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Summer 2015

Interactions between Phospholipids and Polymers with Time Resolved Fluorescence Spectroscopy

Zachary E. Hier
University of Akron Main Campus, zeh1@zips.uakron.edu

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Interactions between Phospholipids and Polymers with Time Resolved Fluorescence Spectroscopy

Zachary E. Hier

Abstract

Interactions between macromolecule species and fluorescently labeled phospholipids were explored in supported lipid bilayers (SLBs). The concentration and the lateral mobility of the labeled phospholipids within the confocal volume were determined via fluorescence correlation spectroscopy. Low molecular weight polystyrene was used for the neutral charged macromolecule. Three separate concentrations of polystyrene were used and a significant change in lateral mobility of phospholipids was found for the SLB doped with all three concentration of styrene from polystyrene (molecular weight = 1000). Polyester with mimic lysine pendant groups was used for the cationic polymer at physiological pH. A large decrease in the mobility of head labeled lipids was found when the polyester was introduced to the bilayer. The same polyester was used on an experiment with SLBs doped with negatively charged, tail labeled phospholipids. However, a faster diffusion coefficient was found for the SLB when introduced to the polycationic molecule. This is contradicting to previous reports but this is just a preliminary observation. More experiments need to be completed to further determine these relationships. Overall, important groundwork has been completed to help describe the dynamics of the lipid-macromolecule interface.

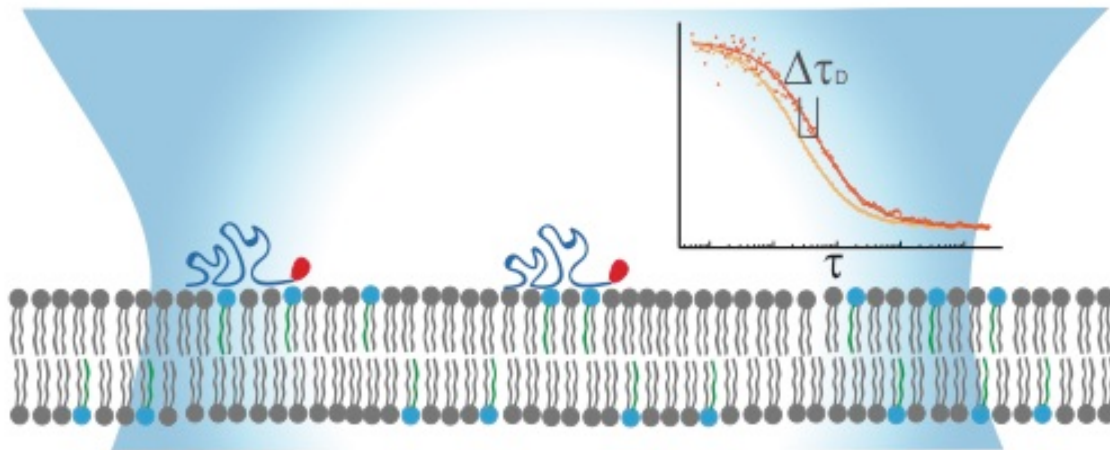


Figure 1. Cartoon of cationic polymer interacting with the heads of phospholipids. Taken from reference 1.

Introduction

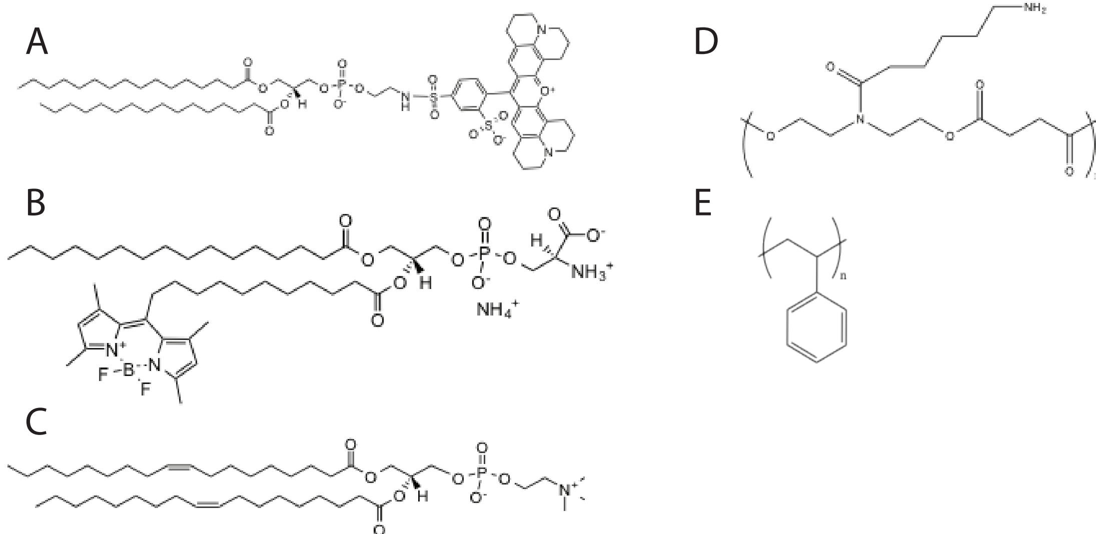
Interactions between phospholipids and proteins play a significant role in cell signaling. Proteins are macromolecules that contain multiple units of amino acids. The amino acids have side groups that control the properties of the protein. There are acidic, basic, and hydrophobic side chains on amino acids. Clusters of basic residues within MARCKs peptide have been assigned to specific interactions with anionic phospholipids within the cytoplasmic leaflet of the plasma membrane.[1,2] Studies have been done concerning the interactions between cationic macromolecules and anionic phospholipids and it is believed that this binding affects the mobility of phospholipids.[1,2,3,4] The dynamics of the macromolecule and membrane interface still remains relatively unknown. Recent work done by Shi et. al, has shown that the electrostatic interactions between polycationic quaternized polyvinylpyridine (QPVP) is dependent on the salt concentration of the buffer.[1] There was a direct correlation between the strength of electrostatic interaction and the charge of the headgroup.[1] We envision that the macromolecule sits on top of the

