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Characterization of Lipid Structure and Fluidity of Lipid Membranes on Epitaxial Graphene and Their Correlation to Graphene Features

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Abstract

Graphene has been recognized as an enhanced platform for biosensors due to its high electron mobility. To integrate active membrane proteins into graphene-based materials for such applications, graphene's surface must be functionalized with lipids to mimic the biological environment of these proteins. Several studies have examined supported lipids on various types of graphene and obtained conflicting results for lipid structure. Here, we present a correlative characterization technique based on fluorescence measurements in a Raman spectroscopy setup to study lipid structure and dynamics on epitaxial graphene. Compared to other graphene variations, epitaxial graphene is grown on a substrate more conducive to production of electronics and offers unique topographic features. Based on experimental and computational results, we propose that a lipid sesquilayer (1.5 bilayer) forms on epitaxial graphene and demonstrate that the distinct surface features of epitaxial graphene affect structure and diffusion of supported lipids.

Graphical Abstract

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Supporting Information Available: Descriptions analysis of graphene quality and details for simulations of lipid sesquilayer on graphene. Supporting figures for traditional FRAP setups, analysis of graphene layer number, optical profilometry of epitaxial graphene samples, Langmuir-Blodgett deposition, contact angle measurements, Raman-FRAP of lipid monolayer, modified Raman spectroscopy setup, and supplementary movies from simulations.

Keywords

epitaxial graphene; lipids; sesquilayer; Raman-FRAP; diffusion

1. Introduction

Graphene is a two-dimensional (2D) material comprised of sp^2 hybridized carbon. The high surface area, single-atom thickness, flexibility, and conductivity have made graphene potentially useful in a multitude of applications ranging from energy storage to biosensors.¹ Biosensors are devices that transduce biological indicators into electrical signals, and these devices are vital in the healthcare industry for their applications in drug delivery and disease detection.² Previous studies have evaluated graphene as a platform for biosensing and demonstrated this 2D material as an enhanced foundation for bioelectrochemical systems due to increased electron transfer, higher sensitivity, and rapid response time.^{3–6} Many biosensors are membrane protein-based and can perform diverse functions, including DNA sequencing and toxin detection, depending on the membrane protein incorporated into the device.⁷ For incorporation of membrane proteins into graphene-based devices, graphene's surface must be modified with lipids to simulate the biological domain of membrane proteins and prevent protein denaturation.

Several studies have examined the structure of supported lipids on various types of graphene and their potential uses as enzymatic devices and ion channels.^{8–15} These studies obtained conflicting results for lipid structure on graphene in liquid with proposed structures including a lipid monolayer^{9, 12–15}, lipid bilayer^{8, 11}, and modified 1.5 lipid bilayer¹⁰. These differences in proposed structure most likely stem from the usage of different techniques to form the supported lipid structures and the utilization of graphene grown from a variety of processes and varying post-growth treatment methods. The techniques used in previous studies include both vesicle fusion and lipid-dip pen nanolithography (L-DPN). The process and resulting supported lipid structures on graphene created by L-DPN are different than the vesicle fusion method for two main reasons: 1) L-DPN deposits the lipids in air while vesicle fusion is executed entirely in a liquid environment. 2) Vesicle fusion is more spontaneous in the lipid structure formed whereas lipid dip-pen is more controlled (*self-assembly vs. directed assembly*).

Furthermore, graphene can be synthesized using several different techniques, including thermal decomposition of SiC, mechanical or chemical exfoliation, reduction of graphene oxide (rGO), or chemical vapor deposition (CVD), with each of these synthesis procedures having advantages and disadvantages regarding cost, graphene properties, and purity.^{16–18} Although CVD graphene is recognized as a viable growth method for large-scale, economic production of graphene, this method requires transfer of graphene to another supporting substrate for characterization, causing potential damage and contamination.^{19–20} Epitaxial graphene grown by thermal decomposition of SiC produces high quality graphene on a usable substrate for electronic devices, eliminating the need for transfer and potentially minimizing risk of introducing defects and contaminants to the synthesized graphene.^{21–22} In this work, we examine supported lipids formed by vesicle fusion on epitaxial graphene

grown on the Si-face of 6H-SiC substrates to investigate lipid dynamics and structure on high quality graphene to gain a better fundamental understanding of lipid interactions with graphene and establish a standardized technique for characterization of both lipid and graphene features.

