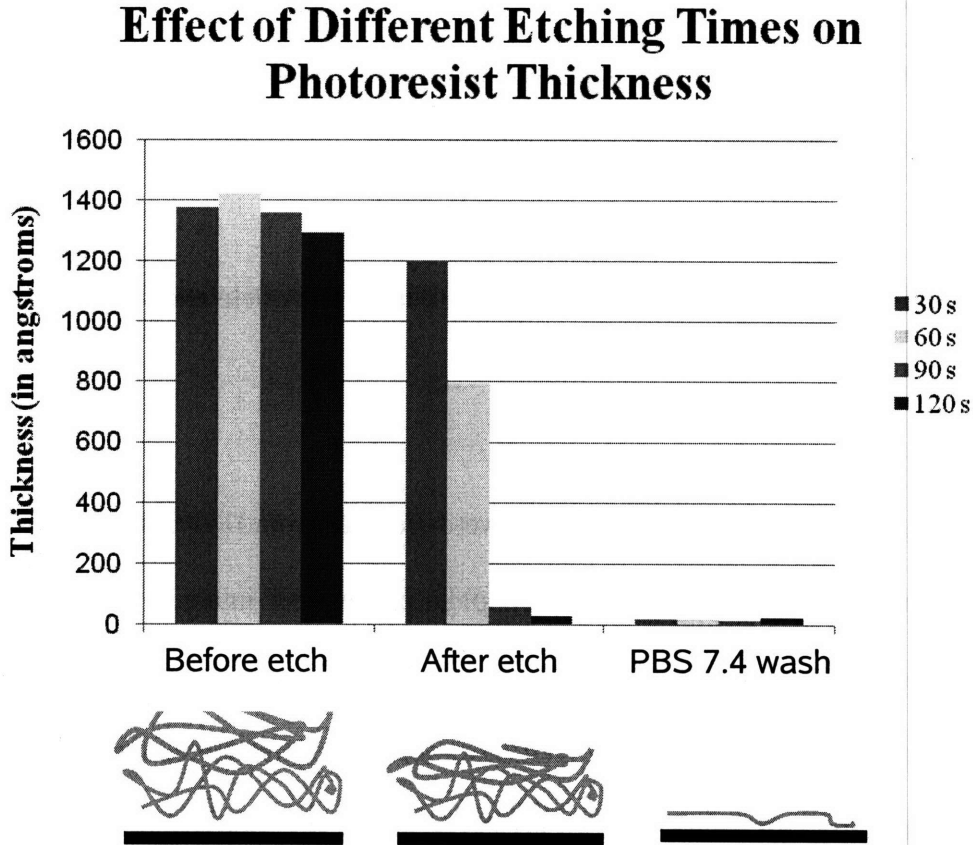
of the slides after liftoff with PBS pH 7.4.

Effect of Different Etching Times on Photoresist Thickness

Figure 9: The effect on photoresist polymer film thickness for etch times of 30 seconds, 60 seconds, 90 seconds, and 120 seconds.

Etching for 30 seconds reduces the average polymer film thickness to 87% of its original

value. Etching for 30 more seconds reduces it to 56% of its original value, and an

additional 30 seconds etch time reduces it to 4% of its original thickness. Finally, etching

for 30 more seconds (for a total of 120 seconds) reduces the film thickness to 2% of its

original value. Developing the UV exposed photoresist film in PBS pH 7.4 further reduces

all four sample thicknesses to about 1% of their original values.

The slides were washed in PBS pH 7.4 to ensure that etching did not prevent polymer

dissolution. Based on this data, an etch time of 60 seconds was adopted for the final

fabrication protocol. After etching for 1 minute, the background is still 700-800 nm in

thickness, and it was assumed that the spots were bare of polymer film. At 90 seconds, the

polymer background is dangerously thin, at 70-80 nm, and so this etch time is too long.

