Development of a Biosensor for Investigating Membrane Curvature Sorting

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Development of a Biosensor for Investigating Membrane Curvature Sorting

A Dissertation

Presented to

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Doctor of Philosophy

by

Joshua C. Black

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Advisor: Michelle Knowles
Abstract

The physical structure of cellular membranes plays a critical role in lipid and protein sorting. A novel biosensor was developed to probe the influence of curvature on sorting. This biosensor mimics large, two-dimensional membranes in dynamic equilibrium, achieves high spatial resolution between curvature and molecules of interest, and has high sensitivity, enough for single particle detection. The biosensor consists of continuous supported lipid bilayer formed over nanoparticles (40 to 200 nm diameter) deposited on a glass substrate. The nanoparticles determine the extent of curvature. This biosensor is the first to observe large-scale 2-dimensional diffusion of biomolecules on a supported lipid bilayer with small radii of curvature in equilibrium with flat areas of fluid bilayer. This will allow correlation between protein function and the physical shape of a membrane. Fluorescence microscopy was used to quantify spatial sorting of lipids and a protein relevant to cardiovascular disease, C-reactive protein (CRP). Two lipids, fluorescein labeled hexadecanoic acid and 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, sense curvature by accumulating in areas over nanoparticles, and both are able to laterally exchange with surrounding lipids. Dynamic fluidity of the bilayer was assessed using fluorescence recovery after photobleaching. Lipids directly at sites of curvature recover more slowly than lipids over flat sections. The spatial distribution of CRP was also assessed. Curvature sensing of CRP is isoform dependent where native CRP does not sense curvature and modified CRP does sense curvature.
Finally, we show that a ribonucleic acid aptamer will bind specifically to modified CRP and not native CRP, enabling isoform specific studies of CRP to be conducted.
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To my King who gives living water, and without whom, I would be nothing.

To my new wife whose love and patience are boundless. With you, I am excited for life to come.

To my parents who love unconditionally and know when to give the swift kick of motivation.

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Chapter One: Introduction

Membrane curvature plays an important role in several crucial cellular functions. Changes to membrane curvature are found everywhere from cell birth to cell death. At mitosis, the central section of the cell pinches in, dividing the cell in half. At cell death, the plasma membrane loses stability, increases permeability, and ejects apoptotic bodies into the intercellular space. These mechanisms in the cell are complex and layered with confounding variables such as protein feedback loops, protein-lipid interplay, lipid domain formation, and much more. The basic research presented here separates these variables to gain fundamental insight into the processes at work. We investigate how lipids and proteins sort with regard to membrane curvature. A novel biosensor was developed that separates curvature generation from other chemical aspects of cellular curvature, which enables us to probe the effects of curvature under controlled conditions. This resulted in new information regarding the curvature sensing properties of various lipids and a relevant cardiovascular marker, C-reactive protein.

Biological Relevance of Membrane Curvature

Membrane curvature is of particular interest in current biology (1,2,3,4,5,6,7,8,9). Changes to curvature can be seen during apoptosis, endocytosis, mitosis/meiosis, extracellular signaling, and many other cellular operations. However, other chemical and physical changes occur during these operations, and often they are inseparable from curvature changes. For example, during apoptosis several changes occur at the cellular
The plasma membrane in particular undergoes multiple changes including increased permeability, changes to leaflet composition, oxidization of lipids, and the formation of areas of high curvature \((10)\). Fundamental understanding of cellular processes relies on the ability to separate these variables and to categorize their individual contributions to cellular activity. Techniques capable of separating these variables from membrane curvature do exist, but each has its limitations. In this introduction, I will establish the biological aspects of curvature and discuss model membrane systems that mimic curvature found in a cell. These model systems served as the basis for our design as we sought to minimize disadvantages and maximize advantages in our system.

Given the varied macroscopic processes that induce changes to curvature, it is not surprising to find areas of extreme membrane curvature throughout the cell interior. Curvature can be transient, as in vesicle trafficking. It can also be in more permanent structures like microvilli. Figure 1 is a graphic \((2)\) that highlights the diversity of curvature within a typical cell. The topology of a budding vesicle shows the different degrees of curvature. Positive curvature creates a convex surface of interaction and has a larger surface area than a corresponding flat membrane. Negative curvature creates a concave surface of interaction with a smaller relative surface area. Also note that if the bending surface is a bilayer, areas of both positive and

![Figure 1: Diagram detailing areas in the cell where extreme membrane curvature can occur. Figure used with permission (2).](image)
negative curvature are present, but on opposite leaflets. The terminology this work uses is to identify curvature based on the surface of interaction. For example, a bilayer where a protein binds to the convex surface would be considered to have positive curvature, even though a concave surface exists on the non-interacting face. A budding vesicle (Figure 2) is a good model process to consider for membrane curvature since it transitions through various radii of curvature (ROC), demonstrates both positive and negative curvature, and at one point has a saddle which includes both positive and negative curvature on the same interacting surface.

These changes to surface area are the main driver for the characteristic instability of lipids in curvature. On the negative face, lipid head groups are forced together and strong electrostatic forces repel the head groups from each other. The positive face forces exposure of the hydrophobic tails to the aqueous solution creating entropic instability. This lipid crowding on the negative face, and the corresponding hydrophobic exposure on the positive face, cause instability in the curved structure. These energetically unstable structures must be 1) maintained by proteins which stabilize the strained morphology, or 2) accommodate a different leaflet ratio of lipids or different types of lipids.

Apoptotic bodies and budding vesicles are ideal examples of membrane curvature. Apoptosis is the process of programmed cell death and has several purposes in
the body, such as maintaining homeostasis and as an immune response (10). During this process, the cells shrink in size, become more dense and irregular in shape. The plasma membrane begins to bleb severely. These blebs later entirely release from the dying cell as apoptotic bodies. The membranes of these bodies remain intact and are later absorbed by surrounding macrophages or other cells where the contents of the bodies are recycled. As a reference for size, endothelial cells create apoptotic bodies with radii of curvature from 500 nm to 2 μm. Smaller ejections (microparticles) were also observed (11).

Comparatively, vesicle sizes are typically much smaller, with radii on the order of 10s to 100s of nanometers. This transport is the primary mechanism in cells to transfer proteins between compartments of the endomembrane system and the plasma membrane, and a primary mechanism for ingesting new material via endocytosis. Due to the large extents of curvature, these vesicles are often accompanied by coat proteins which help stabilize the unfavorable extreme curvature. Triggering of the vesicle formation usually accompanies recruitment of cargo via receptor molecules. After reaching a destination, these coat proteins detach, making the vesicle more fusogenic and easily incorporated into the destination membrane (12,2).

**Causes of Membrane Curvature**

For a membrane to maintain a given structure in the face of this strained morphology, lipid composition and protein coating change to counter and minimize the energetic cost of bending. These two broad effects are not independent from each other, but are discussed here separately for simplicity. A minor contribution to curvature comes from the physical or molecular shape of the lipid (5). A typical saturated lipid like phosphotidylcholine is cylindrical in shape, and this will stabilize zero curvature or a flat
membrane. As a flat membrane begins to curve, defects appear in the positive face (13). A defect is a point in the membrane where the hydrophobic tails are exposed to aqueous solvent, and this arises due to the increase in surface area of the positive face. These defects are stabilized by inverse conical lipids, such as lysolipids or certain dye labeled lipids. On the negative face of a curving membrane, head groups are forced closer together due to the reduction in surface area. This steric hindrance is eased by conical lipids like phosphatidic acid. The smaller head group and broader tail region help ease the steric interaction between neighboring lipids. Differences in numbers of lipids between the two leaflets also act to stabilize highly curved surfaces. In a liposome with 50 nm on the outer diameter and a thickness of 5 nm, the ratio of lipids on the outer leaflet to inner leaflet is 1.23 (2). Lipids also tend to form domains that have bending rigidity that hinder or assist in curvature formation. Cylindrical lipids tend to form ordered domains with high bending rigidity, whereas conical and inverted conical lipids form disordered domains with low rigidity. Though individual lipids have a small contribution to overall curvature formation, curvature could influence the formation of domains and further stabilize the presence of domains (7). All of these changes assist or hinder morphology directly, but certain lipids also act as sites for protein binding, which can further influence curvature stability.

Figure 3: Crystal structure of a BAR dimer with a proposed bilayer conforming to the intrinsic shape. When a bilayer is fitted well to the shape of the protein, more electrostatic interactions are present, thus increasing binding. Figure used with permission (2).
Proteins have several methods to influence curvature. 1) Scaffolding is common in the cell and functions by having a rigid protein structure conform to and support a new membrane structure. Some common examples of scaffolding proteins are clathrin, the dynamin family, and caveolin \((14,15,16,17)\). 2) Many proteins incorporate BAR (Bin-Amphiphysin-Rvs) domains, dimer domains that have a distinctive banana shape. These domains, seen in Figure 3 (2), will sense curvature at low concentrations \((18)\). When the curvature of the membrane matches the intrinsic curvature of the dimer, there is tighter binding. It is expected that the binding energy of BAR domains to curvature matches the energy required to bend the membrane, as has been seen with amphiphysin \((19)\). 3) Commonly operating alongside BAR domains are amphipathic helices, in which the helix is attached to the N-terminus of the BAR domain (the combination dubbed N-BAR). These protein domains will insert into a membrane and increase the surface area and thus generate positive curvature. Figure 4 (2) shows the wedge effect that generates the curvature. The \(\alpha\)-helix structure has one face that is hydrophobic and one face that is positively charged. The hydrophobic face inserts into the tail section of the membrane while the positive face interacts with the head group region \((20)\). The one-sided insertion causes the surface area asymmetry. 4)
Microfilament (actin) and microtubule (tubulin) activity can deform the membrane through changes in bilayer tension \((21,22,23)\). It is interesting to note the complex interplay that occurs between these mechanisms through tension. The cytoskeleton is responsible for maintaining and changing tension, which causes a response in microtubule activity, which affects both tension and feeds back to the cytoskeleton activity \((24)\). The cytoskeleton also plays a secondary role in morphology changes by directing vesicles to sites of fusion and endocytosis. \((25)\) 5) The final mechanism responsible for membrane deformation is the presence of transmembrane proteins. These proteins will typically prefer curvatures that mold around their shape. While this mechanism is known to exist, such as with the voltage dependent \(K^+\)-channel \((26)\), not much is broadly known about this effect since the structures of transmembrane proteins remain elusive.

**History of Fluorescence**

The biosensor was designed with fluorescence microscopy in mind. This method is extremely useful when studying biological systems, which we intend to mimic. Fluorescence is a subcategory of luminescence, which is defined as “a spontaneous emission of radiation from an electronically excited species (or from a vibrationally excited species) not in thermal equilibrium with its environment.” \((27)\). Fluorescence, along with phosphorescence and delayed fluorescence, is a type of photoluminescence. These forms of luminescence arise from excitation due to photon absorption and are distinct from other excitation modes, like chemiluminescence and bioluminescence \((27)\).

The first observation of fluorescence can be attributed to Spanish physician Nicolás Monardes \((28)\) in 1565. He reported a blue color from an infusion of Mexican
wood used to treat urinary diseases. This infusion was rare, thus making it a target of counterfeiting. Dr. Monardes used the blue color as a method of detecting false infusions (28). After Dr. Monardes, others noted similar color inconsistencies from natural substances: 1) the dichroic nature of fluorite by Edward Clarke in 1819 (29), 2) again fluorite by René-Just Haüy in 1822, who (incorrectly) identified the phenomenon as light scattering (opalescence) (30), and 3) red color from chlorophyll extracts by Sir David Brewster in 1833 (31). A major breakthrough in understanding fluorescence came in 1845 by Sir John Herschel. He was the first to note fluorescence from a single substance, not a natural extract (32). Herschel used a prism to illuminate an acid solution of quinine sulfate with blue light (33) to observe emitted light in the blue, green and yellow regions. He failed to note that the emitted light was longer wavelength than the illumination light, and thus attributed the phenomenon, like his predecessors, to scattered light. This crucial observation would ultimately be made by George Stokes.

A turning point in understanding fluorescence, and coining of the term, came from Sir George Gabriel Stokes in 1852. Stokes continued studying quinine solutions and fluorite crystals, but he made the important observation that emitted light is always longer wavelength than absorbed light (34). Using more sophisticated methods than Herschel, Stokes used a prism to separate solar radiation and illuminated a solution of quinine. In the visible region, nothing happened. When the tube was placed beyond the violet region of the spectrum in the ‘invisible rays’ as Stokes describes it, the solution glowed blue. He called it “…literally darkness visible.” (34) Though in his first work Stokes continues the tradition of referring to the phenomenon as a derivative of scattering
in his work, he uses the term *dispersive reflection*, he admits in a footnote his dislike of the term, and he coins the term *fluorescence*:

I confess I do not like this term. I am almost inclined to coin a word, and call the appearance fluorescence, from fluorspar, as the analogous term opalescence is derived from the name of a mineral. (34)

The red-shift that normally accompanies fluorescence is now known as the Stokes law or Stokes shift in honor of Stokes’s contribution. It is useful to note that Edmond Becquerel also observed a red shift of emitted light from calcium sulfide when illuminated with UV light in 1842, 10 years earlier than Stokes (35,32). Becquerel even filed a priority claim against Stokes (36,32) for this observation. However, a primary difference is that Becquerel observed phosphorescence, not fluorescence, and Becquerel misinterpreted Stokes’s observations as short-lived phosphorescence, disregarding the introduction of Stokes’s ideas of the separate phenomenon fluorescence.

The next major advancement in understanding fluorescence came in the late 19th century, along with other observations about the breakdown of classical mechanics and the paradigm shift into quantum mechanics. Eilhard Wiedemann postulated scenarios where the Second Law of Thermodynamics was violated. Fluorescence appeared to allow energy to flow from cold objects to hotter objects (37,38). Additionally, several physicists, including Franz Stenger in 1886 (39,32) and Ernest Merritt in 1904 (40), noted observations where the Stokes law was violated and shorter wavelength fluorescence was observed. A clue was noted by Lommel in 1878 who observed this apparent violation only occurred when absorption and fluorescence curves overlap (41). Resolution to this paradox finally came with Planck theory of quanta and Albert Einstein’s use of this theory for luminescence. He proposed that the curves observed in fluorescence suggest a
statistical process, and that the violation of the Stokes law occurs because the molecular vibration supplies the additional energy needed to blue shift emission. This would satisfy both the First and Second Laws of Thermodynamics. Joseph von Kowalski later proved this in 1910 by showing the violation of the Stokes law for rhodamine is larger at higher temperatures, consistent with Einstein’s calculations (42).

Modern understanding of fluorescence is derived from the work of Francis Perrin in 1922 (43) and Aleksander Jablonski in 1935 (44,45). Perrin introduced what is commonly referred to now as the Jablonski diagram, which included a mechanism for the Stokes shift and a metastable state. The metastable state was an attempt to describe long-lived fluorescence and phosphorescence. However, Perrin did not include a path for radiative transfer from this metastable state back to the ground state. Jablonski provided this information to the diagram, thus cementing his namesake (45). The distinction between fluorescence, a retention of spin multiplicity, and phosphorescence, a change in spin multiplicity, was made by Terenin in 1943 and Kasha in 1944 (45).

Fluorescence has several uses in the modern scientific fields of chemistry, physics, and medicine (45,46). Of particular note for this work is the application of fluorescence recovery after photobleaching (FRAP) to microscopy of supported lipid bilayers. Fluorescence microscopy was first used in 1942 by Coons, Creech, Jones and Berliner to observe fluorescent antibody distribution in mouse tissue (47). From that, cell surface staining was done in 1974 (48), proteins in live cells were seen in 1978 (49), and intrinsic fluorescence (unlabelled protein) imaging done in 1995 (50). Lateral diffusion of objects in a membrane was best described theoretically by Saffman and Delbrück in 1975 (51) as cylindrical objects diffusing freely in 2 dimensions. Experimental work to
observe lateral diffusion can be done by FRAP, and the most complete description of this method is given by Axelrod, Webb, and coworkers in 1976 (52). For supported lipid bilayers, an excellent mimic of 2-dimensional membranes, pioneering work into creation and characterization has been done by Tamm (53,54) and McConnell (54).

FRAP has been used extensively to characterize biological dynamics. The publication by Axelrod, Webb, and coworkers (52) has been cited over 1900 times as of this writing (55). In 1982, Webb and coworkers showed that constraints to lateral diffusion of lipids and receptor molecules are relaxed in blebs of muscle cell membranes (56). The diffusion of voltage-dependent sodium channels is restricted to the axon hillock of neurons with no diffusion to the cell body. This was shown using FRAP in 1988 by Elson and coworkers (57). FRAP has played a crucial role in studying the presence of lipid domains in cells (58,59,60,61), a topic that remained controversial throughout the 1990s and into the 2000s. In recent medical research, FRAP has been used to study interstitial transport of drugs across cancerous tumor boundaries toward target cells (62), identify an association of influenza viral proteins with membrane rafts (63), and image the transcription cycle of HIV-1 (64). Assays including fluorescence remain an integral part of the analytical toolkit used to study biologically interesting phenomena.

The State of the Art in Membrane Curvature Sensing

To get an understanding for the state of the art in curvature sensing assays, I will highlight four different methods already established. As discussed earlier, separating curvature from confounding variables within a cell is difficult. All of these methods seek to control curvature through a variety of variables that mimic cellular conditions. By
discussing these methods and their advantages/disadvantages, I will highlight how the present work maximizes the advantages, minimizes the disadvantages, and introduces new capability beyond what is already known. This biosensor is the first to observe large-scale 2-dimensional diffusion of biomolecules on a supported lipid bilayer with small radii of curvature in equilibrium with flat areas of fluid bilayer. This enables unprecedented observation of the influence of physical structure on dynamic sorting.