Since graphene quenches fluorescence at a rate an order of magnitude higher than gold, commonly used confocal microscope and detector pairs are not sensitive enough to detect fluorescently tagged lipids on epitaxial graphene, limiting the ability to perform Fluorescence Recovery After Photobleaching (FRAP) experiments to analyze mobile fraction and diffusion coefficients of supported lipid structures.²³ To investigate lipid formations on epitaxial graphene, we developed a new correlative technique to examine quality of graphene, lipid characteristics, and lipid-graphene interactions at the same location on epitaxial graphene (EG) samples using a modified Raman spectroscopy setup. By utilizing Raman-FRAP experiments in conjunction with other experimental methods and simulations, our results indicate that a lipid sesquilayer (1.5 bilayer) forms on epitaxial graphene and has unique diffusion characteristics. This technique provides a simple procedure for analyzing lipid functionalization on all forms of graphene, and the results obtained provide valuable information regarding the structure and dynamics of lipid layers on epitaxial graphene.

2. Experimental Section

2.1 Graphene Synthesis.

Few-layer epitaxial graphene was grown on the Si-face of semi-insulating (Va-doped) silicon carbide (SiC) substrate (from II-VI Advanced Materials). The growth process started with an etch using 10% hydrogen at 1500 °C, to remove any subsurface damage caused by the micromechanical polishing of the wafers. The growth was then conducted at 1800 °C in an argon atmosphere at 700 Torr. These conditions have been optimized to get 1–2 layers of graphene; the non-uniformity is attributed to the formation of steps on the SiC during annealing and hence, causing the graphene to be thicker at the step edges than on the step terraces. During this process, the Si sublimates and the C which is left behind reconstructs to form graphene. This epitaxial graphene is characterized by a buffer layer which is partially covalently bonded to the SiC substrate, and the van der Waal bonded graphene layers are on top of this buffer layer.

2.2 Optical Profilometry of SiC after Graphene Synthesis.

The topography of the SiC substrate changes as a result of the silicon sublimation from the Si-face during graphene synthesis. As mentioned, the graphene nucleates at the steps of the SiC surface and extends along the terrace, therefore forming thicker graphene at the steps. The width of the SiC terrace therefore defines the maximum width of monolayer epitaxial graphene. Additionally, the height of the SiC step defines the slope of the surface. Both of these topographical features, terrace width and slope, likely impact the diffusion characteristics of the lipid layer as demonstrated later in this work from computational studies. The topographical features of the SiC were analyzed using a Zygo NexView 3D optical profilometer with both a 2.75x and 50x objective. The results indicate that there is a

wide range of topographical features even across one SiC 5×5 mm sample, with slope magnitudes ranging from 0 to $8.0 \mu\text{m}/\text{mm}$, widths ranging from sub-micron to $\sim 10 \mu\text{m}$, and regions with linear and non-linear step boundaries. This complexity in surface structure is very likely to have contributed to the variance in the lipid diffusion seen in our studies at each analyzed location. Even though the optical profilometry data was collected in approximately the same region as the FRAP analyzed locations, they could not be compared directly because there was not enough information to identify the exact location.

2.3 Synthesis of Lipid Vesicles.

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2- $1,3$ -benzoxadiazol-4-yl) (ammonium salt) (NBD-PE) powders were purchased from Avanti Polar Lipids. Vesicles of 98% POPC and 2% NBD-PE were prepared using the film hydration method. The lipid solution in chloroform was evaporated using a rotary evaporator with a water bath at 40°C followed by further drying in vacuum oven to ensure complete removal of chloroform. The lipid film was rehydrated using 50 mM Tris pH 7.4, 100 mM NaCl, and 30 mM CaCl_2 . Vesicles were extruded through a 200 nm track-etched membrane to create unilamellar uniform sized vesicles. Vesicle size was verified with dynamic light scattering, and their average diameter was determined to be 160 nm.

2.4 Vesicle Fusion for Supported Lipid Structures.

Formation of supported lipid structures on epitaxial graphene and SiO_2 was executed using vesicle fusion. Lipid vesicles of 98% POPC and 2% NBD-PE were incubated on the substrate using a programmed temperature ramp. Vesicles were incubated on substrate for a total of 4 hours, with 30 minutes at 20°C , a slow increase to 45°C for 30 minutes, and a gradual decline back to 20°C . Samples were gently rinsed with DI water to remove any unruptured vesicles before characterization.