**Varying sonication procedure or filtration causes changes in vesicle average effective
diameter.**

To make a functional lipid bilayer, lipid vesicles were prepared from DOPC, rhodamine-

DOPE, and biotin-PEO-DSPE lipid stocks. This lipid mix was dried into a lipid cake, and

then re-suspended in phosphate-buffered saline pH 6.0 (PBS). To stimulate vesicle

formation, the lipid solution was vortexed, sonicated, and then centrifuged. Certain

preparation parameters were varied and their effects on vesicle size noted in order to

determine which produced the smallest vesicles in the least labor-intensive way. The most

desirable vesicles are the ones that are the smallest. This is because these are most likely to

be uniform and unilamellar, and therefore form the most uniform bilayers. Bigger lipid

vesicles are likely to be either multi-lamellar or multi-vesicular (Figure 10), and it is much

less likely that these will burst into neat unilamellar planar bilayers.
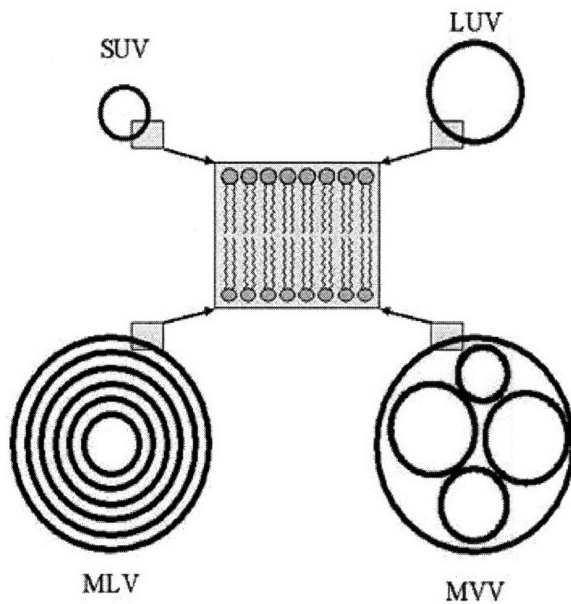
[12]
Figure 10: *Schematic showing small unilamellar vesicle (SUV), large unilamellar vesicle (LUV), multilamellar vesicle (MLV), and multivesicular vesicle (MVV). It would be much less likely an MLV or MVV to burst into a uniform bilayer than a SUV or LUV*

The first parameter tested to improve vesicle quality was sonication power. Four different sonication procedures were tested: sonicating for 5 minutes at 4 watts, sonicating for 5 minutes at 9 watts, sonicating for 5 minutes alternating between 9 watts and 4 watts every 30 seconds, and sonicating for 5 minutes alternating between 9 watts and 4 watts every 15

seconds. The results of these four trials are shown in Figure 11. The smallest average

effective diameter was achieved by alternating between 9 watts and 4 watts, *either* every 30

seconds or every 15 seconds. Sonicating strictly at 9 watts led to the largest average

effective diameter, and sonicating strictly at 4 watts led to an intermediate average effective

diameter. The sonication procedure ultimately adopted was alternating between the two

power levels every 30 seconds.

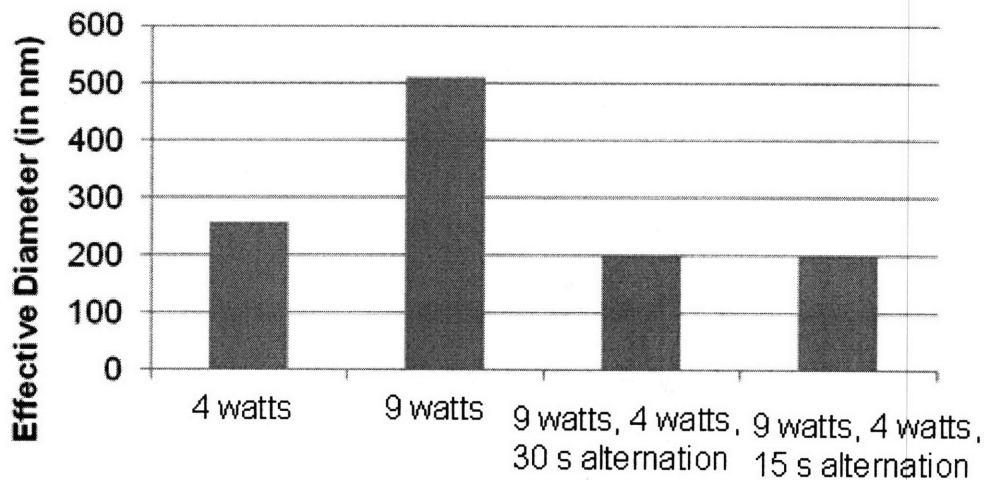## Effect of Different Sonication Procedures on Vesicle Diameter