plasma membrane, resulting in an interaction with the outermost leaflet (refer to Figure 1). Polystyrene with unsaturated amine groups have been shown to have a higher toxicity on sea urchin embryos than polystyrene with carboxylated groups.[5] Additionally, it is believed that polystyrene nanoparticles perturb the membrane and cause a decrease in the lateral mobility of phospholipids.[6] We wanted to extend the observations made by Shi et al by working on more physiological relevant polymers. That is why we decided to work with polyesters with peptide like pendant functional groups that can be used for drug therapy.[7] We also decided to explore neutral polymers that may embed themselves within the plasma membrane and perturb the membrane biochemistry.[6] This study was motivated to gain deeper insight in the effects of macromolecules on lipid mobility.

Materials and Methods

Reagents. 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) and 1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-*sn*-glycero-3-phospho-L-serine (TopFluor-PS) was purchased from Avanti Polar Lipids (Alabaster, AL). Dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Texas Red DHPE) was purchased from Life Technologies (Carlsbad, CA). Polystyrene standard 1,000 (PS1000) was purchased from Sigma Aldrich (St. Louis, MO). Multifunctional polyesters with lysine and aspartic acid pendant mimic groups were synthesized by methods described elsewhere. The molecular structures of DOPC, TopFluor-PS Texas Red DHPE, polystyrene, and the polyester with lysine pendant mimic groups are provided in Scheme 1. Two different concentrations of PBS were prepared: 0.1X

PBS and 1X PBS. Each PBS solution contained 100nM EDTA. All of the water used was Milli-Q water.



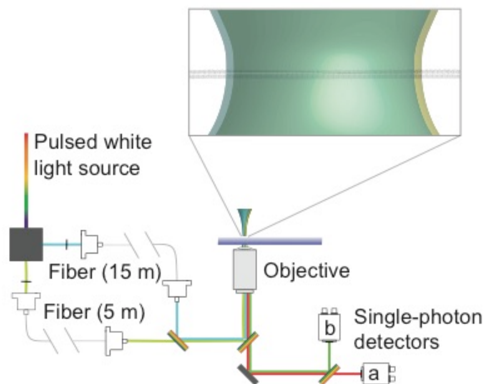
Scheme 1. Structures of phospholipids and polymers used. Figure A shows Texas Red DHPE. Figure B shows TopFluor-PS. Figure C shows DOPC. Figure D shows the polyester with mimic lysine pendant groups. Figure E shows polystyrene.

Preparation of Polyesters with Peptide-Like Pendant Functional Groups. Polyesters with lysine pendant groups were synthesized by a procedure reported elsewhere.[7] These polymers were synthesized and characterized by Abraham Joy and his research group. The polyesters were characterized with NMR and GPC.

Preparation of Phospholipid Vesicles. DOPC and Texas Red DHPE were dissolved in chloroform and DOPC, Texas Red DHPE, and PS1000 were dissolved in chloroform, and DOPC and TopFluor-PS were dissolved in chloroform. Safety considerations while working with chloroform are located in Appendix 1. These solutions were dried under vacuum and subsequently suspended in water, resulting in a 1 mg/mL final concentration. The vesicle suspension was extruded through a

100 nm diameter polycarbonate porous membrane 15 times to yield a small unilamellar vesicle (SUV) suspension.