Liposomes can be particularly useful in curvature investigations because they have a single defined curvature determined by the diameter of the vesicle. Liposomes have been used as models for biological membranes (65). A more recent assay, the single liposome curvature (SLiC) assay, tethers liposomes to a glass surface with sufficiently low density to resolve individual vesicles (66). Figure 5a (3) schematically demonstrates this method. These liposomes are labeled with fluorescent lipids, such as Texas Red DHPE, and the relative fluorescence used to determine size. Using a second, spectrally separated fluorophore on a protein of interest, the amount of binding can be

Figure 5: Details of the SLiC assay. A) Isolated liposomes are immobilized on a glass surface with biotin/streptavidin. B) Confocal image of a heterogeneous sample. Scale bar is 10 μm. C) Total integrated fluorescence for the liposomes indicates size. D) Binding molecule fluorescence over the same area as C. To normalize for the size of the liposome, the density of the binding molecule is calculated by dividing integrated intensity in D over C. Scale bar in C and D is 1 μm. Figure used with permission (3).
quantified. Figures 5b-c show an example confocal image and the ratio used to determine curvature sensing molecule density. In this study (3), researchers used this method to investigate amphipathic motifs binding to curvature. They revealed that binding is mediated by number of binding sites, not increased affinity as would be predicted. The primary advantage of this system is that it uses single liposomes, which illuminates more population information than ensemble studies. A major disadvantage is that the different radii of curvature are isolated. This eliminates dynamic exchange of bound motifs along the surface, which does not mimic membranes in organelles or the plasma membrane.

Following a different track with using liposomes, another method takes giant unilamellar vesicles (GUV) and pulls out long tubes via micropipette aspiration (67). As a tube is pulled from the GUV, the size of the tube is determined by the rigidity of the bilayer and the pulling force exerted on the tube. More rigid bilayers lead to larger tubes, and increasing the pulling force decreases the size of the tube. The radius, $R (m)$, of these pulled tubes is described by the bending rigidity of the bilayer, $\kappa (N \cdot m)$, and membrane tension, $\sigma (N/m)$, as

$$R = \sqrt{\kappa / 2\sigma} \quad \text{(Eqn. 1)}$$

Tension then is dependent on the pulling force, $f (N)$, as

$$f = 2\pi \sqrt{2\kappa \sigma} \quad \text{(Eqn. 2)}$$
Therefore, by controlling the pulling force, the radius can be controlled (59). Figure 6 (59) is a brightfield image of this method in action. The researchers here were able to study lipid sorting into ordered and disordered phases and the ability of those phases to form tubes. After studying rigidity of these phases, the experimenters verified the assumption that tubes are typically pulled out of disordered, less rigid phases. A second study with this method (9) was able to demonstrate N-BAR domains ability to sense curvature at low concentrations but generate curvature at higher concentrations. A major advantage of these methods is that it mimics curvature changes induced by cytoskeletal activity (59). Secondly, tension can be used not only as an inducer of curvature, but a sensor for curvature changes, as in the second study (9). Disadvantages of this method include the need for specialized equipment, the limited ability for lipids to exchange with the ‘bulk’ GUV (9), and like the liposome method, a failure to mimic curvature on large-scale, 2-dimensional surfaces such as the plasma membrane in the areas imaged, the extended tube. Additionally, only ensemble dynamics through fluorescence recovery after photobleaching (FRAP) are accessible in

![Figure 6: A GUV is aspirated to a pipette on one end and attached via biotin/streptavidin to a bead trapped in optical tweezers on the other. In 2-3, the GUV is gently withdrawn and a tube is formed between the GUV and the bead. The tension on the pipette controls the size of the tube. The scale bar is 10 μm. Figure used with permission (59).](image)
this method due to the large percent fluorescent lipid needed (1%), thus single particle tracking is not possible.

Mimicking this large-scale, 2-dimensional surface is the primary advantage of the final two methods presented. Using soft lithography, researchers were able to pattern glass with a regular, wavy surface. Figure 7 (4) demonstrates the topography of this setup. Lipids are added via liposome deposition, a standard supported lipid bilayer (SLB) technique. Using atomic force microscopy (AFM), the glass surface curvature is characterized, which in this study had a maximum radius of curvature of 55nm. The SLB molding around the surface waves is assumed. The wavy nature of the surface allows a continuous transition from positive curvature to negative curvature. Fluorophore labeled proteins are visualized with confocal microscopy and correlated to the AFM images. Mimicking 2D surfaces, transitions between extents of curvature, and presence of a large reservoir of lipids are major advantages of this method. This method also clearly creates areas of negative curvature. Preparation of the surface is the major disadvantage. Preparation requires harsh surface etching (HF, NH₄F, CF₄, and O₂ plasma), high temperatures (200°C), and a
prefabricated polydimethylsiloxane mold. The requirement of this mold makes changing the surface topology in multiple experiments non-trivial. Additionally, the process of determining curvature is separate from the process used to investigate protein location and motion. This requires an additional method to correlate the two. This work interestingly found curvature sensing properties of various proteins (ENTH, N-BAR, and CTB) when the bilayer was saturated, yet single-molecule imaging showed no curvature sensing. This implies a cooperative aspect to curvature sensing.

The final established method I highlight also uses lithography to pattern a glass surface with curved morphology (60). Charged polymer nanoparticles (NPs) will self assemble into ordered arrays on an oppositely charged glass surface. Subsequent thermal treatment will anneal the particles and systematically change the radius of curvature of the particles with respect to the treatment time. An SLB can be formed over this structured surface via liposome deposition. Figure 8 (8) shows scanning electron micrographs of the top (a) and side (b-d). Using FRAP, the researchers observed various diffusion constants of bilayers over the patterned surfaces. Contrary to expectation, they

Figure 8: Series of SEM images showing a sensor made from colloidal lithography. A) Overhead image of 110 nm particles heated at 110°C. B-D are side views of the particles and show the effect of higher temperatures, which causes the radius of curvature to decrease. B) 110°C  C) 114°C  D) 118°C. Figure used with permission (8).
found that more highly curved bilayers had larger diffusion constants. The authors speculated that alleviating of packing constraints at sites of positive curvature was responsible for increased diffusivity. This method is able to easily control the radius of curvature via temperature and simulate a 2D surface. Curvature was not correlated to the spatial organization of the lipids though. Nanoparticles were not fluorescent nor was correlated AFM done, so the influence of curvature at specific sites could not be investigated. Without this, only the general effect of curvature, and not the spatial, morphological influence, can be investigated.

Through our efforts, we seek to further understand the transient interactions between regions of membrane curvature and proteins/lipids. We characterize protein and lipid sorting to curved surfaces, lateral movement, colocalization with pertinent features, and isoform effects. To accomplish this, we have developed a novel biosensor that mimics biological membrane curvature, and demonstrates the application of the biosensor to various lipids and a protein suspected of curvature sensing properties, C-reactive protein (CRP). This new biosensor has notable advantages over the sensors currently in use. 1) Most notably, this biosensor spatially resolves the interaction between protein, lipid, and sites of curvature. It mimics the surface of blebs and budding endocytotic vesicles by having isolated sites of curvature in dynamic equilibrium with a bulk, flat bilayer section. 2) The preparation is quick and simple. 3) Due to the independently resolved extents of curvature, our system can have inherent heterogeneity. This heterogeneity permits molecules of interest to sort dynamically into regions of curvature with a high degree of specificity. This gives us refined information about molecular sorting. 4) Chemical composition of the bilayer is controlled independently from the
5) The sensitivity of the biosensor is high enough to detect single molecules moving on the surface.

This dissertation is divided into six chapters. Chapter two describes the methods and materials used to conduct the experiments. Some terminology is also discussed. In chapter three, we discuss data from characterization of the biosensor and control experiments conducted. Chapter four focuses on the implications of lipid sorting into curved SLBs and the diffusion of curvature sensing lipids. Chapter five discusses our investigations into CRP conformation conversions and curvature sensitivity. Finally, chapter six summarizes the major results.
Chapter Two: Methods

Formation of the biosensor was a modification of established supported lipid bilayer (SLB) techniques (61). Lipid composition was controlled at the time of SLB preparation, and this was separate from preparation of the physical shape of the surface. Surfaces were prepared by a rigorous cleaning method and colloidal deposition of polystyrene nanoparticles (NPs) onto a glass substrate. SLBs were formed around the NPs which caused the bilayers to conform to the NP surface, thus inducing a desired curvature. In future uses of this sensor, proteins and molecules of interest can be added after SLB is formed. This dissertation includes a proof of this concept using C-reactive protein (CRP).

In this chapter, we describe the methods used to create and characterize the biosensor. The primary methods were microscopy techniques (total internal reflection and confocal fluorescence), but other fluorescence techniques were also used to supplement microscope images. These methods were used to assess fluidity of bilayers and to perform controls. These results show our system is congruent with work presented in literature.
Protocol for Biosensor

Formation

Films were prepared by mixing the relevant lipid combination in chloroform. Our typical lipid composition consisted of 98% phosphatidylcholine (PC) lipids harvested from soy (Soy-PC), and 2% fluorescent lipid, percentages by mole. We primarily use three fluorescent lipids: Marina Blue - 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine (MB-DHPE), Fluorescien - 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine (Fl-DHPE), and Fluorescien hexadecanoic acid (Fl-HDA).

Structures of these lipids are shown in Figure 9a-c, respectively. Soy-PC includes a distribution of tail lengths and degrees of unsaturation. A typical carbon-to-degree of unsaturation distribution of tails is 16:0 (14.9%), 18:0 (3.7%), 18:1 (11.4%), 18:2 (63%), 18:3 (5.7%), and unknown (1.3%) (Avanti Polar Lipids, Lot 840054). The structures of the lipids in this mixture are shown in Figure 10a-e, respectively. Stock lipids were

Figure 9: Molecular structures of the three dye-labeled lipids used in this work. A) MB-DHPE, B) Fl-DHPE, and C) Fl-HDA
dissolved in chloroform and stored until use at approximately 10 mM concentration. Total number of moles for each film was 250 nmol. Appropriate volumes to obtain the 98:2 mole ratio were mixed in filtered chloroform and vacuum dried overnight to evaporate the chloroform. Other lipid mixtures could also be obtained by adjusting the 98:2 ratio as needed. Films were then stored for 3-4 months under nitrogen at -20°C. Chloroform was filtered through alumina and stored at -20°C prior to use with any lipid. Inorganic chemicals were purchased from Sigma-Aldrich, lipids from Avanti Polar Lipids, and biological chemicals and proteins from Life Technologies.

Surfaces were prepared via rigorous cleaning and patterned using NPs prior to SLB formation. 8-well dishes were purchased from Lab-Tek (#1.0 or #1.5 glass). First, both sides of the glass plate were immersed in 0.1% sodium dodecylsulfate (SDS, w/v) for one hour. Second, both sides were immersed in 1.0% bleach solution (v/v) overnight. Slides were stored under millipore water for up to 1-2 months. This pre-cleaning is necessary to remove larger contaminants, such as dust and oils. Immediately prior to use, the upper surface was covered in 100μL of 2% Hellmanex (v/v, a proprietary alkaline cleaning solution from Hellma Analytics, Müllheim, Germany) for one hour. At this point, the glass was further patterned using NPs or used for SLB formation as a flat surface.

In this work, three sizes of polystyrene NPs were used: 40 nm, 100 nm, and 200 nm in diameter (Life Technologies). For imaging, NPs were diluted to a level where NPs were not clustered. Dilutions used were 1x10^{-6}, 1x10^{-4}, and 1x10^{-3}, by volume from stock, respectively. Initial stock concentrations were 1% (w/v) solids. This resulted in
approximate bead dilutions for 40, 100, and 200 nm beads as, respectively: $7 \times 10^8$, $4 \times 10^9$, and $5 \times 10^9$ beads/mL. Dilutions were made in a deposition buffer consisting of 30 mM HEPES, 2 mM CaCl$_2$, and 140 mM NaCl at pH 6.4. Diluted NPs were incubated in the wells for 15 minutes, and then the solution was removed. NPs were adhered only lightly to the surface, so we did not rinse. A linker molecule to facilitate NP attachment is not recommended, as this will interfere with bilayer fluidity (Figure 14). This preparation was sufficient for NPs to act as patterns for SLB formation and curvature.

![Molecular shape of the dominant species in soy PC and major constituent of supported lipid bilayers.](image)

*Figure 10: Molecular shape of the dominant species in soy PC and major constituent of supported lipid bilayers.*

We used the following procedure to create fluid SLBs. A dried film, typically 250 nmol, was immersed in 2 mL of deposition buffer and sonicated for 15 minutes using a probe sonicator on low power. The sonicator consisted of a Fisher Prove Dismembrator Model 100 attached to an Ultrasonic Convertor probe head (Serial number: FS3406). Pulsed sonication for 0.1s on and 0.1s off was also used to prevent heat buildup. This method creates a heterogeneous mixture of unilamellar and multilamellar vesicles that will adhere to glass and rupture, forming a lipid bilayer over the glass. This method forms a single bilayer over the glass surface (Figure 19). After sonication, 100 µL of the vesicle solution was added to NP prepared wells and incubated for 1 hour at 37°C. The surfaces were then cleaned of excess liposomes by sequentially adding 100 µL of
deposition buffer, then removing 100 µL. This process left the surface wet at all times, and was repeated a total of three times. The SLB was formed and ready for direct imaging, or for addition of protein. If CRP was added, 100 µL of deposition buffer was accounted for as a dilution factor.

To prove this method creates only a single bilayer, fluorescence of 1-palmitoyl-2-[12-((7-nitro-2-1,3-benzoxadiazol-4-yl)-amino)dodecanoyl]-sn-glycero-3-phosphocholine (NBD-POPC) can be quenched using sodium dithionite. This procedure has been done previously using bilayers formed around silica nanoparticles (68). The SLB was formed in 8-well plates as usual over a surface without NPs with 100 µL of deposition buffer in the well. 10 mM sodium dithionite was prepared in deposition buffer. One frame prior to dithionite addition is taken to get a baseline of fluorescence. 10 µL of dithionite solution (to a final concentration of 5 mM) was added to each well and quickly mixed by pipetting up and down. The first image after addition was taken as quickly as possible, approximately 20 seconds after addition. Five additional images were taken at 1 minute intervals. This prevents photobleaching of NBD and allows for refocus to prevent microscope drift. Mean intensity of fluorescence was measured in a 10µm x 2µm box positioned to avoid liposomes. The curve was normalized to the intensity before addition of dithionite solution.

Preparation of mCRP

Modified CRP (mCRP) was prepared in three ways. Native CRP is purchased purified from human serum from Academy Biomedical Co. The first two methods, urea and heat, denatured CRP to similar molecular weights (MW) when run on a native gel;
both run at smaller MW than native CRP (pCRP) and higher MW than fully denatured CRP (69). All three methods have similar denaturing curves in fluorescence assays. In the first method, pCRP was treated with heat and SDS (mCRP in most sections, mCRP SDS in section: Aptamer Specific to mCRP). pCRP was diluted to a desired concentration (typically 1μM) in 0.01% SDS in deposition buffer. This was heated at 80°C for 1 hour. In the second method, pCRP (typically 1μM) was put in a solution of 8M urea and 10 mM EDTA and incubated at 37°C for 2 hours. Then using 3500 MW cutoff (MWCO) dialysis tube, mCRP was dialyzed against 2 L PBS at 4°C for 2-3 hours. We repeated dialysis a total of 3 times. The final dialysis was run overnight. When needed, mCRP was concentrated using a 10k MWCO, spun at 5000G for 10 minutes. In the final method, using guanidine hydrochloride (GndHCl), pCRP (typically 1 μM) is incubated with 1M GndHCl for 90 minutes at 37°C. These conditions were selected because we observed efficient conversion to mCRP via gel electrophoresis analysis.

A fourth treatment to modify CRP was also done using HCl, though this CRP isoform does not bind C1q. pCRP was incubated in an acidic solution (10 mM 4-(2-hydroxyethyl)-1-piper-azineethanesulfonic acid (HEPES), 0.1 M HCl, pH 2) for 5 min and neutralized with 0.1 M NaOH. A detailed explanation for the use of this isoform is in the section: Aptamer Specific to mCRP, and this isoform is referred to as mCRP HCl.

CRP–aptamer complexes were prepared by incubating the relevant CRP isoform with RNA aptamer in binding buffer (20 mM HEPES, 140 mM NaCl, 50 mM KCl, 0.1 mM DTT, and 5% glycerol at pH 6.5). Incubation was carried out at room temperature for 20 min and analyzed immediately. While calcium is not necessary for aptamer
binding, 2 mM CaCl2 was added to the pCRP sample to retain its folded pentameric structure.

Typically in studies on CRP, this conformational change is often referred to as an “isoform change” (70) (71) (72) (73). This work will continue the use of this terminology, though we recognize that the use of “isoform” is slightly incorrect. The term typically refers to similar proteins resulting from different genes or through alternative gene splicing (74). Technically, the change to CRP discussed here is a conformational change.