Figure 11*: The effect of different sonication methods on lipid vesicle average diameter. The smallest vesicles (approximately 200 nm) are formed by alternating between 9 watts and 4 watts either every 30 seconds, or every 15 seconds.*

The second parameter tested was filtering the vesicles through an 80 nm filter after

centrifugation versus not filtering them. Two controls were used, a 1000 μL sample, and a

500 μL sample, and filtration samples corresponding to these two volumes were prepared.

The results of this comparison are shown in Table 1. Filtration of the 1000 μL sample

leads to a reduction of 7.2 nm in average vesicle diameter, with a final vesicle size of 82.10

nm. Filtration of the 500 μL sample leads to a reduction of 3.16 nm and a final diameter

of 89.47 nm. Because filtering does not lead a much smaller average vesicle size (note the

negligible difference between Control 1 and Filtration 2), in the final fabrication protocol,

the vesicles were not filtered. Also, the effect of centrifugation after sonication on vesicle

effective diameter can be noted from this table. Average vesicle size measured decreases

by more than 100 nm (from about 200 nm to about 90 nm) as a result of centrifugation.

Varying Filtration of Vesicles					
Sample	Volume (μL)	Biotin-PEO-DSPE concentration (mol %)	Sonication and Centrifugation	Filtered	Average Effective Diameter (nm)
Control 1	1000	1	Alternating between "5" and "1" every 30 seconds for 5 min, 5,000 rpm for 5 min	NO	89.30 ± 0.46
Control 2	500	1	Alternating between "5" and "1" every 30 seconds for 5 min, 5,000 rpm for 5 min	NO	92.63 ± 0.68
Filtered 1	1000	1	Alternating between "5" and "1" every 30 seconds for 5 min, 5,000 rpm for 5 min	YES	82.10 ± 1.15
Filtered 2	500	1	Alternating between "5" and "1" every 30 seconds for 5 min, 5,000 rpm for 5 min	YES	89.47 ± 0.88

Table 1: The effect of filtering vesicles (Filtered 1 and 2), versus not filtering them (Control 1 and 2).The average effect diameter is shown in the right-most column.

Increasing pH of the wash buffer results in poor liftoff.

After lipid vesicle incubation, bilayer formation, and streptavidin-488 incubation, the slide

was washed with a PBS buffer of increased pH (7.4 or higher) to remove the background