Preparation of Supported Lipid Bilayers (SLBs). Glass coverslips were sonicated in IPA/water (50:50) solution for 30 minutes, followed by extensive rinsing with water, then sonicated for an additional 30 minutes in water. After sonication, the coverslips were soaked in a 3:1 solution of sulfuric acid and 30% hydrogen peroxide (piranha solution) for 7 minutes and rinsed extensively with water, and dried under a constant stream of nitrogen gas. Safety considerations while working with IPA and piranha are located in Appendix 1. A 30 μL volume of SUV was added to a 30 μL volume of 1X PBS. This mixture was deposited on the clean coverslip via smack and smear method.[8] The coverslip was transferred to an AttoFluor sample chamber (Life Technologies, Carlsbad, CA) and incubated for 2 minutes at room temperature. The sample was washed and rinsed with 30 mL of water and carefully exchanged to the desired buffer. For the experiments with the polyester with the lysine mimic groups and the Texas Red DHPE, the buffer in the sample chamber was exchanged with 15 nM unlabeled polyester in the working buffer. These samples were incubated for 15 minutes at room temperature. For the experiments with the polyester with the lysine mimic groups and the TopFluor-PS, the buffer in the sample chamber was exchanged with 5 nM unlabeled polyester in the working buffer.



Scheme 2. FCS instrumentation setup. Taken from reference 9.

FCS Instrumentation. Fluorescence measurements were taken on an inverted microscope. The setup of the FCS instrumentation is shown in Scheme 2. The laser source used for excitation was a supercontinuum white light fiber laser (Super K NKT Photonics, Birkerod, Denmark). The laser source was operating at a 9.7 MHz pulse rate with pulse duration of 5 ps. One excitation beam was selected from the laser source with bandpass filters and narrowband filters. The green beam passed through a 561 nm filter with a 2.1 fwhm bandwidth (LL02-561-12.5, Semrock, Rochester, NY). A TIRF filter block (zt488/561rpc and zet488/561m, Chroma Technology Corp., Bellows Falls, VT) was used to direct the beam into the optical path of the microscope. A 100X TIRF objective, NA 1.49 (Nikon Corp., Tokyo Japan) was used for imaging and FCS measurements.

The fluorescence signal entered a custom-built confocal detection unit with a 50 μm pinhole (Thorlabs, Newton, NJ). The fluorescence signal was collimated with a 100 mm focal length achromatic lens (AC254-100-A-ML, Thorlabs Inc., Newton, NJ). A 560 nm long-pass beam splitter (FF560-FDi01-25 x 36, Semrock, Rochester, NY) was employed to split the light. The spectral range of the signal was filtered

through a red band-pass filter (612/69 nm, FF01-621/69-25). A 50 mm focal length lens (APAC18, Newport Corp. Irvine, CA) focused the beam onto a single photon avalanche diode detector (Micro Photon Devices, Bolzano, Italy). A time-correlated single photon counting module (Picoharp 300, PicoQuant, Berlin, Germany) collected the data.

FCS Data Analysis. Fluorescence correlation spectroscopy (FCS) enables the dynamics of the SLB from temporal fluctuations in the fluorescence signal within the confocal volume of the microscope. The fluctuations in the fluorescence signal is autocorrelated with the respect to the lag time, τ. The plot of normalized autocorrelation curve versus τ can describe the mobility and the concentration of the fluorescence molecules moving within the confocal volume of the microscope. The autocorrelation curve function is described by the following expression:

$$G(\tau) = \frac{\langle \delta F(t+\tau)\delta F(t)\rangle}{\langle F(t)\rangle^2}$$

The brackets stand for the average and F(t) is the fluorescence intensity at time, t.

The amplitude of the correlation function is directly related to the relative population, N, of the diffusing fluorophore. The amplitude of the respective fluorophore is

$$G(0) = \frac{1}{\langle N\rangle}$$

The FCS autocorrelation curve in two dimensions is described by:

$$G(\tau) = G(0)\frac{1}{\left(1+\frac{\tau}{\tau_D}\right)}$$

where, the dwell time, τ_D, is

$$\tau_D = \frac{\omega_0^2}{4D}$$

D is the diffusion coefficient of the fluorescent molecule and ω_0 is the waist of the laser beam.

A model of anomalous diffusion was used in the calculations. The calculation includes an anomalous correction factor, α:

$$G(\tau) = \frac{1}{\langle N \rangle} \frac{1}{\left(1 + \frac{\tau}{\tau_D}\right)^{\alpha}}$$