2.5 Raman Characterization of Graphene and Lipid-functionalized Graphene.

Both the Fluorescence Recovery After Photobleaching (FRAP) and Raman measurements were performed on a Horiba LabRam HR Evolution with a 488nm Ar ion Melles Griot laser (Figure S1a). The power used for Raman measurements and the photobleaching step in FRAP was 25 mW at the sample (laser threshold tests were performed for EG to ensure this laser power did not alter the material), and the fluorescence was measured at 25 μW laser power, using an Olympus 40x water immersion objective (NA 0.8). Using the DuoScan mode (galvo-mirror positioned above the microscope objective) on the Horiba LabRam HR Evolution, the laser spot was rastered over a circular region with a diameter of $10 \mu\text{m}$ and binning on the Synapse BIUV CCD (2048×512 pixel) was increased to 7 for the FRAP measurements. The FRAP measurements were recorded with 1 second intervals and the photobleach step was performed for 2–3 seconds. Fluorescence was measured for ~ 100 seconds before and after the photobleach step during the FRAP measurement. The only modification required to add FRAP capabilities onto the LabRam was an external neutral density filter that can be inserted and removed from the 488 nm beam path without interruption to the LabSpec software (Figure S1b).

2.6 Langmuir-Blodgett Deposition of Lipid Monolayer on Epitaxial Graphene.

The lipid monolayer on graphene was deposited using a Langmuir trough (KSV NIMA, 75 cm x 33 cm) equipped with a platinum Wilhelmy-type balance. The trough was filled with deionized water, and then 20 μL of the lipid solution (1 mg/mL in chloroform) was spread on the surface of water. The solvent (chloroform) evaporated in seconds, and the lipids formed a monolayer at the air/water interface. The monolayer was allowed to stabilize for 15 minutes. The lipid monolayer was compressed by decreasing the distance between Teflon barriers on the LB trough at a rate of 5 mm/min.

Figure S2 shows the compression isotherm of lipids at the air/water interface. It shows a clear phase transition (liquid to solid) at ~ 23 mN/m where the mean molecular area of ~ 95 $\text{\AA}^2$, and a collapse at ~ 43 mN/m. The lipid monolayer was deposited on graphene at 35 mN/m to make a uniform film. After compression to 35 mN/m, the monolayer was held for 1 hour to stabilize, and the lipids were deposited on the graphene surface by pulling the substrate out from solution at a constant rate (1 mm/min). The surface pressure was fixed at 35 mN/m during the whole deposition process to make the film uniform on the surface. The transfer ratio, the reduced area of lipids at the air/water interface divided by the deposited area was measured at 0.9 (Figure S3), meaning that the surface density of the lipids on graphene is similar to that of solid phase lipids on the air/water interface. The deposited film was dried for one day under ambient conditions.

2.7 Contact Angle Measurements.

Contact angles were measured using the Ramé-Hart standard goniometer (Model 250-F4) setup. To evaluate contact angle of water on samples, sessile drop method was utilized. A drop of water (2 μL) was carefully pipetted onto the substrate, an image was captured using the camera, and DROPimage Advanced software was immediately used for analysis of the water drop. Multiple contact angle measurements were conducted on each sample to obtain an average value (Figure S4 and Table S1).

2.8 Layer-by-layer Construction of Lipid Sesquilayer on Epitaxial Graphene.

Lipid monolayer on EG was deposited as previously explained using a Langmuir-Blodgett trough. Characterization of the lipid monolayer was performed using contact angle measurements and Raman-FRAP (Figure S4 and Table S1). Contact angle measurements were conducted using a goniometer and the sessile drop method. For the formation of the lipid bilayer on top of the pre-deposited lipid monolayer on EG, vesicle fusion was used. Vesicle fusion was executed in the same manner as described for prior samples except that the vesicles were made to be less 90 nm for greater membrane tension and enhanced ability to rupture.²⁴

2.9 Atomic force microscopy.

The atomic force microscopy (AFM) experiments in air and liquid were carried out on a Bruker Icon Instrument in PeakForce tapping mode. Measurements were conducted in air for the Langmuir-Blodgett deposited lipid monolayer using a ScanAsyst Air tip with spring constant of 0.4 N/m. Liquid measurements of the supported lipids on epitaxial graphene were executed using a ScanAsyst Fluid+ tip with a spring constant of 0.7 N/m. For both

experiments in air and liquid, the force utilized was 750 pN or less, Scan rates of 0.5 Hz and 512 pixels/line were used for the images acquired. All resulting images were plane-fit to the first order to adjust for sample tilt. Lipid thickness analysis was conducted using multiple line scans throughout the image of the supported lipid structure on epitaxial graphene acquired from liquid AFM.