polymer film and the streptavidin nonspecifically attached to it. Increasing the pH increases the charge of the polyanionic photoresist polymer and causes its dissolution. Buffers of four different pH's were used to erase the background-7.4, 7.98, 8.48, and 9.05. The hypothesis was that higher pH would lead to a more complete dissolution of the polymer film and a cleaner background after liftoff. The results of these different buffers on liftoff are shown in Figure 12. From these pictures, it can be noted that the cleanest liftoff actually results from using PBS pH 7.4 (the lowest pH tested). The lipid bilayer inside the 10 micron spots appears at its most uniform and the background is the cleanest. In the higher pH liftoffs, the lipid appears splotchy or haloed inside the spots, and the background is obstructed by debris. This may be due to hydrolysis of the polymer and denaturation and hydrolysis of the proteins.
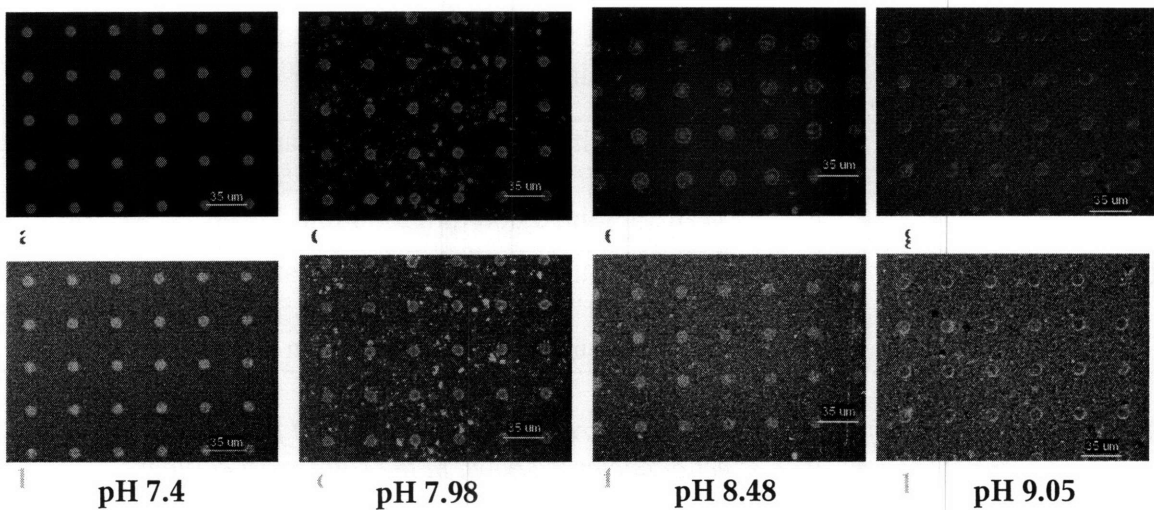

| pH 7.4 | pH 7.98 | pH 8.48 | pH 9.05 |

Figure 12: *Effect of different PBS buffers on polymer background liftoff.* **a**. *lipid at pH 7.4.* **b**. *SAv -488 at pH 7.4.* **c**.*lipid at pH 7.98.* **d**. *SAv-488 at pH 7.98.* **e**. *lipid at pH 8.48.* **f**. *SAv-488 at pH 8.48.* **g**. *lipid at pH 9.05.* **h**. *SAv-488 at pH 9.05.*

Under appropriate conditions, lipids segregate to desired patches, and streptavidin and biotin anti-CD3 selectively bind these patches.