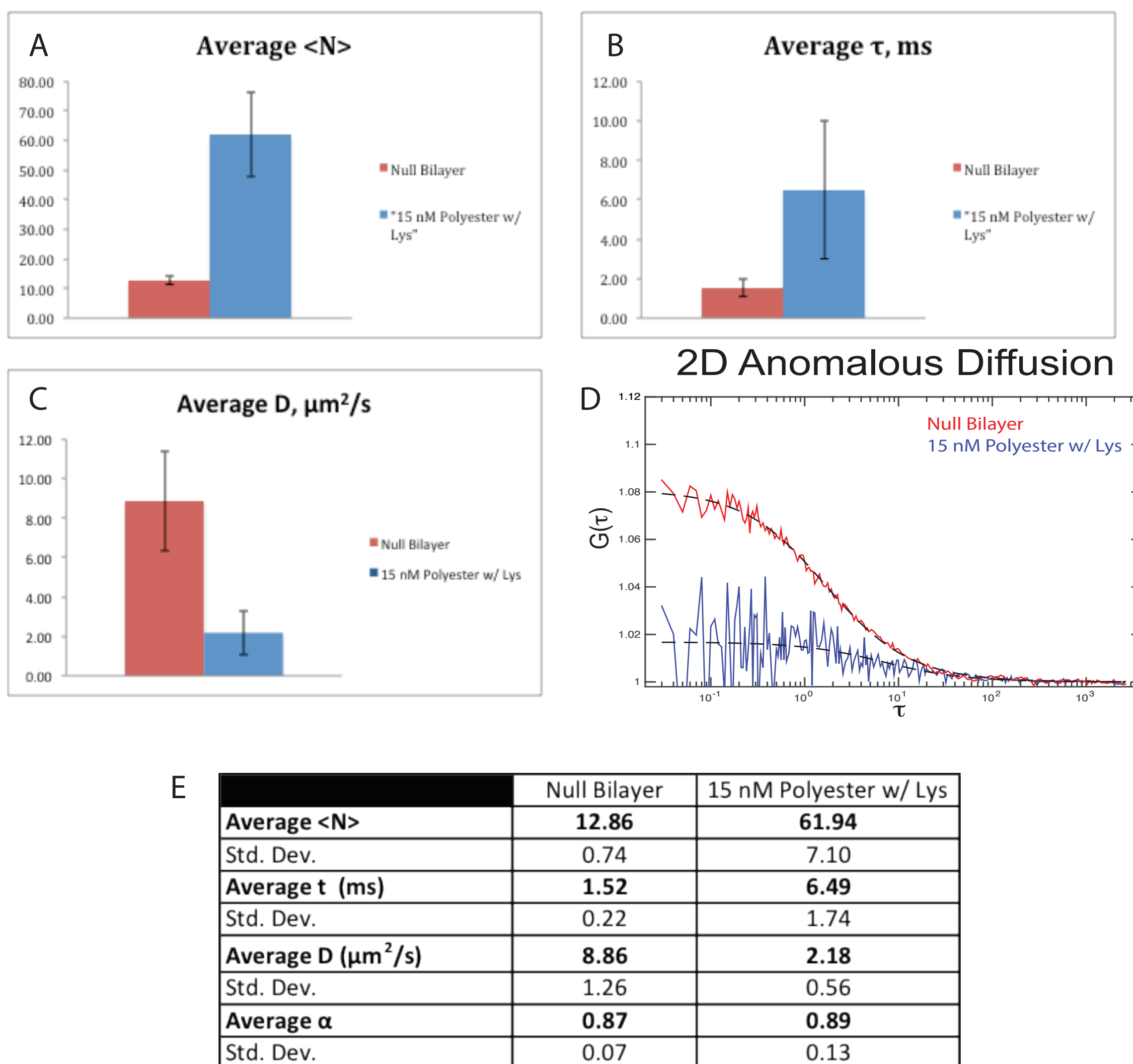
When α is 1, the diffusion is Brownian motion and when α is less than 1, the diffusion is anomalous.

Results

FCS Measurements of Polyester on DOPC SLBs Doped with Texas Red DHPE. Supported lipid bilayers (SLBs) were prepared by rupture of SUV on glass substrate. The lipid vesicles were composed of 99.998% DOPC and 0.002% Texas Red DHPE. Epi-fluorescence imaging indicates that the SLBs were uniform and without major defects. No FRAP experiments were performed because the Texas Red DHPE lipids recovered too quickly to quantify the recovery.

In the following experiment, unlabeled polyester with lysine mimic pendant groups was added to the buffer above the SLBs, where it encounters the lipids in the bilayer. It has been reported that varying ionic strength of the buffer can screen the electrostatic interactions. The electrostatic interaction between the cationic polymer and the anionic head group of the phospholipid decrease as the ionic strength of the buffer increases.[1] In this experiment a 0.1X PBS buffer was used, as it

was the buffer concentration that saw the largest phospholipid and a cationic polymer reported elsewhere.[1]



	Null Bilayer	15 nM Polyester w/ Lys
Average <N>	**12.86**	**61.94**
Std. Dev.	0.74	7.10
Average t (ms)	**1.52**	**6.49**
Std. Dev.	0.22	1.74
Average D (μm²/s)	**8.86**	**2.18**
Std. Dev.	1.26	0.56
Average α	**0.87**	**0.89**
Std. Dev.	0.07	0.13