**Polyacrylamide Gel Electrophoresis**

Native polyacrylamide gel electrophoresis (native PAGE) was used to determine the isomeric state of CRP. Denaturing gels cannot be used, as they denature pCRP, causing it to run at a similar position as mCRP. The procedure to pour a native 10% polyacrylamide gel follows. We prepared a gel apparatus with thickness of 1 mm. For the resolving gel, we mixed 3.35 mL of 30% bis-acrylamide (29:1) solution, 2.5 mL 1.5M Tris (pH = 8.8), and 5 µL 10% (w/v) SDS in a 15 mL tube. This was filled to 10 mL, vortexed, and then we added 10 µL TEMED and 100 µL 10% APS. This final mixture was quickly poured into gel apparatus. We covered the wet gel with a small amount of ethanol and let it stand for 1 hour. Afterwards, we poured off ethanol before addition of the stacking gel. For the stacking gel, we mixed 500 µL 30% bis-acrylamide (29:1) solution, 375 µL 1M Tris (pH = 6.8), and 1.5 µL 10% (w/v) SDS in a 10 mL tube. This was filled to 3 mL, vortexed, and then we added 6 µL TEMED and 30 µL 10% APS. This final mixture was quickly poured into the gel apparatus over the resolving gel
and we inserted the comb. After 30 minutes of standing, the gel was ready for use. We stored them damp at 4°C.

When running the gel, we first prepared 1 L of a running buffer containing 40 mM Tris (4.856 g/L) and 189 mM glycine (14.188 g/L). We assembled the running apparatus as directed using a prepared gel and running buffer. To prepare mixtures for running, we mixed 10 uL of protein with 5 uL of loading buffer (Biorad, 50% glycerol and bromophenol blue). Typical protein concentrations were around 500 nM pCRP (57 µg/mL of CRP). Each well was loaded with 12 µL. We loaded only 5 µL of a protein standard or ladder to a well. The gel was run at 80 V until the sample entered the resolving gel, and then ran at 100 V for approximately 2.0 to 2.5 hours or until the bromophenol blue reached the bottom of the plate.

To fix and silver stain the gel, the following solutions were prepared. All required 25 mL total per gel and were made in Millipore water:

- **Fixing solution**: 50% methanol, 5% acetic acid, 12.5 µL of 37% formaldehyde per 25 mL.
- **Sensitizing solution**: 0.02% sodium thiosulfate. This was made fresh daily, and was made from a fresh 10% solution.
- **Silver stain**: 0.2% silver nitrate
- **Developer solution**: 6% sodium carbonate, 100 µL of 10% sodium thiosulfate per 25 mL, 50 µL of 37% formaldehyde per 25 mL
- **Stopping Solution**: 50% methanol, 5% acetic acid
Once a gel was run, we removed it from the apparatus and gently pried apart the gel plates. First we immersed it in 12.5 mL of fixing solution to separate the gel from the remaining plate. We let it sit for 15 minutes, and then replaced the solution. The gel was washed three times with 50% methanol for 8 minutes each. Then it was immersed in sensitizing solution for exactly 1 minute. This was washed three times with Millipore water for 20 seconds each. The gel was stained using silver stain for 30 minutes. Again, it was washed twice with Millipore water for 30 seconds each. To develop the bands, the gel was immersed in developer solution until bands visibly appeared, typically around a minute. This reaction was quenched with stopping solution by swirling gently. The gel was rinsed with 50% methanol for 10 minutes. One final rinse was done with Millipore water. The gel was imaged immediately, but it can be stored at 4°C in a damp container if desired.

The electrophoretic migration of free 3’Cy5-RNA and bound CRP-3’Cy5-RNA complex were observed using nearinfrared (NIR) imaging of the wet gel using the 700 nm channel on an Odyssey imager and CRP protein was visualized by subsequent silver staining of the same gel. The binding affinity of the RNA aptamer to CRP was analyzed using an EMSA. Increasing amounts of 3’Cy5-RNA (0.48-3.8 μM final concentration) were incubated with CRP (fixed at 23 μg/mL) in binding buffer for 20 min at room temperature before electrophoresis. The CRP–aptamer complex was loaded on a 5% polyacrylamide gel (19:1) in 0.5×TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA) and electrophoresed at 110 V for 40 min at 25 °C.
Confocal Microscopy

Confocal microscopy was performed using an Olympus FV1000 laser scanning confocal microscope using a 100x oil objective (NA = 1.4). An excellent review of confocal microscopy theory was published (75). Briefly, this method of microscopy illuminates a small volume with laser light and collects fluorescence from the same volume, thus reducing out of focus light and increasing signal to noise. In this work, we used three channels of fluorescence. Red channels, typically NPs, were excited with 594 nm diode laser; green channels with 488 nm; and blue channels with 405 nm. Images, sized 512x512 pixels from a spatial area 42x42 microns square, were taken raster-style using a photodiode detector, typically with a dwell time of 12.5 µs per pixel. Laser power, zoom, gain, and detector sensitivity were all adjusted as needed to obtain high quality images. Each channel was collected sequentially to eliminate image bleedthrough. Images were analyzed in ImageJ (76).

Resolution for this microscope is at the diffraction limit, approximately 220 nm for red emission light, centered at 610 nm. For fluorescein, the diffraction limit is approximately 185, centered on 516 nm. The exact equation to calculate the absolute diffraction limit is:

\[ d = \frac{\lambda}{2 \times NA} \quad \text{(Eqn. 3)} \]

where \( \lambda \) is the wavelength of the observed light and NA is the numerical aperture of the collecting objective. For our system, the NA is 1.4. This distance, \( d \), is the minimum size of an airy disc from the peak to the first diffraction minimum. If two emission sources were closer than \( d \) in space, the central maxima of their airy discs would overlap,
making resolution of the sources impossible. We define resolution as the minimum distance the centers of two spots must be from each other to be considered distinct spots. Spots in this work are defined as a 12 pixel area. This is a circle with a 320 nm diameter. Our spot size conservatively presumes a size slightly larger than this absolute optical minimum. If the airy disc is approximated as a Gaussian instead of a Bessel function, our collection diameter would collect 97% for red emission light and >99% for green emission light.

Confocal microscopy was also used for fluorescence recovery after photobleaching (FRAP) studies. In this method, a selected area is illuminated with the appropriate laser at high power, typically 100%, to bleach the local fluorophores. The changes to the spatial distribution of bleached and unbleached fluorophores indicate fluorophore movement, and thus can be used to examine membrane fluidity and protein movement. In this method, frames of fluorescence are taken at regular time points. Each frame consists of a 512x512 set of pixels observing fluorescence from a spatial area 42x42 microns square. For our work, FRAP curves were typically obtained by imaging 3 initial frames, bleaching a selected area for 5 frames or ~500 to 1000 ms, and then imaging 17 or 37 more frames to obtain the recovery. All published work was measured for 37 frames to improve accuracy of data fitting. Pre- and post-bleach frames were imaged at 2µs per pixel. Bleached areas are circles with 125 pixel diameters. Prior to analysis, all FRAP images were adjusted for overall image photobleaching by correcting average intensity to a reference area outside the bleached area. A correction factor was obtained from this outside area by dividing the average intensity at a given time point by
the intensity in frame 1. This factor is the percent drop in intensity due to overall image bleach. The average intensity inside the FRAP area is then divided by this factor for each time point. This corrects all intensities up by a sliding scale based on the total reduction.

**TIRF Microscopy**

Total internal reflection fluorescence (TIRF) microscopy is a technique that selectively illuminates the closest 100 nm of solution to the glass surface with an exponentially decaying evanescent field. The critical angle, \( \theta_c \), for total reflection is dependent on the difference in refractive index between the glass, \( n_2 \), and water, \( n_1 \), solution as:

\[
\theta_c = \sin^{-1} \frac{n_1}{n_2}
\]

(Eqn. 4)

When the ratio of indices is less than 1, total internal reflection occurs. The illumination depth is dependent on the angle of the illumination laser. When this laser is totally internally reflecting, an evanescent field above the glass is created. The rate of decay of this field is dependent on the angle of the illuminating laser, \( \theta \), and decays as:

\[
I_z = I_0 e^{-z/d}
\]

(Eqn. 5)

where

\[
d = \frac{\lambda_0}{4r} (n_2^2 \sin^2 \theta - n_1^2)^{-1/2}
\]

(Eqn. 6)

The term \( \lambda_0 \) is the wavelength of laser light in vacuum. This method has exceptionally high signal-to-noise ratios, making it ideal for single particle tracking. A full review of the technique was published (77). Our experiments were performed on a Nikon Eclipse Ti-U equipped with a TIRF/Epifluorescence illuminator and a 60x TIRF oil objective (NA = 1.49). Images were acquired using an Andor iXon EMCCD with a typical frame
capture time of 200 ms. Two emission channels were available for use on this instrument, red and green. Red channels were excited using a 561 nm diode laser, while green channels were excited using a 491 nm laser. Channels can be imaged simultaneously using a DualView or sequentially using separate filter sets. The former was best for tracking studies while the latter was used in static investigations. Once images were collected, they were analyzed using ImageJ. Protocol for TIRF use is in Appendix B.

CRP binding to aptamer was assessed using TIRF microscopy. Individual chambers were created using CoverWells (Life Technologies, Eugene, OR) that were cut and adhered to cleaned coverslips (No. 1 thickness, Ted Pella). Coverslips were coated with monoclonal, nonspecific anti-CRP antibody (C6, 1:1,000 dilution in 30 mM sodium carbonate/70 mM sodium bicarbonate) for 1 h at room temperature, blocked with 3% BSA for 2 h, and rinsed 3× with 0.01% Tween-20/PBS buffer, and 1× with PBS. CRP (mCRP or pCRP, 6 μg/mL) was preincubated with 0.3 μg/mL of 5′Cy3-RNA in binding buffer for 20 min at room temperature. The samples were then added to each well, incubated for 10 min, rinsed 3× with 20 mM HEPES/140 mM NaCl buffer to remove unbound material, and imaged immediately. Excitation of Cy3 was performed using a fiber launch (Solamere Technology Group, Salt Lake City, UT) equipped with an AOTF to shutter the excitation from a 50 mW, 561 nm diode pumped solid-state laser (Cobalt Jive 50, Cobalt AB, Sweden). Emission was filtered using a 570-nm long pass dichroic (Omega Optical, Brattleboro, VT) and a band pass filter (605/55, Chroma Technology, Bellows Falls, VT). The images were acquired on an Andor iXon EMCCD with an
exposure time of 200 ms and an EMgain of 200 using freely available software (μManager (78)). Each image was filtered using a rolling ball filter (ball radius of 10 pixels) to eliminate background noise and correct for image tilt. The intensity was integrated across the full image to obtain a reading of total fluorescence. TIRF microscopy data are presented as mean ± SE from at least five independent images. A p-value of <0.05 using a Student’s t test was considered as significant.

**Fluorescence Assays**

Steady-state fluorescence techniques were used to illuminate changes to protein structure and binding. The intrinsic fluorescence associated with tryptophan residues in CRP were used for these studies. Both max intensity and peak shift will change in response to the solvent environment the tryptophan is in. As residues transition from a hydrophobic environment to an aqueous environment, the fluorescence decreases and is red shifted. CRP has 30 tryptophan residues in the native, pentameric structure making it a suitable candidate for fluorescence assay development. We used fluorescence scans and anisotropy to investigate the unfolding of CRP. For both assays, CRP was excited at 280 nm and emission collected in a 5 nm window centered on 330 nm. Scans were done on CRP at various stages of denaturing according to the three methods used in this work. In the first method, fluorescence was read at 5 minute intervals from 0 to 90 minutes while heating in 0.01% (w/v) SDS at 80°C. In the second method, fluorescence was read for 1 M increments of concentration from 0 to 8M urea at 37°C for 2 hours. In the final method, fluorescence was read for 0.2 M increments of concentration from 0 to 2.6 M
GndHCl at 37°C for 90 minutes. Fluorescence scans were performed on a Tecan Infinite M1000 plate reader using 200 μL per well.

Anisotropy binding studies were performed on a Perkin Elmer LS55 fluorometer in a quartz cuvette with excitation and emission wavelengths of 555 and 570 nm and slit widths of 2.5 and 10 nm, respectively. Baseline anisotropy of the 5′Cy3-RNA was acquired for 5 min. Changes in anisotropy were measured after normalizing to the initial anisotropy of the 5′Cy3-RNA aptamer in buffer. CRP was then added to the cuvette and incubated for 5 min at room temperature before anisotropy measurements were taken. For the displacement assay, titrations of an unlabeled ssDNA aptamer (79) were added to the CRP-5′Cy3-RNA complex and the measurements were taken immediately. Final concentrations of CRP and 5′Cy3-RNA were 9.1 and 0.59 μg/mL, respectively. Tabulated data were reported as mean ± SD from three independent experiments.

Dot blot assays were prepared to provide direct evidence of aptamer binding to CRP. A 2 μL aliquot of protein was blotted on nitrocellulose membrane, air dried and blocked with 3% BSA+0.05% Tween-20/PBS overnight at 4 °C. After washing 3×with 0.005% Tween-20/PBS, the membrane was probed with 170 nM (2.38 μg/mL) 5′biotin-RNA aptamer for 1 h at 25 °C followed by a 30-min incubation with 1:1,000 streptavidinconjugated IR800 and imaged on an Odyssey imager (Li-COR Biotechnology, Lincoln, NE).

Ministack Analysis

Colocalization has traditionally been a difficult quantity to measure (80). Therefore, we developed a procedure in MATLAB, dubbed Ministack, to compare two
fluorescent channels. In this procedure, one channel was designated as the standard for finding spots. In this work, we used NP channels as spot standards, but any channel with sufficient signal-to-noise and biological justification could act as the spot standard. In the standard image, spots were located by finding the local maximum in a designated size above a threshold. For our work, we choose a threshold of 500 for 200 nm spots, 250 for 100 nm spots, and 100 for 40 nm spots. Size of spots was selected as 9 pixels. This is larger than random noise, but smaller than expected for a diffraction limited spot. A 25x25 pixel area around this maximum is then cut out in the standard channel. Using these maxima, a corresponding spatial location was cut out in the other channels. These two (or more) stacks were then directly compared where each slice pair is a single spatial location. To assess overall colocalization, we averaged each pixel in the 25x25 pixel frame across all slices to give a single, averaged, 25x25 pixel image. These final images showed the average intensity over locations determined by the standard image. If there was colocalization, the second (or third, etc) channels will have increased intensity in the center of the image. Conversely, if there is anti-colocalization, the channels would have had decreased intensity in the center. No preference would have shown an even intensity pattern in the non-standard channels (81). The typical signal-to-noise associated with this method is approximately 300, calculated by taking the mean peak height and dividing by the standard deviation of the background.
Chapter Three: Sensor Characterization

This chapter details the development and characterization of a novel biosensor to investigate the curvature sensing properties of biologically relevant molecules. This tool will enable us to probe the effect of curvature independent from the chemical composition of the bilayer. We achieved three main goals during the development of this biosensor. First, we designed and characterized the lipid covered surface and assessed the fluidity of the lipid bilayer, which is the subject of this current chapter. Second, we investigated the influence curvature has on sorting lipids with different head and tail compositions. Finally, we applied our biosensor to C-reactive protein (CRP), a protein involved in inflammation, and one that potentially has curvature sensing properties. This chapter focuses on the design and characterization of the biosensor.

Using Nanoparticles to Form a Patterned Substrate

Since particles are below the diffraction limit, we first sought to target a specific spot density so that red fluorescent, carboxyl-modified polystyrene NPs are isolated from each other and can be spatially resolved. Stock solutions have rather narrow size distributions. 100 nm NPs have a reported size standard deviation of 5 nm. At high nanoparticle (NP) concentrations, NPs will cluster (82) and form irregular shapes (83) which would give unknown protuberances to the bilayer. Figure 11a and b show a typical pair of images. Target density of NPs is between 0.05 NP/μm² and 0.1 NP/μm². At this density, a majority of spots in the NP channel are isolated NPs, which is
confirmed by a histogram of the average intensities of individual spots (Figure 11c). Intensity indicates particle size, and a monodisperse field of particles will have a narrow distribution. If multiple particles are in a single spot, the intensity of the given spot would be a multiple of the average intensity of a single NP. To confirm we have only single particles, we measured the distribution of spot intensities and analyzed the histogram in two ways.

![Figure 11: A histogram analysis of two images shows that the majority of spots are single NPs. A and B) Two separate images showing the stochastic nature of NP (100 nm) deposition. 42x42 μm². C) Histogram and statistics of average intensity of fluorescence of individual spots.](image-url)
In the first method, we use the t-distribution and a Student’s t-test to check if the observed distribution is binomial with two proposed peaks as multiples of each other. This second peak will be assumed to be at twice the intensity of the median of the primary peak. The median is used instead of the mean to limit the effect of outliers. Additionally, we assume this cluster peak has the same variance as the observed peak. With these assumptions, a Student’s t-test can be performed. This tells us if our observed peak is distinct from the hypothetical 1st cluster peak. If it is not, then we cannot assume that our image has a 1st cluster peak. The calculated test statistic is \( t = 2.83 \), which is larger than the t-statistic (=1.96 for large n, \( \alpha = 0.05 \)). Therefore, our assumption of a binomial distribution is invalid. Another way to conceptualize this result is to consider that only 3.5% of spots are within 1 standard deviation, \( \sigma \), of the hypothetical 1st cluster peak. Yet a third way is to consider that only 2.2% of spots are more than 3\( \sigma \) (the fluorescence intensity of the mean + 3\( \sigma \) is 1513, near the hypothetical 1st cluster peak) from the observed median.