Under optimal plasma etching conditions, vesicle synthesis, and liftoff pH conditions, lipids and the proteins binding to them segregate to the signaling regions defined for them through photolithography. Two different photomasks- one with 100 micron squares and another with 10 micron diameter circles were used as patterns for photolithography and subsequent lipid and protein patterning. In the antigen-presenting cell array substrates resulting from both of these masks, the lipid segregates to the wells formed through UV exposure and plasma etching. This is seen through using the red fluorescence property of the rhodamine-DOPE to image the substrate (Figures 13a, d). Additionally, SAv-488 selectively binds these lipid patches (probably because of the biotin present in them). This is visualized using the green fluorescence of Alexa 488 to image (Figures 13b, c, e, f). Although the background appears relatively SAv-488 (green fluorescence) free in both lipid arrays, the background appears much cleaner in the array of 100 μm squares.
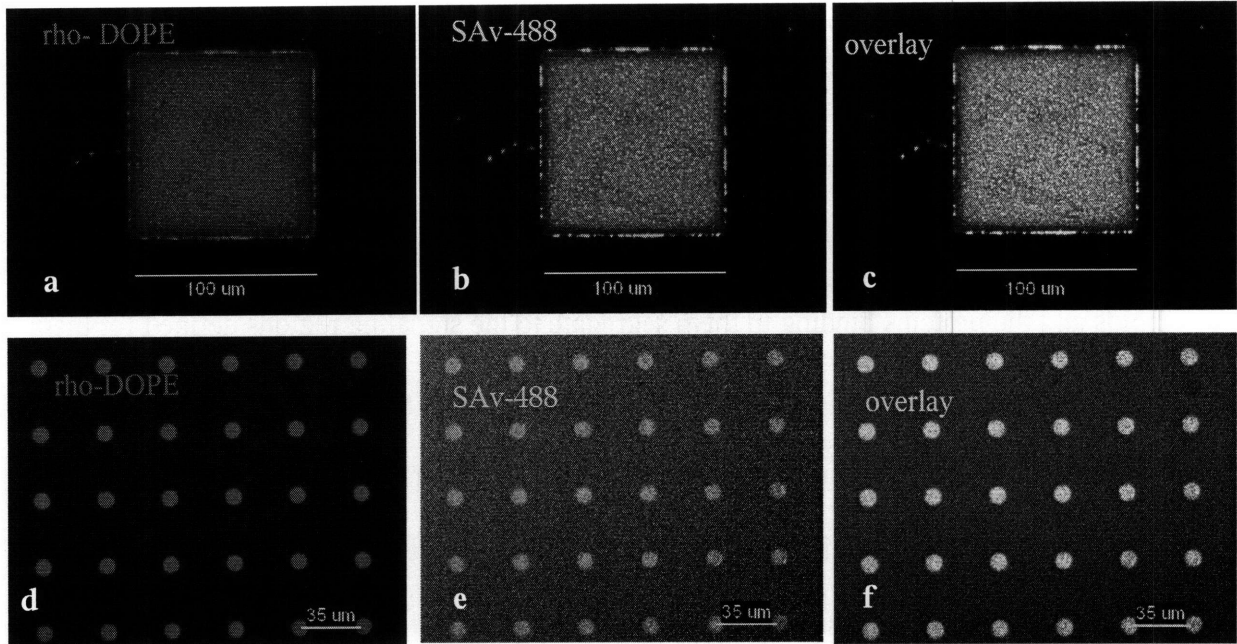
Figure 13: *100 µm squares and 10 µm diameter circles APC arrays substrates.* ***a.*** *lipid bilayer location (in red) on 100 µm squares substrate, imaged using rhodamine fluorescence.* ***b.*** *SAv-488 location (in green) on 100 µm squares substrate, imaged Alexa 488 fluorescence.* ***c.*** *overlay of images a and b.* ***d.*** *lipid bilayer location (in red) on 10 µm circles substrate, imaged using rhodamine fluorescence.* ***e.*** *SAv-488 location (in green) on 10 µm circles substrate, imaged Alexa 488 fluorescence.* ***f.*** *overlay of images d and e.*

Recovery time for lipid bilayer after photobleaching is 30 seconds.

To test the mobility of the lipid bilayer, fluorescence recovery after photobleaching (FRAP) was performed on lipid bilayers formed in 100 µm x 100 µm squares. (10 micron spots are too small to perform FRAP.) In FRAP, a small spot of the lipid bilayer is bleached for a very short amount of time. The bleaching results in a reduction of fluorescence in that spot. If the lipid bilayer is fluid, the bleached lipids will migrate out of that spot, and other lipids will migrate into it. The migration into the spot of these still-fluorescent lipids will cause fluorescence recovery in the spot. The results of FRAP are shown in Figure 14. As can be seen from the graph (Figure14a), the fluorescence is at a

maximum at time 0 seconds (Figure 14i). The spot is bleached for the first 9 seconds, and

after this period, the fluorescence is at a minimum (Figure 14bii). The fluorescence in the

spot returns at time 30 seconds (Figure 14biii). This rapid return of fluorescence inside the