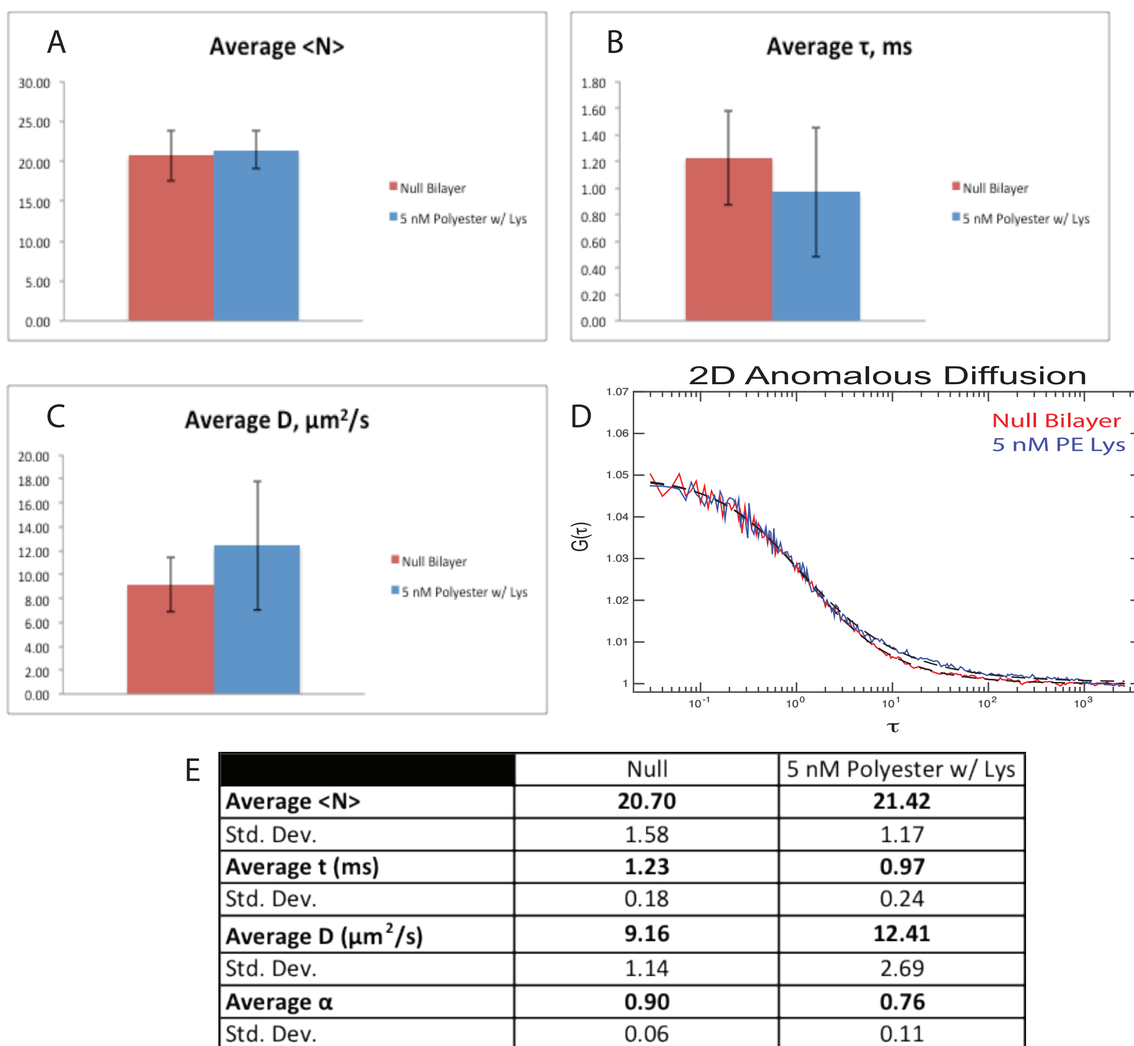
Scheme 3. Figures A, B, and C show the graphical results from the Texas Red DHPE and the polyester with lysine side group experiment for <N>, τ, and D with 2x the standard deviation error bars. Figure D shows the autocorrelation curve. Figure E contains the experiment results summarized.

The results obtained from this study are located in Scheme 3. The mobility of the phospholipids decreased drastically when the SLB was exposed to the polyester (2.18 ± 1.12 μm²) compared to the null SLB (8.86 ± 2.52 μm²). Additionally, the mean τ of the SLB exposed to the polyester is statistically different from the mean τ

of the null SLB (p-value = $4.26\times10^{-6} < 0.001$). This result was expected for a few reasons. First, the labeled lipid is head-grouped labeled. Therefore it is more likely that there would be some type of interaction between the polyester and the labeled lipid because of the bulky dye. This is associated with the large decrease in the counts per second (CPS) recorded per molecule. The large decrease in CPS per molecule due to the large clustering of the labeled lipids within the confocal volume. When the dipole moment of the fluorescent molecules are aligned, will result in self-quenching, also known as concentration quenching.[10]

FCS Measurements of Polyester on DOPC SLBs Doped with TopFluor-PS. Supported lipid bilayers (SLBs) were prepared by rupture of SUV on glass substrate. The lipid vesicles were composed of 99.995% DOPC and 0.005% TopFluor-PS. Epi-fluorescence imaging indicates that the SLBs were uniform and without major defects. No FRAP experiments were performed because TopFluor-PS is not known to interact with the glass substrate.