Secondly, we fit this same histogram to two separate models, once as a single Gaussian and once as a sum of two Gaussians. The first model fit very well. The calculated mean fluorescence was 847 ± 12.2 (95% confidence interval) with an \( R^2 \) of 0.927. The second model failed to converge on a solution. Even if the second model was constrained where the parameters of one Gaussian matched those from the first model, no solution was found. With these two methods, we can conclude that the curvature of the lipid bilayer is determined from single particles and fluorescently marked by the locations of these NPs.
Fluid Lipids on a Patterned Surface

Once NPs were deposited, SLBs were formed over top. For this sensor to be a useful mimic of biological activity, the lipid bilayer that is formed must be fluid both in the flat sections and on top of nanoparticles. Additionally, the presence of the NPs should not perturb the fluidity. Fluorescence recovery after photobleaching (FRAP) is an ideal tool to investigate these requirements. For this method, fluorescence is measured for a specific area with a known shape that has been photobleached by high intensity laser light. Fluid bilayers recovered fluorescence exponentially. Fluorescence recovery kinetics are well established, and an elegant mathematical description has been done previously to relate recovery to lipid diffusion rates \((52, 84)\). For circular beam profiles with known size, the determination of diffusion constants is simplified. The diffusion constant, \(D\), is related to the square of the bleach radius, \(r\), the half-time, \(\tau_{1/2}\), and a constant representing motion with a specific bleach shape, \(\gamma_D\), as

\[
D = \left(\frac{r^2}{4\tau_{1/2}}\right)\gamma_D
\]

(Eqn. 7)

For circular bleach areas, \(\gamma_D = 0.88\). Half-time is the time required for the recovery to equal half of its equilibrium value and can be calculated from a fit to the observed recovery. Fitting to a single exponential can give a characteristic time constant, \(k\), as

\[
F_t = F_0 + (F_\infty - F_0) \times (1 - e^{-kt})
\]

(Eqn. 8)

\(F_{t,0,\infty}\) are values of fluorescence at a given time, initially, and equilibrium, respectively. Then half-time is directly calculated from the time constant as

\[
\tau_{1/2} = \frac{\ln (2)}{k}
\]

(Eqn. 9)
Using these values in equation 1, \( D \) can be calculated. Figure 12 shows a representative FRAP series. Here MB-DHPE is recovering over 200 nm polystyrene NPs. Frames were taken at 1 frame per 0.5 s.

![Figure 12: Typical series of FRAP images. A) NP (200 nm) image. B) Series of MB-DHPE lipid images where the bleach frame is at \( t=0 \) s. Images are corrected for overall photobleach, which are then used to calculate relevant fluidity parameters. All images are 20x20 \( \mu m^2 \).](image)

The above technique was used on a variety of nanoparticle sizes. Figure 13 details results showing that NP size has no discernible impact on diffusion constants or percent recovery. Three different NP sizes were used: 40, 100, and 200 nm in diameter. The SLB formed for these data was 98% Soy-PC and 2% MB-DHPE. Compared to recovery over a flat surface, diffusion constants for all particle sizes were not significantly different from recovery over a flat surface (P-value > 0.19, N=15, ANOVA). These diffusion constants are of the same order as diffusion constants of lipids seen in a typical plasma membrane. Above the liquid crystal transition temperature, lipids in a plasma membrane have diffusion constants approximately 1 \( \mu m^2/s \) (85). In SLBs, diffusion constants are approximately 2.0 \( \mu m^2/s \) (53) (8). Table 1 shows diffusion constants of various molecules in different environments. Our sensor is slightly faster than what is reported for lipids in the plasma membrane, but this is expected due to the lack of transmembrane and insertion proteins that slow membrane
diffusion (86). Other work with SLBs without proteins (53) report diffusion constants within error of our observations. Percent recovery had no significant differences for any particle size either (P-value > 0.06, N=15, ANOVA). Recoveries were high, over 90% for each surface, and are similar to what is seen in a typical plasma membrane (85). This indicates that the presence of NPs does not change the mobile fraction.

**Figure 13:** Presence of NPs does not change fluidity parameters. A) Recovery curves are fit to a single exponential recovery. B) The mobile fraction is unchanged and has low variance for each set of trials. C) Diffusion constants were more varied between trials and showed a higher variance within trials. No statistical significance was observed between trials. Error bars are standard error.
It is worth considering what the minimum detectable difference is between two mean diffusion constants or two percent recoveries. This will give an estimate of how sensitive our method is to changes in fluidity compared to a flat bilayer. A full description of this type of calculation can be found in many introductory statistics books. A useful reference is published (87). The minimum detectable difference in mean diffusion constant, $\theta$, is dependent on the total sample size, $N$ (or $n_1 + n_2$), the standard deviation, $\sigma$ (assumed equivalent for two values), the ratio of sample sizes, $r$ (or $n_1/n_2$, as a value greater than 1), and the test statistics for confidence levels selected for Type I and Type II statistical errors, $t_\alpha$ and $t_\beta$, respectively. These are related as,

$$\theta = \frac{(r+1)(t_{\alpha/2}+t_{\beta})\sigma}{\sqrt{rN}} \quad \text{(Eqn. 10)}$$

Calculating $\theta$ is a relatively straightforward operation when $\sigma$ is known from our observed data. Conservatively, we choose $\alpha$ and $\beta$ values equal to 0.05. These are commonly accepted minimum levels for statistical significance. Therefore the test statistics are 2.145 and 1.761, respectively. The average $\sigma$ for $D$ between all sample types is 0.961 $\mu m^2/s$, and $n = 15$ for both samples. Using these values, $\theta$ is calculated to be 1.37 $\mu m^2/s$. For sufficiently large data sets ($n = 30$ and $t$-values become $z$-values), $\theta$ lowers to 0.913 $\mu m^2/s$. A comparable analysis for percent recovery can be done using an average $\sigma$ of 10.2%. Using our conditions of $n = 15$, $\theta$ is 14.5%. For sufficiently large data sets, $\theta$ lowers to 9.69%. The moderately sized $\theta$ values reflect the inherent variability present in SLBs (53). This analysis indicates our biosensor is moderately sensitive to changes in fluidity though even large data sets would be unable to detect fine differences in fluidity parameters.
Table 1: Comparison of diffusion constants from this work to literature observations. Uncertainty is standard deviation (95% margin of error in parentheses).

<table>
<thead>
<tr>
<th>Membrane lipids</th>
<th>Experimental D ($\mu$m$^2$/s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC bilayer w/ NBD-DMPE</td>
<td>$0.94 \pm 0.27$</td>
<td>(53)</td>
</tr>
<tr>
<td>POPC bilayer w/ NBD-PC (C-6)</td>
<td>$2.61 \pm 0.49$</td>
<td>(53)</td>
</tr>
<tr>
<td>Soy-PC w/ MB-DHPE w/o NPs</td>
<td>$3.70 \pm 0.81$ (0.41)</td>
<td>This work</td>
</tr>
<tr>
<td>Soy-PC w/ MB-DHPE w/ 40 nm NPs</td>
<td>$3.18 \pm 0.93$ (0.47)</td>
<td>This work</td>
</tr>
<tr>
<td>Soy-PC w/ MB-DHPE w/o 100 nm NPs</td>
<td>$3.73 \pm 0.93$ (0.47)</td>
<td>This work</td>
</tr>
<tr>
<td>Soy-PC w/ MB-DHPE w/o 200 nm NPs</td>
<td>$3.98 \pm 1.57$ (0.79)</td>
<td>This work</td>
</tr>
</tbody>
</table>

A continuous lipid membrane can be formed over NPs of different sizes allowing a variety of membrane curvatures to be measured simultaneously. In Figure 14, a continuous supported lipid bilayer with 2% MB-DHPE was formed over 100 nm and 200 nm NPs. Here, red fluorescent 100 nm NPs were mixed with green fluorescent 200 nm NPs. However, both could be labeled with the same dye and the size could be determined based on fluorescence intensity (66), allowing another color channel to be available. The fluidity of lipids on a mixed curvature membrane was similar to flat membranes; both the mobile fraction (Figure 14b) and rate of diffusion (Figure 14c) are unaltered. These are also compared to a surface treated with poly-l-lysine. This treatment, applied after cleaning but before lipids, involves adding concentrated poly-l-
lysine to the well. The linked lysine residues are highly positively charged and prevent movement of biomolecules. This creates a nearly immobile surface.

![Figure 14](image)

*Figure 14: Multiple sizes of NP (Green, 100 nm and Red, 200 nm) are imaged in the same sample (panel A, 4x4 μm²). As expected, mobile fraction (B) and diffusion constants (C) are unchanged. These are also compared to poly-lysine, which makes a bilayer immobile. This caused a noticeable drop in both parameters.*

Determination of diffusion constants is quite sensitive to the number of frames used to fit against. Initially, we fitted our FRAP data against 17 frames of recovery. Fit conditions were satisfied, but the calculated half-times for these fits had unacceptably large margins of error, $w$ (calculated for 95% confidence intervals). Fitting the single exponential to 37 frames of recovery reduced the margin of error noticeably. For example, an average fit to 17 frames for a flat MB-DHPE bilayer had an average $w$ equal to 0.22 μm²/s. Increasing the fit to 37 frames drops the half-time margin of error to 0.14 μm²/s (n=5 for both). This change caused our recovery time to increase from 8.8 s to 19.2 s. The only disadvantages of using more frames are microscope drift at long times and overall photobleaching at long times. If either the microscope drifts out of focus or the lipids are completely bleached, a recovery curve is impossible to obtain. Small deviations in these conditions can be corrected by using an area that is not exposed to the high intensity laser of FRAP, and determining an overall bleach factor. This factor is then used to correct the FRAP area for these deviations.
Surface Characterization and Lipid Contouring

We designed the membrane curvature biosensor to contain regions of membrane curvature in continuum with flat membrane. NPs of different diameters are deposited on a borosilicate glass surface to give a controlled surface topology. To form a supported lipid bilayer, liposomes are sonicated into solution and allowed to fuse on the glass surface (61). This creates isolated areas of curvature defined by single NPs. Liposome coverage far exceeds the coverage of NPs, so continuous bilayers are formed from this process. Figure 15 shows a schematic of this biosensor, and the following section details our characterization of this surface.

Our method is based on previously established SLB techniques, and these methods require extremely clean surfaces. Figure 16 is a pair of AFM images showing the change in surface topology before and after the cleaning treatment, which includes cleaning with bleach, SDS, and Hellmanex, an alkaline cleaning agent. The dirty image was taken on glass straight from the box. These images were taken and processed using wet AFM techniques. Surface features prior to cleaning are around 100 nm in height and are numerous. After cleaning, feature size drops to 3 nm and the frequency of features also drops. Note that intensity scales for the images are not the same. Line scans below each image are scaled to the same value and clearly show the reduction in surface topology after cleaning. Additionally, this cleaning method is not so harsh that new
features form, like troughs or large shallow pools. With a cleaned, low topology surface, the next concern would be to ensure the SLB surrounds deposited NPs.

Figure 16: AFM images show a significant drop in surface roughness after cleaning treatment with Hellmanex. Dirty glass (left) has features nearly 100 nm in height. Cleaned glass (right) reduces feature size to 3-5 nm. Note that contrast scales on the two images change to illuminate the different feature topography.
To detect changes to bilayer morphology in the presence of NPs, 1 micron particles were deposited. These particles are larger than the diffraction limit, so a bilayer coated to the surface of the NPs can be directly observed. Typically, our studies use a maximum diameter of 200 nm. A rare opportunity to image three clustered 1 μm with confocal microscopy is seen in Figure 17. In the red channel (Panel A), polystyrene NPs form a triangle pattern. The lipid channel shows the MB-DHPE lipids forming three distinct circles showing the lipids intercalate between the NPs. The diminished fluorescence in the middle of each ring shows that the lipids surround the NP. Panel B shows the FRAP of these lipids imaged at 2 frames per second. After bleach to background levels, new unbleached lipids refill the bleached areas and show the same pattern of fluorescent lipids wrapping around the particles. Intercalated lipids exchange with external lipids. This pattern of recovery is seen for other particles of this size.

Lipids are also observed above the particles. Figure 18 shows another 1 μm polystyrene NP at three different heights. In the center of the particle, designated 0 nm, a line scan shows increased intensity on the sides, at approximately 0.9 and 1.9 microns,
and reduced intensity in the middle. Taking an image 500 nm below this point shows the lipids converging around the base and the background intensity increasing, indicative of fluorescent lipids on the surface of the surrounding glass. 500 nm above the midpoint shows the background at non-fluorescent noise levels, less than 100 a.u., and increased, even intensity above the particle. Fluorescent lipids are surrounding the NP at all points in the z-stack. In conjunction with previous work (88), these results demonstrate that the SLB wraps around and above the NPs, and these lipids are fluid while surrounding the NPs.

![Figure 18: Lipids cover NPs as well as surround them. Lipids surround the NP at the center (center panel). Above the 1 μm particle (right panel), a line scan shows that lipids cover the top of the particle. Below the particle (left panel), lipids are seen merging with the surrounding flat expanse of lipids. The red line shows the position of the line scan.](image)

Using targeted fluorescence quenching of NBD, we have shown that the SLB formed for this biosensor is a monolayer, though not perfectly continuous. DHPE labeled with NBD [2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl] can be quenched using sodium dithionite (68). This is a useful method to determine if our sensor has only a
single bilayer instead of multilayers. Figure 19 shows the integrated fluorescence reduction after addition of sodium dithionite. Before the first minute, fluorescence drops to 50% the initial value, then the rate of drop slows. This initial drop indicates the upper leaflet of the bilayer is quickly quenched. If there was two bilayers, the initial drop in fluorescence would have been to approximately 75% of initial fluorescence, and even less for more multilayers. However, the fluorescence continued to drop after this initial reduction, indicating that the bilayer formed is not a complete barrier. Trans-bilayer transport of the doubly charged dithionite is highly unlikely, but pores could make the bilayer permeable. This biosensor also creates a bilayer on a large scale, several millimeters. It is highly likely that imperfections over this incredibly large distance permit dithionite to get under the bilayer.

Fluorescent imaging in multiple channels can potentially be contaminated by crosstalk and bleedthrough between the various image channels. To address these concerns, we conducted a series of experiments to assess the image contamination caused
by these two phenomena. In our context, crosstalk occurs when the excitation laser of one fluorophore overlaps significantly with the excitation spectrum of a second fluorophore. Thus the second fluorophore’s emission appears to be that of the first fluorophore. Bleedthrough occurs when the emission of a fluorophore excited with a matched laser is sufficiently strong to get past the emission optics of a competing channel and appear as legitimate fluorescence. Figure 21 is a matrix showing various cross excitations to determine the impact of crosstalk and bleedthrough. Here we used 200 nm fluorescent NPs with either red (580/605 nm) or green (505/515 nm) emission as proxies to determine the relative contribution for each channel. These dyes are proprietary information of Life Technologies, but the spectra are known (Figure 20). Though this green emission/excitation is different from the fluorophores we will use (Alexa 488 and

![Figure 20: Spectra of green dyes used in this work. All are very similar. Green NPs have a peak that is slightly red-shifted, making them more susceptible to crosstalk from the red channel.](image-url)
Fluorescein), the spectra are quite similar. The red-shift of the green NPs compared to Alexa-488 make the NP more susceptible to cross-talk. Images are background subtracted and fluorescence measured as average fluorescence for a 7-pixel diameter circle at the spot location. Nanoparticles are the most intense fluorescent objects used in this work, so these numbers represent the maximum bleedthrough and crosstalk that could occur. In the first column, this is expected fluorescence for a NP with matched excitation light and matched emission channel. “Matched” refers to the red channel/fluorophore excited by a 594 nm laser and the green channel/fluorophore excited by a 488 nm laser. This is used as a reference for how much that emission light will contaminate other channels. In the second column, bleedthrough light is assessed by observing fluorescence with matched excitation but mismatched emission channel. Here, red has very weak bleedthrough to green (0.18%) but green has significant bleedthrough into red (25%). This is expected given the overlap in spectra between these two fluorophores. In the third column, crosstalk light is assessed by observing fluorescence with mismatched excitation light and a mismatched emission channel. Again red has very weak crosstalk with green (0.36%). Green’s crosstalk is also weak (1.8%), but more significant than red. Based on these data, we choose to image sequentially, and thus eliminate bleedthrough as a source of contamination because the laser and the channel are always matched for this imaging sequence.
From these data, we chose red as our NP to mark curvature since it has the least crosstalk. This ensures we would not mistake lipid colocalization with NP locations. Therefore green will be used to identify molecules of interest, and we possibly can mistake molecule clusters for NP locations. This is unlikely due to the low crosstalk percentage and relatively high red emission. Taking a ratio of average green crosstalk fluorescence with average red matched fluorescence, the ratio is still only 1.9%. With matched red fluorescence 50x brighter than the crosstalk from the brightest green object possible, other green molecules of interest will not be mistaken for a NP. It should be noted that this analysis can only ever be a proxy. Exact bleedthrough and crosstalk contamination by color. Excitation/emission wavelengths (nm) are shown for each image. Red NPs (top row) have very little contamination into other channels. Both types of contamination are less than 0.5%. Green NPs (middle row) have more significant contamination, particularly as bleedthrough. Ratio to matched fluorescence is shown (bottom table). Images are 2x2 μm² and scaled equally.

Figure 21: Bleedthrough and crosstalk contamination by color. Excitation/emission wavelengths (nm) are shown for each image. Red NPs (top row) have very little contamination into other channels. Both types of contamination are less than 0.5%. Green NPs (middle row) have more significant contamination, particularly as bleedthrough. Ratio to matched fluorescence is shown (bottom table). Images are 2x2 μm² and scaled equally.