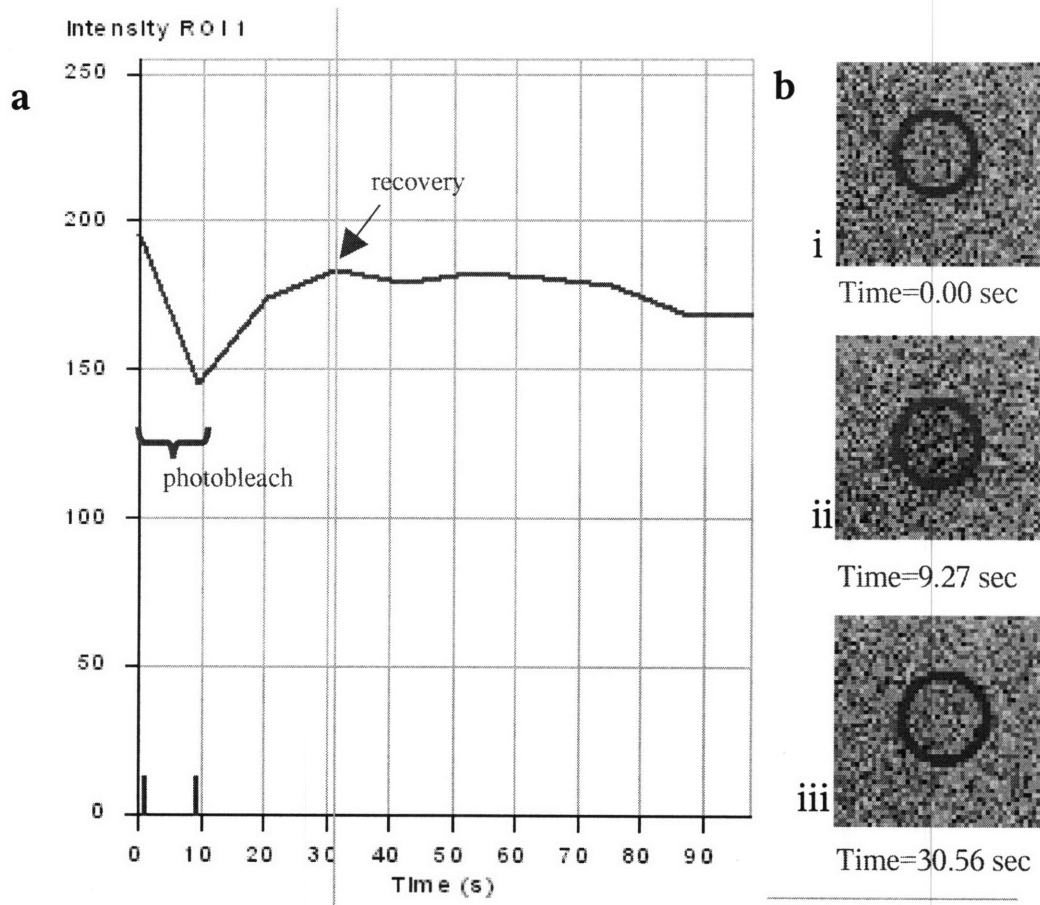photobleached spot indicates that the lipid bilayer is mobile.

Figure 14: *Results of Fluorescence Recovery After Photobleaching (FRAP).* ***a.*** *Graph of fluorescence vs. time inside photobleached spot. The fluorescence starts at a maximum, then reaches a minimum at 9.27 seconds. Fluorescence recovery occurs at 30.56 seconds.* ***b.*** *images of photobleached spot from location inside a 100 μm square.* ***i.*** *Spot at 0.00 seconds.* ***ii.*** *Spot at 9.27 seconds.* ***iii.*** *Spot at 30.56 seconds.*

Cells interact with anti-CD3 patterned onto the lipid bilayer.