In the following experiment, unlabeled polyester with lysine mimic pendant groups was added to the buffer above the SLBs, where it encounters the lipids in the bilayer. In this experiment a 0.1X PBS buffer was used, as it was the buffer concentration that saw the largest phospholipid and a cationic polymer reported, elsewhere.[1]



	Null	5 nM Polyester w/ Lys
Average <N>	**20.70**	**21.42**
Std. Dev.	1.58	1.17
Average t (ms)	**1.23**	**0.97**
Std. Dev.	0.18	0.24
Average D (μm^2/s)	**9.16**	**12.41**
Std. Dev.	1.14	2.69
Average α	**0.90**	**0.76**
Std. Dev.	0.06	0.11

Scheme 4. Figures A, B, and C show the graphical results from the TopFluor-PS and the polyester with lysine side group experiment for <N>, τ, and D with 2x the standard deviation error bars. Figure D shows the autocorrelation curve. Figure E contains the experiment results summarized.

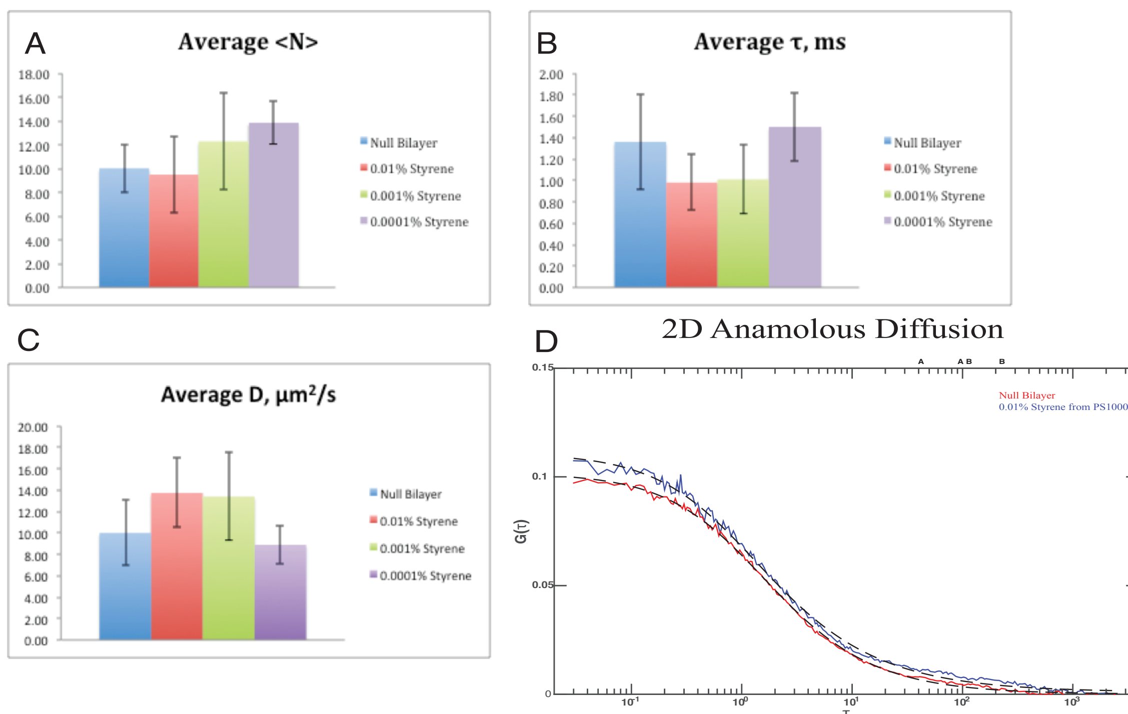
The results from the following experiment are located in Scheme 4. There was a change in the diffusion coefficient, D, when the polymer was introduced to the bilayer. The null SLB (9.16 ± 2.28 μm^2) diffused slower across the confocal volume in comparison to the SLB with the 5 nM polyester with lysine buffer solution (12.41 ± 5.38 μm^2). This is interesting considering that in previous experiments reported elsewhere showing that a polycationic molecule caused a decrease in mobility of the

TopFluor-PS.[1] A two-sample t-Test assuming equal variances (determined from the results of the F-Test) indicate that the τ of the null and the experimental bilayer are significantly different (p-value = 0.003 < 0.01). This may mean that the polyester with lysine pendant groups speed up the diffusion of monovalent phospholipids. Also, the average α-value of the SLB exposed to the 5 nM polyester with lysine is lower than the average α-value of the null SLB. This is an indication that there is some type of interaction occurring in the SLB exposed to the polyester because there is a deviation from the Brownian motion. Further experimentation will have to be explored to find if there are any trends with increasing and decreasing negative charge of the phospholipid head group. However, the standard deviations of both the null SLB and the experimental SLB are large, and indicate experimental error. Another possible explanation for the large standard deviation is that the surface of the substrate was not actually found and that the size of the laser beam was different for each area. This would result in changes in the average fluorophore population, τ and D. A way to lower the standard deviation is by using a technique called Z-Scan FCS, where the z-position of the objective is varied with respective to the substrate so the true z_0 position is found.