<table>
<thead>
<tr>
<th>Color</th>
<th>Matched</th>
<th>Bleedthrough</th>
<th>Crosstalk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>100%</td>
<td>0.18%</td>
<td>0.36%</td>
</tr>
<tr>
<td>Color</td>
<td>100%</td>
<td>24.67%</td>
<td>1.81%</td>
</tr>
</tbody>
</table>
values are dependent on fluorophores used and the relevant microscope settings, most importantly laser power and emission windows on the confocal. Given the low values obtained, the similarity between proxy fluorophores and experimental fluorophores, and similar microscope settings, we are confident this analysis of crosstalk and bleedthrough is consistent for our experimental conditions. Therefore, our conditions will be as follows: NPs are red (brightest), laser powers < 5%, small, nonoverlapping emission windows, and sequential imaging to eliminate bleedthrough.

**Controlling Chemical Composition**

A primary goal of this biosensor is to be able to control the chemical composition of the bilayer independent from the curvature. By mixing lipids in chloroform prior to drying, different lipids are intermingled into a single bilayer and deposited after resuspension. Initially, this was assumed, but Förster resonance energy transfer (FRET) verified our lipids were intermingling during preparation. FRET is a process where energy is nonradiatively transferred from a donor molecule to an acceptor molecule. Donor molecules have excitation/emission bands that are higher in energy than the acceptor molecules. The emission band of the donor overlaps with the excitation band of an appropriate acceptor molecule. This overlap permits nonradiative transfer if the molecules are close enough (<10 nm) and the donor is in an excited state. Molecules need to be extremely close, typically 3-5 nm for most efficient transfer. This has interesting implications for FRAP. Prior to bleaching, little to no emission would be seen in the donor channel. If the acceptor molecules are bleached, and thus unable to accept the energy transfer, the donor emission would go up during the bleach frame. Subsequent
frames would show a decrease in this donor emission. This technique is called fluorescence loss after photobleaching (FLAP). If this fluorescence loss exists, then both lipids are in the same bilayer.

To test our ability to mix and control the bilayer chemical composition, we tested the addition of a second fluorescent lipid after a film of initial composition was created. Bilayers were initially mixed and dried with 97.9% PC and 2% MB-DHPE (donor) via chloroform evaporation, and then 0.1% Fl-HDA (acceptor) was added via DMSO and sonicated in deposition buffer. Percentages are final molar percents. Our FRET studies of this mixture are shown in Figure 22. When imaging these bilayers in the MB-DHPE channel, nearly no fluorescence was seen. However, bleaching in the FL excitation band at 488 nm caused a sudden increase in fluorescence in the MB emission channel. This indicates that the molecules were experiencing FRET, but the destruction of acceptor molecules by bleaching temporarily stopped the transfer.
To explore mixed mobility of lipids on curvature, we conducted an analysis of variance (ANOVA) analysis of the FRET and FLAP data. This type of analysis would indicate if the curvature had a different effect on diffusion when molecules are mixed as opposed to being in a bilayer containing only one type of fluorescing lipid. Differences when two types of lipid are present would arise if lipid domains form when one lipid is present.

Figure 22: FLAP and FRET of mixed MB-DHPE and Fl-HDA bilayers. A) Loss and recovery curves of MB-DHPE (blue) and Fl-HDA (green). Mobility is similar for multiple extents of curvature. B) MB-DHPE (acceptor) fluorescence increases when Fl-HDA (donor) molecules are bleached. C) Fl-HDA fluorescence is lost when bleached. Scale bar is 3 μm.

To explore mixed mobility of lipids on curvature, we conducted an analysis of variance (ANOVA) analysis of the FRET and FLAP data. This type of analysis would indicate if the curvature had a different effect on diffusion when molecules are mixed as opposed to being in a bilayer containing only one type of fluorescing lipid. Differences when two types of lipid are present would arise if lipid domains form when one lipid is
not miscible with the other domain. Diffusion would be hindered. An examination of the diffusion constants when these lipids are solo in a PC bilayer versus mixed in a PC bilayer was inconclusive. Table 2 summarizes the diffusion constants. An analysis using a 2-Way ANOVA revealed MB-DHPE diffusion was unperturbed by the presence of additional lipids, specifically Fl-HDA. We defined 2 sources of variation: one as a change in curvature (flat, 40 nm, 100 nm), second as a change in mixing (mixed or not). The interaction P-value for these variables was 0.7106, as expected for independent variables. The high P-value indicates that the variance is very likely due to random variation (71.1% chance). This indicates that the effect of mixing the lipids has the same effect for MB-DHPE on any ROC. This insignificance permits further interpretation of the two sources of variation. Neither curvature nor mixing had a significant impact on diffusion constants (P-values > 0.40). Diffusion of MB-DHPE is neither perturbed by curvature nor mixing. However, a similar analysis of Fl-HDA for these two variables revealed inconclusive results. The interaction variable was significant (P-value = 0.0497), which does not permit interpretation of individual effects. This indicates that the two variables for Fl-HDA are not independent and would imply that curvature affects the mixing properties of the lipids, and thus the diffusion. As the ROC increases, diffusion of the sensing molecule (Fl-HDA) is more hindered at lower ROC than it is when diffusing alone for those same ROCs. This result is intriguing, but should be taken with caution. The FRET data here has a low number of trials, and the recovery curve is only fit to 17 frames. Both of these lead to very wide confidence intervals which decrease the
likelihood of finding small variations, and fitting to a small number of frames can lead to erroneous results entirely.

Table 2: Comparison of diffusion constants for two lipids. Analyzing by a 2-Way ANOVA, MB-DHPE is unchanged by the presence of FRET. Fl-HDA is inconclusive as indicated by the nonlinear changes in a mixed bilayer.

<table>
<thead>
<tr>
<th>Diffusion Constants (μm²/s ± 95% Confidence)</th>
<th>Flat</th>
<th>40 nm</th>
<th>100 nm</th>
<th>n</th>
<th>Fit Frames</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB-DHPE (solo)</td>
<td>3.70 ± 0.41</td>
<td>3.72 ± 0.47</td>
<td>3.73 ± 0.47</td>
<td>All = 15</td>
<td>37</td>
</tr>
<tr>
<td>Fl-HDA (solo)</td>
<td>2.57 ± 0.14</td>
<td>2.32 ± 0.09</td>
<td>2.09 ± 0.11</td>
<td>All = 5</td>
<td>37</td>
</tr>
<tr>
<td>MB-DHPE (mixed)</td>
<td>3.57 ± 0.40</td>
<td>3.38 ± 0.34</td>
<td>3.41 ± 0.48</td>
<td>Flat = 5</td>
<td>17</td>
</tr>
<tr>
<td>Fl-HDA (mixed)</td>
<td>1.31 ± 0.16</td>
<td>1.18 ± 0.20</td>
<td>1.24 ± 0.24</td>
<td>Flat = 4</td>
<td>17</td>
</tr>
</tbody>
</table>

To demonstrate that our biosensor is broadly useful, we incorporated lipids that mimic the intracellular membrane. The intracellular leaflet of the plasma membrane in a healthy cell is often markedly different than the extracellular leaflet. For example, in endothelial cells, nearly all phosphatidylcholine and sphingomyelin lipids are on the outer leaflet, while phosphatidylethanolamine and phosphatidylserine (PS) lipids are on the inner leaflet (89). The early stages of apoptosis will cause PS lipids flipping to the outer leaflet and be exposed to extracellular proteins (10). We were able to form stable bilayers using 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) at relatively high concentration. Table 3 details the diffusion constants and percent recoveries of bilayers with increasing mole percent DOPS. MB-DHPE is kept constant at 2%, and soy-PC is
reduced proportionally (Ex: For 5% PS, there is 2% MB-DHPE and 93% soy-PC).

Recoveries were fit to 17 frames.

**Table 3: Diffusion constants (<D>) and percent recoveries (<%R>) for bilayers formed with DOPS.**

<table>
<thead>
<tr>
<th></th>
<th>Diffusion Constants (μm²/s ± 95% Confidence)</th>
<th>Percent Recoveries (% ± 95% Confidence)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% PS - &lt;D&gt;</td>
<td>4.62 ± 0.18</td>
<td>5.45 ± 0.20</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>92 ± 2</td>
<td>88 ± 2</td>
<td>5</td>
</tr>
<tr>
<td>10% PS - &lt;D&gt;</td>
<td>5.16 ± 0.29</td>
<td>5.86 ± 0.60</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>86 ± 2</td>
<td>94 ± 2</td>
<td>5</td>
</tr>
<tr>
<td>20% PS - &lt;D&gt;</td>
<td>5.72 ± 0.19</td>
<td>5.46 ± 0.25</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>91 ± 1</td>
<td>93 ± 1</td>
<td>5</td>
</tr>
</tbody>
</table>

We demonstrate that our biosensor forms fluid bilayers in the presence of relatively high PS concentrations and that the negative charge does not interfere with bilayer fluidity or formation. In future experiments, this will enable us to explore the effect of PS on curvature sensing, which mimics aspects of early apoptosis. PS also has a strong negative charge, which could cause significant changes to protein recruitment.

**Conclusions from the Development of the Sensor**

The first major contribution of this work is the development of the biosensor itself. This tool consists of isolated nanoparticles covered in an SLB with defined curvature independent from composition of the SLB. Use of this tool enables efficient investigations into membrane curvature sensing. The current art of curvature sensing has
some disadvantages, and this new biosensor overcomes many of these hindrances. We demonstrate the dynamic equilibrium between lipids at a site of curvature and lipids on a flat surface. The majority of our studies were done after one hour of incubation time. Given the diffusion constants observed, this is long enough for the system to reach equilibrium. Therefore, a binding molecule, whether lipid, protein, or anything else, can bind anywhere on the continuum of lipids, then migrate to sites of ideal curvature. Good spatial resolution is obtained from imaging individual NPs. Fluorescent NPs identify sites of curvature, and easy alignment between fluorescent channels makes spatial correlation between species of interest and curvature straightforward. In addition to the studies in presented here, this is ideal for investigating single particle motion, which is being pursued by other lab members.

Composition of the bilayer is controlled separately from the curvature of the bilayer. We demonstrated surfaces patterned with one type and two types of NPs, but this diversity can be scaled up to include a distribution of particles sizes. Using fluorescence intensity as an indicator of size (66), a surface containing a range of particle sizes can be examined as a continuous surface with a wide variety of curvature. The inherent polydispersity from using silica nanoparticles would be ideal for use with this biosensor. We mixed DOPS into soy-PC bilayers at varying mole fractions. This is controlled during lipid film preparation and demonstrates our ability to control composition independent of curvature control. Other lipids can be added via DMSO after film formation.
Analysis of the effect of mixing was only partly conclusive. For MB-DHPE, a molecule that does not sense curvature, we conclude that the presence of mixing has no impact on fluidity, with curvature or without. On the other hand, the observed diffusion for a sensing molecule, Fl-HDA, does seem to show curvature affecting molecule mixing. We speculate that the cause of this interaction term could be from the change in spatial distribution of sensing molecules versus non-sensing molecules. Sensing molecules, having an equilibrium favoring curvature, would outcompete non-sensing molecules at those sites. Sensing molecules would displace non-sensing molecules causing an exclusion of non-sensing molecules at sites of curvature, which would impact the ensemble diffusion of both molecule types. Figure 23 schematically depicts this speculation. This interaction would depend on the molecule’s sensitivity to the particular ROC. More sensitive molecules would have large effects on mixed diffusion. We recognize that this line of research needs to be explored more fully. Longer recovery times for mixed bilayers need to be measured, and single-particle studies would help illuminate any areas of exclusion that exist. Overall, our results show that FRET occurs between MB-DHPE and Fl-HDA, and this indicates that lipids are within 10 nm of each other on the supported bilayer.
Figure 23: Potential mechanism for mixed bilayers. Sensing molecules, in orange, equilibrate toward more curvature association. As these molecules redistribute (A to B), they displace non-sensing molecules, in blue. This displacement would hinder diffusion of sensing molecules, causing curvature to affect sensing molecules differently than non-sensing molecules.

Spatial dynamics are easily studied using this biosensor. Curvature is well defined by the Gaussian fluorescence intensity distribution of the bright NPs, and multiple channels of fluorescence permit many different species to be studied. Designed with TIRF microscopy in mind, the biosensor has been used to address dynamics on two levels, ensemble and single molecule. FRAP is well defined as a method to assess mobility, and single particle tracking is an emerging technique able to study particle motion in fine detail. Both levels are accessible using this biosensor. We foresee this substrate as being highly useful when measuring transient interactions between lipids or proteins and regions of membrane curvature.

A major disadvantage of this biosensor is that it is still hindered by the diffraction limit. Resolution below ~300nm is impossible using direct imaging techniques. Therefore, it is impossible to distinguish between binding to negative curvature at the wings of a NP or the positive curvature on top of a NP. A way to overcome this limitation is by using superresolution techniques (such as stochastic optical reconstruction microscopy (STORM) (90)). Use of total internal reflection fluorescence
microscopy on the biosensor gives a signal-to-noise ratio sufficiently high for these techniques, and this is ongoing research in the lab.
Chapter Four: Lipid Sorting

Before tackling the effect of curvature on proteins, we assessed the effect curvature had on lipid partitioning for some known curvature sensitive and curvature insensitive molecules. Using the ministack method we developed, we assess curvature sensitivity by comparing lipid fluorescence at sites of curvature. Molecules that sense curvature have increased fluorescence spatially coincident with nanoparticle locations; those that do not will have uniform fluorescence across the field of view.

Lipids Partition to Curvature

We use DHPE-MB as a bilayer marker. While curvature does not affect the rate of diffusion or percent mobile fraction, it remains to be seen whether DHPE-MB itself has curvature sensitivity. Using a mixture with 98% soy-PC and 2% MB-DHPE, images were taken of a fluid SLB over 100 nm NPs. Full images were scanned at 12.5 μs/pixel. In Figure 24, ministack analysis shows that at sites of curvature, DHPE-MB does not have increased fluorescence and thus no curvature sensitivity. The averaged image is nearly uniform in intensity and a line scan across the center shows no noticeable increase. A line scan through the center of the image shows no

Figure 24: DHPE-MB does not sense curvature. Ministack analysis of lipids (right) shows no increase at 200 nm NP locations (left). Line scan is below. Red line indicates scan. Image size is 2x2 μm².
increase at sites of curvature. This supports DHPE-MB as an appropriate choice as a reporter for soy-PC fluidity.

Two other lipids have previously been shown to have curvature sensitivity, Fl-DHPE and Fl-HDA (3). When included in the SLB each at 2% by mole, deposited over 100 nm NPs, and imaged, both of these lipids were more abundant at sites of curvature than in flat areas of the bilayer. Fl-DHPE and Fl-HDA both show colocalization with regions of membrane curvature after a ministack analysis. Red fluorescent NPs (Figure 25a) are imaged followed by imaging of Fl-DHPE (Figure 25b) and an overlay reveals that most NPs accumulate Fl-DHPE (Figure 25c). To quantify the ability of regions of membrane curvature to bind lipids, a colocalization method was developed that involved finding sites of curvature using an existing automated algorithm (81) then cropping a 25 x 25 pixel area around that region from both the green and red channels. If a green spot appears in the center, an interaction exists between the two differently colors species. The average cut out image for both lipids clearly reveals that Fl-DHPE and Fl-HDA prefer sites of curvature (Figure 25d). The intensity can be quantified by plotting a line scan. The normalized line scan conveys the enrichment of the green fluorescent lipids for sites of curvature (Figure 25e). Fl-DHPE is more enriched than Fl-HDA, a single tailed lipid, which is consistent with previous work (3). High lipid fluorescence not at sites of curvature likely are lipids associating with non-fluorescing nanoparticles.
Figure 25: Fluorescein labeled lipids accumulate in regions of curvature. A-C) Images of the NPs (A) and Fl-DHPE (B) and an overlay (C) are shown. Images are 6.5x6.5 μm². D) To quantify the colocalization, the red NPs are located using an automated algorithm and a 2x2 μm² region is cropped around each NP. The same region is cropped out of the lipid images. An average of all cropped images shows the accumulation of Fl-DHPE and Fl-HDA at regions of curvature. Additionally, a randomized image of Fl-DHPE shows no accumulation. (C) A normalized cross section of Fl-HDA, Fl-DHPE and a blank (containing no green fluorescent lipids) shows that the accumulation is not bleedthrough. Fl-DHPE is more sensitive to curvature than Fl-HDA.
As an additional negative control, we randomized the NP images for the data from Fl-DHPE lipids. This scrambles any correlation the NP and lipid channels originally had. If the increased fluorescence at NP locations is real and not an artifact of our ministack method, scrambling the NP locations should give an even field of view in the final averaged cutout. Using a plugin for ImageJ, JACoP (91), the NP image (Figure 26a) was divided up into 4x4 squares and randomized. Figure 26b shows this new randomized image with an overlay on the original image (Figure 26c). Both the original and randomized images were used as a basis for determining NP locations for the ministack procedure. The matrix of final, averaged images (Figure 26e-h) shows the results of this analysis. The randomization has no effect on the average NP image, which is to be expected. Since the NP image is used to find spots, it should resolve into a single spot after image averaging. However, the average lipid images show a significant change. After randomizing the NP image, there is no increase in lipid fluorescence at NP locations (Figure 26h, also in 25d).
Labeling only one leaflet of the bilayer results in the same spatial distribution as has been shown. Our current method to mix lipids together via sonication will disperse fluorescent lipids to both leaflets of the SLB. Using a method originally developed for cells (92), we asymmetrically labeled SLBs with Fl-HDA using fatty acid free bovine serum albumin (BSA). By preloading BSA with Fl-HDA then incubating this BSA with the SLB, this method inserts HDA into only the outer leaflet of the SLB after the SLB is formed on the glass substrate. Transbilayer flip-flop is a concern, since flip-flop for fatty acids is significantly faster than typical phospholipids (93). Uncharged hexadecanoic acid flips with a half time of 3.5 s in egg-PC and cholesterol mixed GUVs. However,

Figure 26: As a negative control, randomizing the image eliminates accumulation seen at sites of curvature. A-C) When overlaying an original NP image (A) with a randomized counterpart (B), there is no colocalization in the overlay (C). D) Lipid images remain unchanged. E-H) A matrix of Ministack analyses show a loss of accumulation when the randomized image is used to determine NP locations. Image size for A-D is 42x42 μm², and E-H size is 2x2 μm².
fluorescein is only fluorescent when charged, which causes a significant barrier to flip-flop. Figure 27 show the results of these experiments. Increases for 200 nm NPs were the most significant, with a 32.2% increase in fluorescence from baseline to the peak of the line scan. This is similar to the increase shown in Figure 25e for Fl-HDA present in both leaflets over 200 nm NPs. 100 nm and 40 nm also increased 11.6% and 1.7% at the peak, respectively.