The end purpose of creating a lipid bilayer substrate is to mimic an antigen-presenting cell (APC) and then observe how this synthetically created "cell" substrate interacts with real helper T cells. To do this, the 10 micron lipid spots have to be functionalized with ligand that the cells can recognize and interact with. The substrates were incubated with biotin-anti-CD3 for this express purpose. Afterwards, cells were placed on the substrate, and their behavior observed. Images of this procedure are shown in Figure 15. The cells were only on the substrate for 20 minutes. Although it appears that there may be some cell-substrate contacts forming, these pictures are in no way definitive.
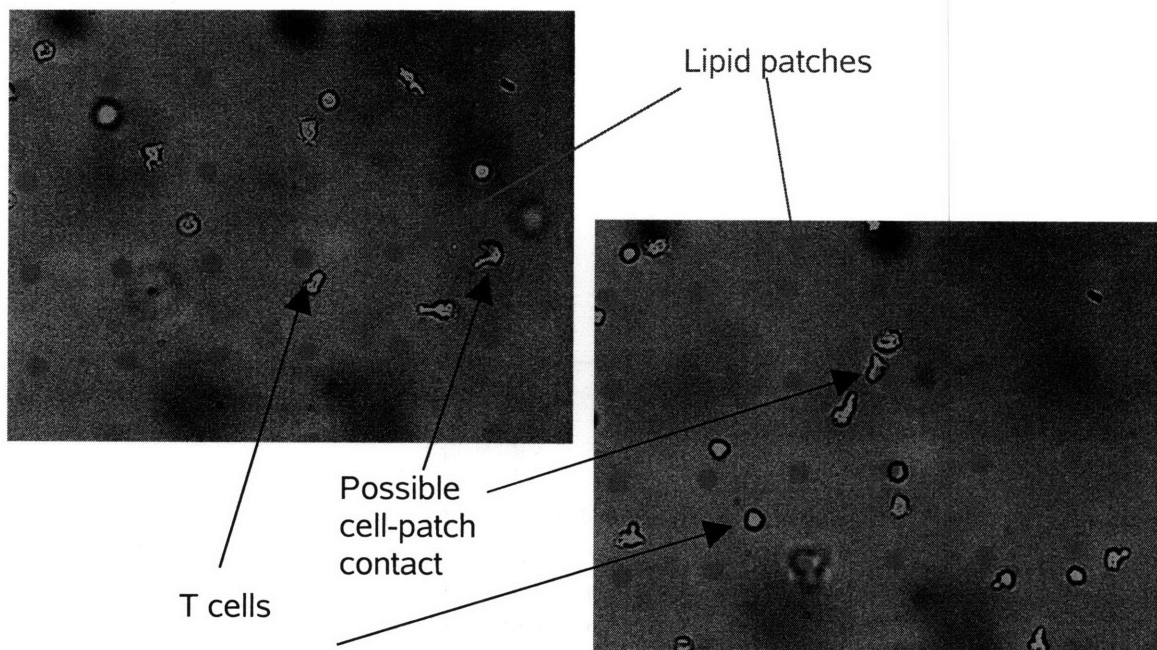

Figure 15: *Helper T cells interacting with lipid-SAv-ligand patches (red) on antigen-presenting cell array at about 10 minutes after incubation.*

Discussion

The goal of this work was to create an antigen- presenting cell substrate with mobile signaling ligand. This is an important step in making synthetic antigen- presenting cell arrays more physiological. To do so, photolithography, along with combined patterning of lipids and proteins, was employed. Important concerns were whether the lipid and signaling ligand (biotin anti- CD3 ϵ) could be patterned selectively into signal spots, whether the lipid bilayer would be fluid, and whether T cells would interact with the synthetic antigen-presenting cell array.

The creation of this substrate was based on the photolithographic process developed by Doh and Irvine[10]. The process relies upon the novel terpolymer PNMP, which as mentioned, is photo-sensitive, pH-sensitive, and may be biotinylated. This approach led to an antigen-presenting cell array with signal protein immobilized on spots in the foreground and adhesion protein immobilized on the background. One of the really superb aspects of this process is that it does not require the harsh solvents or extreme processing conditions of other photolithographic processes with photosensitive polymers. This ensures that the proteins patterned onto the substrate will not be denatured, and that the T cells allowed to interact with the substrate will not die from toxicity. The weakness of this process is that the protein ligand in both the signal and adhesion regions is fixed. Our work sought to make this substrate more physiological by adding mobility to the signaling ligand patches. To do this, we combined patterning of proteins and lipids.

We found that under appropriate substrate and vesicle synthesis conditions, lipid bilayers form exclusively in the defined signaling regions. Moreover, streptavidin-488 which is used as a bridge between the lipid and the signaling ligand also segregates to these signaling regions. From this, it was assumed that biotin-anti CD3ϵ, the signaling ligand, also binds to these regions exclusively.

Further, we showed that the lipid bilayer is in fact mobile in these regions, experiencing fluorescence recovery within 30 seconds of photobleaching. This fluidity of the bilayer is extremely important because it makes the mobility of the signaling ligand in the lipid patches possible.