The results of this experiment compared to the experiment done with Texas Red DHPE and 15 nM polyester with lysine mimic groups are interesting. The change in the average <N> in the confocal volume is different. This could be attributed to the fact that the TopFluor-PS is not head group labeled like the Texas Red DHPE and therefore there is less steric bulk at the head group of the TopFluor-PS compared to the Texas Red DHPE. Also, it has been shown in other experiments

that polycationic molecules do not sequester the monovalent PS.[1,2] The next step of experimentation would include making SLBs doped with PIP_2, a multivalent lipid, and introducing the polyester with the lysine side groups.

FCS Measurement of SLBs Made from SUVs Doped with Polystyrene. Three separate concentrations of styrene in the form of polystyrene (MW = 1000) were used in the composition of SUV. The concentrations included 0.01%, 0.001%, and 0.0001% styrene. The lipid composition in the SUV was 99.998% DOPC and 0.002% Texas Red DHPE. Higher concentrations of styrene could not be experimented on because of a solubility issue. Additionally, a polystyrene MW=10,000 was tried but this composition with lipids could not be extruded. Sonication of the SUV doped with polystyrene MW = 10,000 was attempted; however the styrene would not dissolve in the lipid/water mixture during rehydration. The null bilayer data was collected on separate days and were compared to see if there was any noticeable difference in the population and mobility.



E

	Null	0.01% Styrene	0.001% Styrene	0.0001% Styrene
Average <N>	**10.02**	**9.49**	**12.28**	**13.88**
Std. Dev.	1.01	0.68	1.11	2.17
Average t (ms)	**1.36**	**0.98**	**1.01**	**1.50**
Std. Dev.	0.22	0.13	0.16	0.16
Average D	**10.00**	**13.73**	**13.40**	**8.89**
Std Dev	1.52	1.62	2.04	0.91
Average α	**0.84**	**0.68**	**0.65**	**0.82**
Std Dev	0.08	0.06	0.04	0.06

Scheme 5. Figures A, B, and C show the graphical results from the polystyrene (MW=1000) experiment for <N>, τ, and D with 2x the standard deviation error bars. Figure D shows the autocorrelation curve. Figure E contains the experiment results summarized.

The results of the experiments are located in Scheme 5. A two sample t-Test was completed for each SLB doped with various concentrations of styrene with respect to the null SLB comparing the value of τ. A significant difference was found between the mean τ of the null bilayer and the SLB doped with 0.01% styrene (p-value = 0.0001). Additionally, there was a significant difference found between the mean τ of the null bilayer and the SLB doped with 0.001% styrene (p-value =

0.0006) and the mean τ of the null bilayer and the SLB doped with 0.0001% styrene (p-value = 0.017). Also, the average α-value for the SLBs doped with 0.01% styrene and the 0.001% styrene decreased in comparison to the null bilayer and the SLB doped with 0.0001% styrene. The decrease in α-value means that there is a deviation in Brownian motion and might indicate an interaction. Rossi et al found that polystyrene partitions the liquid-ordered domains regardless of chain length.[6] This would result in a change in lipid concentration where polystyrene is present in the bilayer. The partitioning of the domains would affect the τ of the fluorescently labeled species.

It is interesting to note that the SLBs doped with 0.01% and 0.001% styrene had an increase in the diffusion coefficient, where the SLB doped with 0.0001% styrene had a decrease in the diffusion coefficient. The experiments should be repeated to determine the change in mobility for the SLB doped with 0.0001% styrene as this SLB deviated from the trend set by the SLBs doped with 0.01% and 0.001% styrene. This may have been caused by the SUV used to make the SLB was a day old and may have resulted in a poor bilayer.

There were experimental challenges faced. The true concentration of the polystyrene in the SUV may not be accurate be determined because the polystyrene may interact with the polycarbonate membrane filter in the extruder. This interaction, however, will never be determined. Also, since the polystyrene was not labeled, we have no idea of knowing if the polystyrene remains within the SUV after extrusion and even after making the SLB via rupture of SUV.