![Figure 27: Asymmetric labeling of the lipid bilayer does not change curvature sensing. A) An example overlay of the Fl-HDA channel and the nanoparticle channel shows strong colocalization in the merged image. B) Ministack analysis of Fl-HDA. With increasing ROC, the amount of colocalization increases. All images are 2x2 μm² and each channel is scaled the same. C) Line scans of the Fl-HDA ministacks quantify sensing ability by plotting fluorescence intensity against position.](image)

**Recovery Directly over Nanoparticles is Slower than Flat Areas**

Finally with regard to lipids, we have observed lipid recovery directly over NPs. There was a concern that since the NPs are below the diffraction limit, recovery could be
only surrounding the particles, and the lipids on top would not be mobile. Using images from the Fl-DHPE experiments, lipids show recovered fluorescence at NP locations after 2 minutes of recovery time, instead of the usual 15 seconds. In panel A of Figure 28, an example NP with associated recovery is shown. The increased fluorescence that is seen before bleaching is also seen after bleaching. If these lipids were not mobile, they would not be able to recover with unbleached lipids further away (> 2 μm). In panel B, the slower recovery is seen in the plotted fluorescence against a longer time scale than seen previously. Fitting to a single exponential, the observed diffusion constant for this fluorescence directly over the sites of curvature was 0.64 ± 0.17 μm²/s (95% confidence interval). The <R²> for this fit was 0.835 (n=5).

To elucidate the mechanism for this reduction in diffusion constant, we chose to treat these data as containing two exponentials with respective k values, k_l and k_b, where k_l is the k value for the lipids, and k_b is the k value for any additional bilayer properties (Ex: defect sites or microdomains). The model these data were fit to is:

\[
F_t = F_0 + (F_\infty - F_0) * P_f * (1 - e^{-k_l t}) + (F_\infty - F_0) * (1 - P_f) * (1 - e^{-k_b t}) \quad (Eqn. 11)
\]

F_t, 0, \infty are fluorescence intensity values at a given time, initially, and equilibrium, respectively. P_f is a partition term or the fractional contribution from the faster exponential (k_l), which makes 1 minus P_f the fraction contribution from the slower exponential (k_b). Therefore, we fit the same data as in Figure 28b to two exponentials, but constrained one parameter, k_l, to a constant value, 0.206 s⁻¹, which corresponds to a
\[ D_1 = 1.72 \mu m^2/s. \] This value is the constant observed for Fl-DHPE on a flat bilayer.

Figure 29 shows recovery over flat glass side by side with recovery over a NP. With this constraint, the new fit converged for 4 of 5 curves with an \( <R^2> \) of 0.880 and the average

---

**Figure 28:** Lipids over NPs are in dynamic equilibrium with the surrounding lipid pool. A) After bleaching, fluorescence recovers (left panels) at NP locations (right panel). Scale bar is 1 \( \mu m. \) B) Directly over NP, fluorescence intensity (\( <F> \)) recovers after bleaching at \( t=0s. \)
\( k_b \) for these fits was 0.036 s\(^{-1}\). Table 5 summarizes the parameters from this model and other models. This is an improvement, but an analysis using an F-test is needed to determine if this is a meaningful improvement in lieu of the change in model parameters. When increasing the number of fit parameters to a data set, the goodness of fit will increase and the degrees of freedom will decrease. The F-test will robustly account for this decrease in degrees of freedom and allow a comparison between models. This test is similar to the Student’s t-test as it is a test of significance, and the F-test becomes a unidirectional t-test when only one parameter is compared.

The observed diffusion constant for \( k_b \) was \( D_b = 0.28 \pm 0.20 \) \( \mu \text{m}^2/\text{s} \). The F-test analysis would indicate that, if an improvement is seen, the new effect is hindering recovery.

Using an F-test, we determined that this additional exponential term improves the fit in a statistically significant way. Our null hypothesis was that the fit to two exponentials shows no improvement over a fit to one exponential. Table 4 summarizes the calculations for determining the F-statistic, which was calculated to be 7.77. The null model (one exponential) had 34 degrees of freedom and the alternative model (two exponentials) had 33 degrees of freedom. Using these values, the calculated
P-value for this comparison was 0.0087, or a less than 1% chance that the improvement in fit happened by random chance.

Table 4: Statistical calculations showing the improvement in fit from a 1-exponential fit to a 2-exponential fit. This F-statistic corresponds to a P-value of 0.0087.

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Variance</th>
<th>F-Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Improvement (Difference)</td>
<td>0.0777</td>
<td>1</td>
<td>0.0377</td>
<td>7.77</td>
</tr>
<tr>
<td>2-Exponential Fit (Alternative)</td>
<td>0.1601</td>
<td>33</td>
<td>0.00485</td>
<td></td>
</tr>
<tr>
<td>1-Exponential Fit (Null)</td>
<td>0.2378</td>
<td>34</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In addition to the analysis above, we considered alternative analyses to support our conclusion. 1) First, we used a 2-exponential model that had no constraints, and fit this model to the data. Only 3 out of 5 curves converged on a best solution, but these three had a good averaged $R^2$ value of 0.877. Time constant parameters from this model (Table 5) are in broad agreement with the parameters from the constrained model. However, large confidence intervals, particularly for the partition term, $P_f$, make an assessment of close agreement impossible. 2) Secondly, we assigned a model that included three exponentials and added two parameters, a third time constant, $k_3$, and a second partition term, $P_2$. This model failed to converge to a solution for any curve due to the parameters being statistically interdependent. This result strongly implies that the 3-exponential model is overcomplicated for this data and that a 3rd exponential is not present. To further confirm this conclusion, we assigned a model using four exponentials. This also failed to converge on a solution. 3) Finally, we assigned a model
using a distribution of exponentials and fit this to the curves. In this model, 100 evenly spaced (on a log scale) time constants are selected. The upper and lower bounds are calculated as the inverse of the largest and smallest observed spacing between time points in the observation range of the recovery: lower bound from the time between two data points, and the upper bound from the time between the first and last data points. Each of the 100 exponentials has a partition term, \( P_i \), and this partition term is varied until the sum of the squares is minimized or the best fit is obtained. This method failed to converge on a best solution due to the large number of fitting parameters. The model fits 100 variables while the recovery curve only has 37 data points. Even if a solution would be found, this model would significantly overfit the data observed.

**Table 5:** Parameters to various models considered regarding fluorescence recovery over single nanoparticles. Uncertainty is ±95% Confidence Intervals.

<table>
<thead>
<tr>
<th>Model</th>
<th>( k_1 ) (s(^{-1}))</th>
<th>( k_b ) (s(^{-1}))</th>
<th>( k_3 ) (s(^{-1}))</th>
<th>( P_f )</th>
<th>( P_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1-Exponential Fit</strong></td>
<td>0.077±0.020</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>2-Exponential Fit</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with ( k_i ) constraint</td>
<td>[0.21]</td>
<td>0.028±0.024</td>
<td>N/A</td>
<td>0.51±0.17</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>2-Exponential Fit</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>without constraint</td>
<td>0.30±0.14</td>
<td>0.018±0.016</td>
<td>N/A</td>
<td>.37±.52</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>3-Exponential Fit</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distribution of Exponentials</td>
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</table>

**Conclusions from Lipid Sorting**

Our work has shown that bilayer curvature can provide a means for sorting lipids. Curvature based sorting was tested on two fluorescein labeled lipids (Fl-DHPE and Fl-HDA) using the membrane curvature substrate designed and characterized here. On the
substrate containing 100 nm diameter nanoparticles coated with a lipid bilayer, Fl-DHPE binds with a higher density than Fl-HDA. This trend is observed in other work, where liposomes \((d \sim 100 \text{ nm})\) bind approximately twice the amount of Fl-DHPE compared to a 12 carbon fluorescein labeled fatty acid \((3)\). Based on this previous work, the number of carbons was proposed to be directly related to the density of lipids that bind to membrane curvature, with longer chain lipids binding at higher density to regions of curvature. Our work supports this by showing a slight increase in binding for Fl-DHPE, a lipid with two 16 carbon tails, relative to Fl-HDA, which has only one. We have also shown that this sorting occurs when both leaflets are loaded with a sensing molecule or when only the outer leaflet is loaded with a sensing molecule. These results also address potential criticisms regarding the lipid directly interacting with the nanoparticle and affecting spatial distribution. Loading the bilayer asymmetrically eliminates fluorescent lipid-NP interactions.

Our data clarify a controversy between other research \((5,3)\) and fills in a gap. Theoretical work \((5)\) indicates that lipid shape has a minimal effect on sorting into curvature. In this work the curvature coupling constant, \(\Gamma\), was measured for a series of NBD labeled phospholipids. NBD-DHPE has a coupling constant of \(-2.13 \text{ nm}\), indicating a preference for negative curvature. The following equation can be used to predict the enrichment of lipids at on the outer leaflet of a liposome of curvature, \(H\):

\[
n_o = \left(\frac{1}{2} + \frac{D}{2}H\right) + \Gamma H
\]  
(Eqn. 12)

Where \(D\) is the thickness of a lipid bilayer and \(H\) is the curvature. \(H = 0.018 \text{ nm}^{-1}\) and \(D\) is 3.6 nm for PC bilayers, 49% of the DHPE lipids should be on the inner leaflet and 51%
on the outer leaflet. More are on the outer leaflet merely because of the surface area
difference. If a lipid shows no curvature preference, this would be 47% and 53%,
respectively. Our observations show this difference to be much larger.

There are three possible reasons for our disagreement with this prediction. 1) The
shape of our substrate is not spherical. Within a diffraction limited area, the outer leaflet
contains both positive and negative regions of curvature. Our dye labeled lipid is added
during sonication and can be on either side leaflet. It is possible that Fl-DHPE binds to
regions of negative curvature on the inner leaflet as it curves around the top of the
nanoparticle and on the regions of negative curvature on the outer leaflet as the lipid
bilayer transitions from the nanoparticle to the flat supported lipid bilayer. Under the
fluorescence microscope this would appear as one spot, colocalized with a nanoparticle.
2) The fluorescein label is large relative to the NBD label, which could cause the
molecular shape of Fl-DHPE to be slightly different than that of NBD-DHPE, altering the
curvature preference of the lipid. Fluorescein is more hydrophilic, and due to the
difference in structure, we speculate has significantly larger hydrodynamic radius. Our
data do show a difference between DHPE when labeled with fluorescein versus marina
blue, indicating that fluorophore choice has an influence. However, NBD, MB and
Fluorescein head group labeled lipids are likely above the surface of the membrane, so
the mechanism remains unclear. 3) The concentration of Fl-DHPE (2%) is much higher
than previous work on NBD-DHPE (0.01%), which brings out different features in
curvature sensing. Recent work has suggested that cooperative lipid behavior can
facilitate curvature sensing (4).
Given the symmetry to our sensor design, the first reason seems the most likely explanation for our differences. This biosensor design neatly removes the theoretical differences associated with differences in surface area. Since we see lipid increase at sites of curvature, our data supports the presence of another mechanism separate from differences in surface area. Defect sites only occur at sites of positive curvature, and are a likely explanation to the differences observed in this work.

Curvature sensing lipids (Fl-HDA and Fl-DHPE) recover more quickly on flat surfaces compared to these lipids directly over sites of curvature. Our analysis supports a separate mechanism that is hindering lipid diffusion at sites of curvature that is not individual lipid diffusion. We propose two potential mechanisms for this hindered diffusion. First, defect sites (3) are consistent with these observations. Defects are areas in the lipid membrane where head groups separate to reveal the hydrophobic tails. Movement of these sites can be conceived as similar to movement of positively charged holes in a doped semiconductor. If this comparison is valid, then defect sites would have an independent diffusion constant that is hindered by the neck of the curved bilayer where there is significant negative curvature.

Second, we consider that the extended recovery time is due to microdomain formation at sites of curvature. This would limit the exchange from bleached fluorophores on top of the particle and fresh fluorophores in the flat areas, thus potentially causing the observed reduction in diffusion constant. Indeed, large ordered domains do have diffusion constants on the order of what is calculated here (~0.1 μm²/s), but these domains are 0.3 to 3.0 μm (94). Diffusion of domains is inversely proportional
to radius. If microdomains are causing the effect seen in the current work, we predict the size boundary to potentially range from 25 nm up to 300 nm (the diffraction limit). Using the well known Saffman-Delbrück model (51), the calculated diffusion constant of the domains range from 0.5386 μm²/s to 0.3263 μm²/s, respectively. This range is similar to our observed $D_b$, though the largest calculated microdomain is still faster than our observation. This size is also very near the diffraction limit, meaning we would likely see individually moving spots replace bleached fluorescence if this is the cause of our observed $D_b$.

In conclusion to this section, we have shown that lipids sort to different areas of our biosensor according to the curvature of that bilayer. In congruence with previous work, through using our novel ministack procedure, Fl-DHPE was more sensitive than Fl-HDA to sensing curvature. We also have shown that recovery at sites of curvature is hindered, and this hindrance could be another diffusing entity.
Chapter Five: C-Reactive Protein Senses Curvature

Introduction of CRP

C-reactive protein (CRP) is a positive acute phase protein that participates in activating the classical compliment immune response which, among many things, clears damaged tissue and dying cells. It was first discovered by Tillett and Francis in 1930 (95), and is named because it precipitated C-polysaccharide derived from pneumococcal cell walls. Phylogeny is highly conserved, and there is no known deficiency in humans. It modulates complement activation in both apoptotic and necrotic cells (96,70). Inflamed tissue can show a thousand-fold rise in CRP levels from basal levels (97), which makes it risk factor for cardiovascular disease (CVD) and other inflammatory diseases (98). There is growing evidence that CRP actively participates in vascular events and is not simply a marker. In fact, CVD relevant studies of CRP include the following: it has been shown to induce apoptosis in human endothelial cells (99), induce production of pro-inflammatory cytokines (100), increase foam cell production (101), increase phagocyte activity (96), destabilize plaque (102), and interestingly, stabilize plaque (96). Other activities have also been identified (103). Isoform conversion has been a subject of particular interest and has been proposed to be responsible for conflicting observations of pro- and anti-inflammatory effects of CRP (104,105,106,107).
The structure of CRP is well characterized by X-ray crystallography. Native CRP is a homopentamer forming a pentagon with C5 symmetry. The crystal structure is shown in Figure 30 (108). On one face of the pentagon are binding sites for calcium and phosphatidylcholine (PC). PC is one target for CRP and is found in all plasma membranes in the body, though it does not bind ubiquitously. This binding is dependent on calcium concentration, and the calcium site is near the PC site. The opposite face binds C1q (109). CRP is approximately 10 nm across the face and 3 nm thick. Monomers are held together noncovalently, and each monomer has C1 symmetry.

Isoform conversion has been implicated previously (97) as a mechanism for CRP binding to endothelial cells. This work has shown the breaking up of native CRP (a pentamer) into subunits, deemed mCRP\textsubscript{m} (a monomer or modified subunit that is membrane bound). Subunit conversion occurred at monolayers and in liposomes of egg PC/lyso PC, and at the plasma membrane of Jukart T cells. Conversion also occurs with the disruption of the single, intra-subunit disulfide bond (110). This disruption retains monomer activity, but prevents pentamer formation. Intrinsic tryptophan fluorescence was shown to decrease in the presence of egg PC/lyso PC liposomes. This indicates the loss of tertiary structure in pCRP as tryptophan residues were exposed to aqueous solvent. Secondary structure was conserved, as demonstrated by circular dichroism experiments (97). Using electron microscopy, the circular structure of pCRP was observed converting to individual subunits over the course of 48 hours on egg PC/lyso PC monolayers. Furthermore, the neoepitope expression ratio remained constant, suggesting the membrane bound mCRP\textsubscript{m} retained the conformation of pCRP. After
detachment from the membrane, these mCRP\textsubscript{m} molecules showed antigenicity equivalent to mCRP, and caused endothelial cells to generate interleukin-8 and chemokine ligand 2 more efficiently than pCRP. Interleukin-8 and chemokine ligand 2 are proteins that induce an immune response targeted to the sites of protein generation. When reacted with C1q, this membrane-bound conformer showed increased binding to C1q, suggesting more efficient complement activation. This work indicates strongly that the plasma membrane plays a significant role in CRP isoform conversion and CRP function.