Finally, we found results suggesting that the antigen-presenting cell substrate synthesized is in fact bioactive and T cells do interact with it. However, this aspect of the substrate definitely needs to be better characterized.

Our system represents an improvement over several existing systems. One of these is the system developed by Orth et al, also for micron scale patterning with lipid bilayers, and also involving polymer lifotff. The polymer used is Parylene C, which is not photo-sensitive or biotinylated. The end result of this is that although lipid is patterned into micron scale patches, the background is bare glass, with no functionalization capabilities[13]. By contrast, our system is capable of patterning adhesion ligand into the background.

Of course, the substrate we fabricated does have some caveats. The biggest is that we used biotin-anti-CD3 ε as our signaling ligand instead of the physiological signal ligand peptide-MHC. Peptide-MHC is extremely difficult to synthesize, whereas biotin-anti-CD3 ε is much more readily available. CD3 ε is part of the T cell receptor complex, so this antibody induces a response from TCRs by crosslinking them. The TCR response to biotin-anti-CD3 ε is actually much stronger than its response to peptide-MHC. This means that TCR binds biotin-anti-CD3 ε in situations when it wouldn't bind peptide-MHC. If the release of cytokines and other factors from the T cell was examined, its immune response might be much greater than in the presence of peptide-MHC. Our system should try to incorporate peptide-MHC as the signaling ligand towards making a more physiologically accurate and relevant system.

Another caveat is that we did not actually pattern the adhesion ligand onto the substrates we fabricated because we were more concerned with characterizing the formation of the lipid bilayer. The process to pattern adhesion ligand onto the background is known and will be described here. Adhesion ligand can be added to the background by using streptavidin as a bridge between the biotinylated polymer and biotinylated anti-human Fc antibody. A fusion protein of ICAM-1 (adhesion ligand) and human Fc could then be patterned on top. (Pure ICAM-1 is not commercially available.) For reference, look at Figure 6.

The final caveat of our system is that the bioactivity has not been characterized. The adhesion ligand background allows T cells to crawl over the substrate using their LFA-1

receptors. If they encounter a signal spot with biotin anti- CD3 ε, they can either stop and interact with it, potentially forming an immunological synapse, or they can continue crawling. Hopefully, adding the adhesion background to our substrate will allow cells to interact with it for longer and crawl over it to find signaling ligand. We can then see if immunological synapses form. If they do, this substrate will be an ideal platform for further investigating different aspects of the IS. The substrate could be changed to present ligand in different densities or in different shapes or patterns. Different types of signal and adhesion ligand could be put on the same substrate to see how T cells respond. The end goal in these manipulations would be elucidating which factors are necessary for IS formation, why the IS must form for an immune response, and why certain complexes within it act the way they do.

Additionally, our system might have applications beyond studying the immunological synapse. A corollary application would be the study of soluble (instead of membrane bound) factors such as cytokines or growth factors. Nam et al developed a fluid bilayer system in order to do this, but their system is only capable of patterning one ligand onto its surface. Ours can currently pattern two and may be able to pattern more through further iterations of UV exposure through a photomask and elevated pH wash [14].

A truly powerful system would be one which presented a multitude of ligands to stimulate a variety of T cell responses, or responses from several different types of immune system cells. As suggested by Dustin and Groves [3], a system such as this could be developed into a diagnostic tool to evaluate the health of the immune system and the presence or

absence of certain cell types. Therapeutically, this system could produce antitumor T cells

or regulatory T cells which could them be introduced back into the body to aid the immune

response.

Conclusions

Photolithography with a pH sensitive, photoresist polymer was used for combined patterning of lipids and proteins. It was found that lipid bilayers would segregate to desired regions defined by UV exposure, and that streptavidin- 488 and biotin anti CD3 would also segregate to these regions. Additionally, the lipid bilayer was fluid in these regions and also potentially bioactive. The purpose behind designing this substrate was to make a synthetic surface to mimic an array of antigen- presenting cells. This surface would have mobile signal ligand (biotin anti- CD3) through presence of the lipid bilayer.

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