Conclusions. We investigated the interactions of various lipids with two different polymer types. There was a significant difference between the lateral mobility of SLBs doped with polystyrene and null SLBs. The SLB with 0.0001% styrene should be remade to see if the bilayer used for experimentation was flawed. The result obtained is in agreement with the simulation reported by Rossi et al where the polystyrene causes partitions in the plasma membrane and therefore affects the mobility of phospholipids. The SLBs doped with 0.01% and 0.001% styrene showed an increase in lateral mobility. For the second polymer type investigated here, there was a drastic change in the mobility and the concentration of labeled Texas Red DHPE when 15 nM polyester with polyester containing pendant lysine mimic groups. The clustering of the labeled DHPE resulted in a decrease in CPS/molecule due to concentration quenching. However, an interesting result was obtained when a SLB doped with TopFluor-PS was introduced to the cationic polyester with lysine mimic groups had an increase in the diffusion coefficient. In the near future, synthesis of the polyesters will include a fluorescent label to conduct pulse interleaved excitation fluorescence correlation spectroscopy (PIE-FCCS). This will allow for the concentration and mobility of the two populations (polymer and phospholipid) to be determined.

Acknowledgements

I want to thank Professor Adam W. Smith (Univ. of Akron, Chemistry Department) for his guidance with this project. Additionally, I want to thank Professor Abraham Joy and his group (Univ. of Akron, Polymer Science) for synthesizing and providing the polyester with lysine pendant functional groups.

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Appendix 1
Safety Considerations

There are numerous hazardous chemicals involved in this research including but not limited to isopropanol and chloroform. Isopropanol is a flammable material and produces hazardous vapors and skin irritation. The flammable solvents should be used far away from open flames. This chemical may be used outside of the hood if used from a squirt bottle. The squirt bottle should be stored away from sinks and in a secondary confinement bin. Chloroform must be used inside of a fume hood. Chloroform creates hazardous vapors, causes skin irritation, and is carcinogenic. Solvent quantities greater than 500 mL should be tightly sealed and stored in a cabinet designated for flammable materials. Eye protection and latex or nitrile gloves should be used when working with these organic solvents. All solvents should be used in well-ventilated areas, usually a fume hood. Any processes including these solvents should be labeled with the chemical name(s) and user name if left unattended. Waste should be deposited in the designated organic solvent waste bottles in the fume hoods. If the waste bottle is full, loosely cap the waste bottle, label as full, and notify the laboratory safety crew for pickup.

Piranha is used to clean the glass substrates in our experiments. This solution contains a 3:1 mixture of concentrated sulfuric acid with hydrogen peroxide. The hazardous chemicals involved in making piranha are concentrated sulfuric acid and hydrogen peroxide. When working with piranha, the reaction may accelerate out of control. This includes the piranha foaming out of its container and onto the deck as well as an huge explosion. Piranha burns organic compounds therefore if there is sufficient fuel such as isopropanol, there will be a large heat

generation. Users of piranha are required to read and sign the standard operating procedure of Dr. Adam Smith's lab. There is a designated acid hood for the piranha to be used. There are special handling procedures and storage requirements for piranha. When piranha is being used only glass containers, preferably Pyrex, should be used. Containers being used should be visibly labeled with a warning sign. Organics should not be brought into the acid hood to prevent explosive reactions. Sulfuric acid and hydrogen peroxide should be mixed in the flow hood with the sash between you and the solution. Also, at a minimum, you should be wearing a lab coat, a face shield, and gloves. An acid smock and thick acid gloves are recommended. When the piranha solution is being prepared, the hydrogen peroxide should always be added to the acid, this will help prevent having a high percentage of hydrogen peroxide and therefore reduce the chances of piranha violently boiling over. The piranha solution is very energetic (exothermic) and has the potential for explosion. The solution is very likely to become hot (> 100 °C). Substrates should be rinsed with water and dried before being placed in the piranha solution. It is very important not to add any water, organic compounds, or bases to the piranha solution as it will accelerate the reaction and increase the possibility of explosion. The hot piranha solution should never be stored. The hot piranha solution should be labeled in an open container until cool. When the solution is cool, dispose of properly in the waste container with a funnel. Small spills of piranha should be neutralized with sodium bicarbonate. If piranha comes in contact with the skin, the affected area should be immediately rinsed with large amounts of water for at least 15 minutes. If piranha comes in contact with the eyes, the eyes should be irrigated

for at least 30 minutes while keeping the eyelids apart and away from eyeballs during irrigation. An ice pack should be placed on the eyes until the emergency room is reached. If the piranha solution is inhaled, conscious persons should be assisted to an area with fresh, uncontaminated air. Medical attention should be sought in any event of respiratory irritation. In the case of a large exposure, the victim should be removed from the contaminated area, placed under a safety shower, and the emergency personal contacted immediately.

The same care and caution used when working with electrical devices and power tools should be used when working with lasers. Special attention should be placed upon making sure the laser beam does not get redirected into eyes. The human body is susceptible to the output of certain lasers. Exposure to the lasers can result in skin and eye damage. Therefore careful attention should be paid to not allow the laser to focus on the eyes or skin to prevent damage.