Additional research \textit{(71)} has investigated the interaction between mCRP and lipid microdomains in endothelial cells. Using human aortic endothelial cells (HAECs), researchers demonstrated an increased affinity to the cell surface and increased internalization speed of mCRP over native CRP. mCRP also showed a factor of 20 increase in maximal protein binding to the cell surface over its native counterpart. Binding was concentration dependent, saturable, and fit well to a single-site binding model. Using egg-PC monolayers as a substitute lipid membrane, the researchers observed an increase in surface pressure upon addition of mCRP, which indicates the protein inserts into the monolayer. As further evidence, they showed colocalization between fluorescein tagged mCRP with fluorescent markers that bind to lipid raft microdomains both in the model eggPC system and in HAECs. The results of this study indicate lipid rafts as viable binding domains for mCRP. Cholesterol is a possible binding partner and CRP is thought to have two cholesterol binding domains \textit{(111)}. Taken together, these studies reveal interesting new questions to be answered regarding CRP’s interactions with apoptotic cells.
Modified CRP shows a stronger link to pro-inflammatory effects than native pCRP \(105,112,113\). Modified CRP is generated \textit{in vivo} from pCRP at membrane surfaces in activated platlets \(112\), apoptotic blebs \(96\), and oxidized low density lipoprotein \(97,114\). The antigenicity of the two isoforms of CRP are distinctly different \(115,116,117\), and only modified CRP shows binding to C1q and complement activation \textit{in vivo} \(73\). In addition to its chemokine production from endothelial cells \(97\), mCRP can interact with neutrophils \(104\), platelets \(118\), and macrophages \(119\), cells that play a key role in inflammatory responses. In neutrophils, mCRP was shown to upregulate adhesion molecules, which results in increased adherence to endothelium \(113\). Slowing of neutrophils in blood vessel flow and adhesion to blood vessel walls is an early phase of inflammation \(120\). This process is mediated by a class of molecules called cell adhesion molecules \(120\). Conversely, pCRP inhibits activation and adherence of neutrophils \(121\). pCRP functions to inhibit neutrophil chemotaxis, though some evidence indicates mCRP does this as well, using different receptors \(106\). mCRP interaction with platelets caused an upregulation in P-selectin, another adhesion molecule, which subsequently increases neutrophil aggregation \(118\). pCRP attenuates these changes in platelets, and further inhibits platelet aggregation directly by inhibiting phospholipases and reducing platelet aggregation caused by platelet aggregation factor \(122\). One study suggested \(123\) that structurally modified CRP promotes the uptake of LDL into macrophages. Macrophages are thought to become foam cells by taking up low density lipoprotein (LDL) \(106\), a critical event in atherosclerosis. It is unclear however if this structurally modified CRP is mCRP. A second study \(124\) demonstrated that
native pCRP bound to LDL does not promote uptake. Taken together, these results indicate that mCRP has a more pronounced pro-inflammatory effect than native pCRP. However, as is seen with neutrophils, the role of CRP isoforms can be a complex topic.
There are several ways that CRP could interact with the cell surface. Small molecules and proteins have been identified as targets for CRP, such as PC headgroups, low density lipoprotein (LDL), oxidized LDL, lysoPC, and FcγRIII (71). Although CRP has a PC binding site, it will not bind PC on a flat bilayer. One study demonstrated CRP
will bind to highly curved, PC coated gold nanoparticles (6). Here, CRP with Ca$^{2+}$ present caused a shift in the localized surface plasmon resonance signal, indicating that CRP directly modified the lipid structure surrounding the nanoparticle. There was a clear cutoff in size, where CRP bound to curved PC membranes that were presented on structures smaller than 30 nm in diameter. A consequence of this study is that CRP will recognize a PC lipid membrane in the absence of other protein receptors or lipid oxidation when at regions of high curvature. This result opens further questions regarding CRP since it confounds our understanding of CRP’s specificity. Phosphocholine is a ubiquitous group found on the surface of all cells in the body, not just apoptotic ones. Lipid rafts, often rich in PC lipids, are also not specific to apoptotic cells. Some other mechanism other than presence of a ligand must dictate specificity. These results imply CRP’s interaction could be related to the physical structure of the binding site, a possible mechanism by which CRP interacts with the curved blebs of apoptotic cells.

**Figure 31**: CRP curvature sensing is isoform specific. Green spots are CRP; red spots are NPs. A) mCRP colocalizes with 100 nm particles. Yellow shows strong colocalization. B) pCRP does not colocalize with curvature. Scale bar is 5 μm.
Human C-Reactive Protein Isoform Recognizes Curvature

Given the implications of CRP binding curvature, the biosensor we developed was an ideal method for determining if isoform changes would cause a change to CRP’s curvature sensitivity. Upon treating CRP with 0.01% SDS and 70°C heat, CRP monomerized. This treatment of CRP does not eliminate binding to C1q, the next protein in the classical complement pathway, which indicates mCRP is still an active form (125). mCRP preferentially bound to sites of curved bilayer whereas untreated pCRP bound to flat areas of bilayer. A representative confocal image for mCRP is shown in Figure 31a. In the overlay, areas of yellow show strong colocalization. In contrast, in Figure 31b, confocal images for pCRP are shown. These have no observable overlap in the overlay.

Figure 32: Comparison of ministacks of pCRP and mCRP at two extents of curvature. A) Matrix of averaged ministacks for CRP isoforms, autoscaled. In both cases, mCRP colocalizes to curvature, demonstrated by the increased intensity in the center of the image. Because pCRP has reduced intensity in the center, this might indicate curvature avoidance, but the high contrast makes these data inconclusive. The red line indicates where the line scan was taken. B) Normalized fluorescence intensity for line scans for the 4 ministacks. mCRP (in blue) increases 300-400% from the background level. pCRP (in green) is flat relative to the background with a possible dip in the center. Images are 1x1 μm².
image. A comparison of ministack images in Figure 32 shows that even under this sensitive technique, no pCRP intensity is seen at locations of curvature. Line scans of the mCRP data show a 300-400% increase in intensity at locations of curvature. Averaged images of pCRP potentially show avoidance of the curved regions (Figure 32a), but the change is extremely small once normalized to the overall image intensity. Other methods were explored to evaluate colocalization, but these methods were found insufficient for a variety of reasons. In particular, an intriguing thresholding method (80) showed promise initially. Briefly, this method assesses colocalization by comparing the pixel intensity of two channels in a scattergram. It incrementally decreases a threshold for each channel until the Pearson coefficient of the pixels below the thresholds is 0. Then pixels above the threshold are considered colocalized. Unfortunately, this method ultimately proved too sensitive to background noise. This is because individual pixels were examined, not features. Due to the high sensitivity required to image CRP on the surface, pixel intensity for the background was sufficient enough to be considered correlated in this method.

**Single Particle Tracking**

Finally, we investigated the mobility of CRP at the single particle level using TIRF microscopy. Confocal microscopy revealed the preference of curvature at the bulk level, but recent work has shown that ensemble and single particle measurements do not necessarily align (4). Both m- and pCRP have a mixture of mobile and stationary particles on the surface of the biosensor. Figure 33 shows a typical track of mCRP. Full movies of trackable particles can be obtained in TIRF, and these data could elucidate transient interactions with curvature. However, several areas of troubleshooting remain
to be explored. These data are encouraging by showing proof of concept for single molecule tracking using the biosensor, and this line of research is being pursued by other members of the Knowles group.

**Characterization of Human C-Reactive Protein**

There are at least two distinct isoforms of CRP, and we have begun studying the different structures that arise as CRP unfolds. In Figure 34, native pCRP is denatured using two separate methods, and the absolute tryptophan fluorescence measured. Using a plate reader, we examined the fluorescence from the amino acid tryptophan. This fluorescence is very sensitive to solvent environment changes, and fluorescence reduces when moving from a hydrophobic environment to a hydrophilic environment. CRP has 30 tryptophan

![Figure 34: Representative track of pCRP moving on the surface of an SLB.](image)

![Figure 33: Two separate methods of denaturing CRP indicate a stable isoform exists between the native form and a fully denatured state.](image)
residues, and is a good candidate for a tryptophan fluorescence assay. With increasing concentration of guanidine hydrochloride or after 45 minutes, there is a distinct central plateau in fluorescence indicating a stable transitional state. This central isoform does still bind to C1q (69), and is thought to be the active, modified form of CRP (mCRP). Equimolar free tryptophan has a normalized fluorescence of approximately 50%.

CRP was detected through primary and secondary antibody binding. We initially considered directly labeling CRP with a dye, but this process caused CRP to misfold to an unidentified, broad band on native gel. Therefore we selected an antibody pair to visualize CRP. Primary mouse anti-CRP was purchased from Santa Cruz Biotechnology and secondary goat anti-mouse immunoglobulin G with Alexa-488 was purchased from Life Technologies. Several primary antibodies were tested with varying success. The final candidate, clone 8 (C8) from Santa Cruz, was the most consistent. Dot blots (Appendix B) can be used to determine if an antibody (or pair) will successfully bind to a specific protein. This is a qualitative assay where the protein is known. An example dot blot in Figure 35 shows that this antibody pair we used does bind sufficiently to CRP. The center spot is where CRP was dotted onto nitrocellulose, and after treatment with two antibodies (primary C8 and secondary), fluorescence is seen at the protein spot.

Figure 35: Dot blot of CRP shows the antibody pair selected binds to CRP.
Figure 36: SLB prevents adherence and binding of non-membrane binding entities. Without an SLB, secondary antibodies bind to bare glass (A). With an SLB, neither primary/secondary antibodies (B) or primary/secondary antibodies with BSA (C) will significantly bind to the surface. Contrast is scaled the same for all images.

Without the presence of CRP, the antibody pair does not bind to the surface of the biosensor. Figure 36 shows confocal images of the antibodies without a bilayer present, with a bilayer, and with a bilayer and BSA. Without a bilayer present, antibodies stick to the surface of the glass. With a bilayer or with BSA present, no fluorescence is seen in the protein channel indicating no binding. Therefore in the presence of CRP, we know that when fluorescence is seen at the bilayer surface, it is due to CRP binding, not antibodies only.

**Aptamer Specific to mCRP**

While antibodies are an excellent method for visualizing CRP when the isoform is known, a tool is needed to identify the isoform when it is not known. CRP is notorious for displaying contradictory inflammatory effects, and this is attributed to the difficulty in identifying which isoform is at work (106). In collaboration with researchers at the University of Colorado – Denver (69), we identified an RNA aptamer that specifically binds to mCRP and not pCRP. The aptamer (Integrated DNA Technologies, Inc) was end-labeled with either Cy3 on the 5’ end (5’Cy3-RNA) or Cy5 on the 3’ end (3’Cy5-
RNA) and stocks of aptamers were reconstituted with diethylpyrocarbonate (DEPC) treated water to remove nucleases and stored at -20°C until use. The sequence for the RNA aptamer used in this study is as follows: 5’-GCC UGU AAG GUG GUC GGU GUG GCG AGU GUG UUA GGA GAG AUU GC-3’.

Figure 37: RNA aptamer will selectively bind to mCRP. A) Control image with no CRP present. B) mCRP is captured on the surface with a nonspecific antibody and aptamer is present. C) pCRP is similarly captured in the presence of aptamer. D) Comparison of integrated fluorescent intensity shows increased aptamer binding to mCRP compared to pCRP or the control. Data is mean ± SEM. Using Student’s t-test, **P-value <0.01 and #P-value <0.05. Comparison of pCRP to the control showed similar integrated intensities (P-value > 0.20). Scale bar is 40 μm.
Binding between 5′Cy 3-RNA and surface-bound CRP was analyzed using TIRF microscopy. TIRF permits the selective excitation of 5′Cy3-RNA attached to the surface while excluding 5′Cy3-RNA in the bulk solution. Therefore, binding events can be detected with low background noise by comparing the intensity and distribution of fluorescence spots on the surface. Integrated fluorescence intensities (F) of each sample were subjected to image processing and plotted (Figure 37). Anti-CRP antibodies were used as a capture method to avoid pCRP conversion associated with direct capture. The integrated intensity of the 5′Cy3-RNA control on the anti-CRP antibody coated coverslip was 18.8 ± 0.51 (×10⁶). A significant increase in intensity was measured (ΔF = 3.2×10⁶, P-value < 0.01, Student’s t-test) when mCRP-5′Cy3-RNA was added to the antibody-coated coverslip as evident from the increase in spot density on the surface (Figure 37b). In contrast, addition of pCRP-5′Cy3-RNA (Figure 37c) caused a slight decrease in fluorescence intensity (ΔF = 1.1×10⁶, P-value > 0.2, Student’s t-test), and the spot density on the surface was comparable to the 5′Cy3-RNA control (Figure 37a), demonstrating that 5′Cy3-RNA binds to mCRP but not pCRP.

The specificity of the aptamer for mCRP was also supported by anisotropy and gel electrophoresis work done by our collaborators at the University of Colorado at Denver (69). We demonstrated that an aptamer previously isolated against CRP (126) has a strong affinity for the mCRP isoform and does not associate with pCRP under the same conditions. A solution binding analysis using fluorescence anisotropy was used to identify which isoforms of CRP bind to the aptamer, 5′Cy3-RNA (Figure 38). Fluorescence anisotropy measures the change in rotational diffusion rate of a fluorescent
probe and has been used to measure the interactions of biomolecules (127,6,128).

Labeled aptamer should freely rotate and have a lower anisotropy than if it is bound to CRP. Addition of mCRP (labeled mCRP-SDS in Figure 38 and mCRPSDS for the rest of this section for clarity between this and mCRP_HCl) to the 5'Cy3-RNA aptamer resulted in a significant increase in anisotropy (Table 6), with a Δr of +0.097±0.019. This is compared to a CRP denaturing method that eliminates the ability to activate compliment, mCRP_HCl (129). Addition of mCRP_HCl to the 5'Cy3-RNA aptamer also resulted in a significant increase in anisotropy (Table 6), with a Δr of +0.107±0.016. In contrast, when the same amount of pCRP was added to the 5'Cy3-RNA aptamer the Δr was +0.014 ± 0.007 showing that the much larger pCRP had negligible affinity for the aptamer. The final concentrations of 5'Cy3-RNA and CRP were 0.59 and 9.1 μg/mL, respectively. In an additional control, no binding was observed between the aptamer and BSA (Figure 38) confirming that the interactions observed were specific.
Figure 38: Solution binding analyses between 5′Cy3-RNA, CRP, and C1q using fluorescence anisotropy. Baseline anisotropy readings of 5′Cy3-RNA (0.59 μg/mL) were acquired prior to the addition of (green) mCRP_{SDS}, (red) mCRP_{HCl}, (yellow) pCRP or (blue) BSA (each at 9.1 μg/mL) at the first indicated time point. At the second indicated time point, an aliquot of C1q (5.8 μg/mL) was added to the CRP-aptamer complex and evaluated for complement activity.
Table 6: Degree of binding between 5’Cy3-RNA and CRP (Δr1) and between CRP and C1q (Δr2) determined by the change in anisotropy. n=3, *P<0.001 compared to BSA control.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Δr1 (Mean ± SD)</th>
<th>Δr1 (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCRP&lt;sub&gt;SDS&lt;/sub&gt;</td>
<td>0.097* ± 0.019</td>
<td>0.099* ± 0.015</td>
</tr>
<tr>
<td>mCRP&lt;sub&gt;HCl&lt;/sub&gt;</td>
<td>0.107* ± 0.016</td>
<td>0.013 ± 0.009</td>
</tr>
<tr>
<td>pCRP</td>
<td>0.014 ± 0.007</td>
<td>0.013 ± 0.008</td>
</tr>
<tr>
<td>BSA</td>
<td>0.018 ± 0.005</td>
<td>0.009 ± 0.005</td>
</tr>
</tbody>
</table>

Although the RNA aptamer recognizes both forms of mCRP, the bioactivity of each mCRP form was noticeably different. Since modified CRP has been shown to activate complement in vivo (79), binding studies between mCRP and C1q were further evaluated by anisotropy to assess the biological activity of the each mCRP (Figure 38). The mCRP<sub>SDS</sub> isoform bound to C1q, resulting in a significant increase in anisotropy, with a Δr of +0.099±0.016 in the presence of 0.01% SDS (Figure 38). This confirms that mCRP<sub>SDS</sub> is functional even though it is not in the pentameric form. In contrast, mCRP<sub>HCl</sub> did not interact with C1q (Δr = +0.013 ± 0.009) (Figure 38) and adding the same concentration of SDS to mCRP<sub>HCl</sub> did not restore C1q binding, consistent with acid treatment resulting in complete and irreversible denaturation. The final concentration of C1q in each case was 0.58 μg/mL. In addition, ELISA confirmed that mCRP<sub>SDS</sub> bound C1q better than either pCRP or mCRP<sub>HCl</sub> (Figure 39). The binding between C1q and mCRP<sub>SDS</sub> was twice as strong compared with that of the mCRP<sub>HCl</sub> by ELISA. As expected, no binding was observed between pCRP and C1q by ELISA. The strong binding between C1q and mCRP<sub>SDS</sub> suggests that CRP heated in a minimal amount of
SDS is likely to retain a folded structure as do other proteins at low SDS concentration (130). On the other hand, the acid treatment (mCRP_HCl) rendered the CRP irreversibly denatured (116,129) and without the ability to activate complement, which is in agreement with previous observations (129). These results demonstrate that the mCRP_{SDS} remains biologically active and expresses the neo-epitopes responsible for C1q binding which occurs through the Asp 112, Tyr 175, and His 38 residues on CRP (109). This also suggests that the aptamer specifically recognizes a portion of CRP that is

Figure 39: Binding of different CRP isoforms to immobilized C1q. ELISA wells were coated with C1q (1μg/mL) and incubated with various modifications of CRP at increasing concentrations. The degree of CRP binding was detected using biotinylated polyclonal anti-CRP antibody (1:5000) with a streptavidin-HRP conjugate (1:10,000) and the absorbance of TMB substrate was measured on a plate reader at 450 nm with background subtraction at 620 nm. mCRP_{SDS} was formed by heating with 0.1% SDS.
blocked when protomers are adjacent to each other, and that the mCRP<sub>SDS</sub> shows similar antigenicity to the modified CRP found in tissues and human arterial intima (73,131) that are relevant to biological function in complement activation. Because of its high affinity to C1q, we consider the mCRP<sub>SDS</sub> as similar to the biologically relevant form of modified CRP. Although both mCRP<sub>SDS</sub> and biologically modified CRP bind to C1q, additional study will be required to fully understand the antigenicity of mCRP<sub>SDS</sub>. Since mCRP<sub>SDS</sub> appears to be a better model of modified CRP than does mCRP<sub>HCl</sub> and because modified CRP and mCRP<sub>SDS</sub> both have an intact C1q binding site (97), we therefore sought to further characterize the interactions of the aptamer probe with mCRP<sub>SDS</sub>.

To provide direct visual evidence of the protein–aptamer complex, we performed a dot blot assay and adapted standard PAGE methods for electrophoretic mobility shift assay (EMSA) to create an in-gel assay using a 3’Cy5-RNA and NIR imaging. The 5’biotin-RNA showed strong affinity for mCRP, with relatively weak binding to pCRP, and no affinity for BSA (Figure 40a). To further observe the specificity of aptamer for mCRP<sub>SDS</sub> over pCRP, the proteins were separated by PAGE and visualized using silver stain (Figure 40b) and near infrared (NIR) imaging (Figure 40c). When resolved on a 10% polyacrylamide gel containing 0.005% SDS, pCRP showed an apparent MW of 140 kDa and mCRP<sub>SDS</sub> had an apparent MW of 48 kDa (Figure 40b). This high MW is caused by the nearly native conditions used and is in agreement with a previous study using the same gel conditions (132). NIR imaging of the same gel revealed a distinct band at 48 kDa in the mCRP<sub>SDS</sub> and 3’Cy5-RNA co-incubated sample (Figure 40c). This suggests that mCRP<sub>SDS</sub> forms a complex with 3’Cy5-RNA that has a similar mobility to
the protein alone. In contrast, no additional bands were observed in the pCRP + 3’Cy5-RNA lane (Figure 40c), indicating that 3’Cy5-RNA did not form a persistent complex with pCRP. To further evaluate the complex formation of mCRP<sub>SDS</sub>-3’Cy5-RNA, EMSA was performed using increasing concentrations of 3’Cy5-RNA into a fixed amount of mCRP<sub>SDS</sub> and resolved on a 5% 0.5× TBE acrylamide gel (Figure 41). NIR EMSA gel showed increasing mCRP<sub>SDS</sub>-3’Cy5-RNA complex formation with increasing 3’Cy5-RNA concentration and a difference in mobilities of the free 3’Cy5-RNA and the mCRP<sub>SDS</sub>-3’ Cy5-RNA complex bands (Figure 41a), confirming the formation of an mCRP<sub>SDS</sub>-3'Cy5-RNA complex. Silver staining of the same gel also confirmed that the mCRP<sub>SDS</sub>-3’Cy5-RNA complex migrated differently than free mCRP<sub>SDS</sub> (Figure 41b), and the mCRP<sub>SDS</sub>-3’ Cy5-RNA complex bands from both techniques overlapped with the 3’Cy5-RNA bands observed by NIR imaging (Figure 41c). In contrast, migration of pCRP did not change in the presence of 3’Cy5-RNA and no complex was observed between pCRP and 3’Cy5-RNA. Both dot blot and EMSA results clearly showed that 3’ Cy5-RNA interacts only with mCRP<sub>SDS</sub> and not pCRP, as indicated by a distinct mCRP<sub>SDS</sub>-3’Cy5-RNA complex band, visible by NIR imaging (Figure 41b). The main advantage of this adapted EMSA method is that both the protein and protein–aptamer complex bands can be resolved on the same gel without the need to perform a Northern blot or the use of radioisotopes.
Figure 40: CRP-RNA complex formation. A) Dot blot assay was carried out by blotting CRP and BSA onto nitrocellulose and detected using a biotinylated aptamer (177nM) followed by streptavidin conjugated-IR 800 (1:1000) and imaged on an Odyssey NIR imager. CRP was preincubated with 3’Cy5-RNA before resolved on a 10% polyacrylamide gel containing 0.005% SDS. Protein bands were visualized by B) silver staining and the gel was imaged for 3’Cy5-RNA and CRP-3’Cy5-RNA complex using C) NIR imaging.
Figure 41: EMSA of mCRP and pCRP using 3'Cy5-RNA probe. A) In-gel visualization of 3’Cy5-RNA and 3’Cy5-RNA-mCRP complex using NIR imaging, and B) visualization of CRP bands by silver staining of the same gel. C) Superimposed image of the NIR image and silver stain gel, highlighting the overlapping areas of the 3’Cy5-RNA-mCRP complex.
Increasing concentrations of mCRP<sub>SDS</sub> were added to 5′ Cy3-RNA to obtain a binding constant for RNA aptamer and mCRP<sub>SDS</sub> using anisotropy (Figure 42a). The starting concentration of 5′Cy3-RNA was 0.68 μg/mL and aliquots of mCRP<sub>SDS</sub> were titrated into the solution. The titration curve showed a saturation binding equilibrium between 5′ Cy3-RNA and mCRP<sub>SDS</sub>, starting with an initial anisotropy value of 0.299±0.009 for the 5′Cy3-RNA probe, followed by a gradual increase in anisotropy, and finally reaching a maximum anisotropy of 0.381±0.007. The data were fit using nonlinear least squares regression (GraphPad Prism 5). From the fit curve, the CRP concentration at half-maximum was 4.34 μg/mL. This corresponds to a K<sub>d</sub> of 187.7 nM (using the MW for mCRP of 23 kDa), comparable to that of the previously reported K<sub>d</sub> for this aptamer (133).

Figure 42: Quantitative binding studies between RNA-aptamer and mCRP<sub>SDS</sub>. A) Saturation binding curve of 5′Cy3-RNA aptamer (0.68 μg/mL) with increasing mCRP<sub>SDS</sub> concentrations. B) Competitive displacement assay with increasing concentration of ssDNA. Titrations of ssDNA were added to a solution containing mCRP<sub>SDS</sub>-5′Cy3-RNA complex (18 μg/mL mCRP<sub>SDS</sub> and 0.68 μg/mL 5′Cy3-RNA) Data represents mean ± SE, n=3.
In addition to the RNA aptamer, a second aptamer against CRP has been recently selected (79). Because the ssDNA aptamer was selected against CRP bound to magnetic beads (79), we expected the ssDNA aptamer could also be mCRP specific and possibly compete for the same binding site as the RNA aptamer. To determine if the DNA aptamer also binds mCRP, we performed a competitive displacement study using the CRP-specific ssDNA aptamer as a competitor (Figure 42b). Increasing amounts of ssDNA aptamer were added to the mCRP$_{SDS}$-5′Cy3-RNA complex until the anisotropy reached the baseline value. The initial concentrations of the mCRP$_{SDS}$ and 5′Cy3-RNA were 18 μg/mL and 0.68 μg/mL, respectively. The initial anisotropy of the mCRP$_{SDS}$-5′Cy3-RNA complex was 0.381±0.006 (the maximum anisotropy value) and no significant changes in anisotropy were detected at lower titrations of ssDNA. After an initial lag phase, a decrease in anisotropy was observed and the drop in anisotropy proceeded to a plateau at a final minimum anisotropy of 0.288±0.01. The competitive displacement data were fit to a dose-dependent Hill equation and the concentration of ssDNA needed to displace 50% of the 5′Cy3-RNA from the mCRP$_{SDS}$-5′Cy3-RNA complex (EC50) was 0.83 μg/mL (GraphPad Prism). A complete displacement of the 5′Cy3-RNA was achieved at ssDNA concentrations greater than 1.6 μg/mL, or approximately 2X molar excess of the 5′Cy3-RNA. In an inhibition assay, when ssDNA aptamer was added to mCRP$_{SDS}$ prior to the 5′Cy3 RNA aptamer addition, no change in anisotropy was observed (data not shown). This competitive displacement assay suggested that the ssDNA has strong affinity to mCRP$_{SDS}$, suggesting that both aptamers may bind to the same epitope on the mCRP$_{SDS}$ consistent with the ssDNA also being
specific to mCRP. This further highlights a general problem with using SELEX to obtain aptamers for proteins that are prone to aggregation or conformational changes. The bulk of the work done in this section was done with Dr. Min Wang.

**Conclusions on CRP Sorting**

The discussion about CRP isoforms has remained an active topic for the past decade (105,104,106,107). Using a variety of methods to denature CRP, our data further confirm that (at least) a three state model for denaturing is appropriate (Figure 34). CRP’s ability to bind to C1q after denaturing to this second state, but not binding in the native state, implies that it is this non-native isoform that is functional at the surface of a cell.

The difference in curvature sensitivity between isoforms also has implications to the active form at the cell surface. As an indicator for apoptotic cell removal, CRP must be able to distinguish between healthy and apoptotic cells. A defining characteristic of apoptotic cells is the physical change to the plasma membrane, notably the changes to extents of curvature. Our data indicate that the change in CRP to this second, C1q-binding isoform will bind to a soy-PC bilayer and recognize curvature. Interestingly, CRP will bind to the membrane as a native, pentameric form, but either has no preference or weakly avoids curvature. After conversion, CRP strongly binds to curvature. This suggests that small scale curvature, where radii are less than 100 nm, plays a major role how CRP differentiates between healthy and apoptotic cells. Other work indicates that pCRP will bind curvature only below ROC of 30 nm (125).
Finally, we identified an aptamer that will successfully differentiate between mCRP and pCRP. By using this aptamer, isoforms can be distinguished in a variety of situations. Our biosensor is one such opportunity, but this aptamer could be used in cellular studies as well. Some studies (134) indicate that CRP could change isoform at the surface of cells. This aptamer would be ideal to investigate these changes.
Chapter Six: Summary

This work has outlined the development of a biosensor able to investigate the curvature sensing properties of lipids and proteins. The biosensor consists of nanoparticles noncovalently adhered to a glass surface with a supported lipid bilayer formed over them. This induces curvature in the bilayer independent of bilayer composition. Using fluorescence microscopy, the amount of sensing can be assessed through colocalization with nanoparticles and FRAP. This unique approach has several advantages over other methods currently in use. 1) Most notably, our sensor allows lipids at sites of curvature to be in dynamic and ready equilibrium with lipids not at curvature. 2) This permits us to make conclusions based on a mimic that models large, flat membranes. This is ideal for modeling the plasma membrane or large organelles. 3) Our sensor also offers significant control over the composition of the bilayer that is independent from the induced curvature. This permits targeted investigations that could help unravel the interplay between curvature and lipid sorting. 4) The heterogeneity of this setup allows specific sorting to ROC after initial binding to the bilayer. 5) The preparation is simple and quick. 6) And finally, the sensitivity of this assay is high enough to permit single-molecule studies. This sensitivity is crucial to being able to probe kinetic effects such as cooperativity and nonergodic systems. Rare, transient interactions between biomolecules could be observed.
Curvature influences lipid and protein interactions with bilayers. We have shown that lipids partition differently in the presence of curvature and that these lipids do exchange with lipids in flat regions, though more slowly. This supports other work indicating the presence of defect sites at areas of curvature, and we propose that these defect sites have an influence on lipid diffusion. We have also shown that curvature will influence the isoform-dependent binding of CRP. Pentameric CRP will bind to phosphocholine bilayers, but will not bind to curvature sites. Monomeric (or modified) CRP will bind preferentially to sites of curvature. This implies a mechanism for CRP recognition of apoptotic cells instead of healthy cells.

**Future Work**

Several opportunities exist to pursue work using this biosensor. Currently, work is being done to investigate the dynamics of single lipids and to discern any cooperative effects that result. We speculate, with others (4), that cooperative effects cause differences between single particle motion and ensemble motion. Single particle studies could also illuminate any corralling effects that curvature could impart on lipid motion. Further investigations into mixtures of lipids, via FRET, FLAP, and FRAP, would provide insight into how compositions of lipids change the fluid parameters. Phase transitions have an effect on lipid sorting (59), which has implications for micodomain activity, vesicle trafficking, and other membrane deforming processes.

CRP recognition of apoptotic cells is being further explored by other group members. Isoform conversion could have significant impacts not just on membrane sorting, but also on activation of other proteins. One candidate is C1q. This is another
complement protein and known binding partner to CRP. It is the next protein in the pathway to complement activation. C1q’s binding to the biosensor could be explored in the presence of either isoform of CRP. C1q can bind to either PS or CRP, and it would be interesting to explore the curvature recognition of this protein in the presence of various binding partners.
References

32. Valeur, B.; Berberan-Santos, M. N. A brief history of fluorescence and phosphorescence before the emergence of quantum theory. *Journal of Chemical
Education 2011, 88, 731-738.
105-113.


69. Wang, M.; Black, J. C.; Knowles, M. K.; Reed, S. M. C-reactive protein (CRP)
aptamer binds to monomeric but not pentameric form of CRP. *Analytical and Bioanalytical Chemistry* 2011, 401 (4), 1309-1318.


137. National Science Board. *Science and Engineering Indicators 2008*; National Science


Appendix A: Acronyms

AFM – Atomic force microscopy
ANOVA – Analysis of variance
BAR – Bin-Amphiphysin-Rys
CRP – C-reactive protein
CVD – Cardiovascular disease
DEPC – Diethylpyrocarbonate
DMSO – Dimethyl sulfoxide
DOPS - 1,2-dioleoyl-sn-glycero-3-phospho-L-serine
Fl -HDA – Fluorescien hexadeacanoic acid
FLAP – Fluorescence loss after photobleaching
FL-DHPE – Fluorescien 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine
FRAP – Fluorescence recovery after photobleaching
FRET – Föster resonance energy transfer
GUV – Giant unilamillar vesicles
HAEC – Human aortic endothelial cells
MB-DHPE – Marina Blue 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine
mCRP – Monomeric CRP or modified CRP
MWCO – Molecular weight cutoff
NBD - [2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl]trimethylammonium
NP – Nanoparticle
PAGE – Polyacrylamide gel electrophoresis
PC – Phosphocholine

pCRP – Pentameric CRP or native CRP

ROC – Radius of curvature

SDS – Sodium dodecyl sulfate

SLB – Supported lipid bilayer

SLiC – Single liposome curvature

STORM - Stochastic optical reconstruction microscopy
Appendix B: Protocols

Dot Blot Protocol

Buffer:

TTBS: 50 mM Tris, 0.5 mM NaCl, 0.05% Tween-20, pH 7.4

Protocol:

1. Cut a piece of nitrocellulose into a circle that will fit into the smallest cell culture dish
2. Dot onto the nitrocellulose an appropriate concentration. Use approximately 10 uL. You can blot various concentrations to gauge what’s needed for a Western. A suggested concentration is less than 50 nM.
3. Incubate on the membrane at room temperature until the blots are dry. This is about an hour.
4. Block the membrane with 5% dry milk in TTBS for 1 hour. Pour off the buffer. Keep the membrane wet for the remainder of the procedure.
5. Incubate in primary antibody for 1 hour at 1:5000 dilution at RT in TTBS.
6. Wash the membrane 3 times (10 minutes each) in TTBS on a rocker.
7. Incubate in secondary antibody for 1 hour at 1:5000 dilution at RT in TTBS.
8. Wash the membrane 3 times (10 minutes each) in TTBS on a rocker.

TIRF Startup Protocol

Startup

1. Turn on power strip behind the laser system.
2. Wait for 2 lights to be lit steadily (no blinking) on each laser power supply. These should be “Power” and “Temp Lock.”
3. Turn on both keys if using both lasers.
4. Turn on the computer.

5. Run the Micromanager program. Hit ok to load configuration.

6. Wait for the camera to cool down to -70°C. This should take 3-4 minutes. This can be monitored from the Device/Property Manager under the Tools menu.

7. Once camera is cooled, the system is ready for imaging.

8. The TIRF field is controlled by the knob on the front side of the black launch behind the microscope. This adjusts the angle of the laser and, in turn, the TIRF field. Clockwise increases laser angle of incidence; counterclockwise decreases laser angle.

Other Comments

- The objective is an oil objective. One drop of oil is good for 3-5 coverslips. To clean, use ThorLabs lens paper with isopropenol. NOT KIMWIPES.

- Close the shutter on the camera (controlled from Micromanager) if the overhead lights are turned on for lengthy periods (more than a minute).

- Do not use EM Gains above 300 if it can be avoided. Most standard imaging (exposure times of 100ms+) do not need large EM Gains.

- DO NOT SATURATE THE CAMERA. This can quickly destroy the camera. If it is saturated, reduce EM Gain and exposure time.

- If there is a problem, contact Michelle in Mudd 101 (Phone:1-6698, 503-267-2430), or Josh or Phillip in Mudd 259 (Phone: 1-2984)

Shutdown Protocol

1. Shut down Micromanager.

2. Turn off both keys on the laser power supplies.

3. Wait for 2 steady lights (no blinking). These should be “Power” and “Temp Lock.”

4. Turn off the power strip behind the laser system.

Turn off the computer.