2.10 Simulations of Lipid Sesquilayer on Graphene.

All-atom molecular dynamics simulations of the 1.5 lipid bilayer structure were performed using the program NAMD. The Inorganic Builder plugin of VMD was utilized to create atomic scale models of graphene and the carbon nanotube. Graphene sheets were created by stacking layers of graphene and removing carbon atoms in the y direction. To create the lipid sesquilayer, a single leaflet from a pre-equilibrated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer was placed on top of either stepped and flat graphene sheets, with the lipid tail ends being 0.1 nm away from graphene. Then a POPC bilayer was placed on top of the already positioned lipid monolayer such that the center-of-mass distance between the phosphate atoms of the leaflets was 1.0 nm. A typical simulation system contained a rectangular patch of a graphene membrane, three layers of lipids arranged parallel to the graphene sheet, and a volume of water that, under periodic boundary conditions, separated the bare surface of the graphene membrane from the outer lipid layer. Additional details for the simulation setup are included in the supporting information.

2.11 Frequency Modulated Kelvin Probe Microscopy (FM-KPFM) –

The surface potential of epitaxial graphene as a function of layer number was obtained on an AIST-NT scanning probe micrometer coupled to the Horiba LabRam HR Evolution, enabling co-localized FM-KPFM/Raman scans. The FM-KPFM scans were performed in a 2-pass modality (1st pass scans topography, 2nd scan records the FM-KPFM) with a 10 nm lift for the 2nd pass, lock-in amplifier AC voltage modulation $\sim 2\text{--}5$ V and frequency modulation set to ~ 1 kHz. The scan resolution was set to 512×512 pixel for the 27×27 μm map with a scan rate of 0.2 Hz. An APPNano ACCESS Non-Contact gold coated probe (tip radius nominally 6 nm, nominal resonance frequency ranging 200–400 kHz and a nominal spring constant ranging 25–95 N/m) was used for the measurements. These tips provide optical access to the tip, which are optimal for the co-localized KPFM/Raman scan. The work function of the tip was not calibrated for these measurements, instead we just measured the contact potential difference between the work function of the tip and the work function of the epitaxial graphene.

3. Results and Discussion

3.1 Characterization of Lipid Diffusion of Supported Lipids on Epitaxial Graphene using Modified Raman Spectroscopy Setup

To examine both graphene and lipid features in the same region, a slightly modified Raman spectroscopy setup was utilized (Figure 1a). Raman spectroscopy is a frequently used, non-destructive technique to examine graphene features, such as defects, strain, and number of graphene layers.^{25–28} Both fluorescence and Raman scattering lead to shifts in the wavelength of the incident light, and they can be measured with the same optical set up.

Additionally, Raman spectroscopy is a low-light level technique, requiring sensitive detectors. Consequently, this provides the sensitivity to measure the quenched fluorescence of the fluorescently tagged lipids on EG, overcoming graphene's strong quenching ability previously observed with a traditional Fluorescence Recovery after Photobleaching (FRAP) setup (Figure S5). Performing both Raman spectroscopy and FRAP on the same instrument provides ability to conduct versatile analyses for both materials and biological research (Figure 1). In FRAP experiments, the measurement of diffusion of fluorescently tagged lipids (98% POPC and 2% NBD-PE) was initiated by photobleaching a micron-sized region and monitoring fluorescence recovery. Diffusion coefficients and mobile fractions were extracted from resulting FRAP curves to gain information about lipid fluidity and coverage on substrates (Figure 1b).^{29–30} The following equations were used to determine mobile fraction and diffusion coefficient of supported lipids from FRAP data. The fluorescence intensity was normalized by accounting for both the intensity before photobleaching and immediately after photobleaching as shown in Equation 1, where F_t is the fluorescence intensity at time t , F_0 the fluorescence intensity following photobleaching, and F_i the fluorescence intensity prior to photobleaching.

$$F(t) = \frac{F_t - F_0}{F_i - F_0} \quad (1)$$

The resulting FRAP curve was fitted to an exponential, and the diffusion coefficient and mobile fractions were extracted using:

$$F(t) = A(1 - e^{-t/\tau}) \quad (2)$$

Where A corresponds to the mobile fraction and τ relates diffusion coefficient through $\tau_{1/2}$, which is defined as the half time for recovery (Equation 3).

$$\tau_{1/2} = \frac{\ln(0.5)}{-\tau} \quad (3)$$

For calculation of diffusion coefficient, the radius of the bleached spot, r , is related to laser characteristics and $\tau_{1/2}$ shown in Equation 4.

$$D = \frac{0.25r^2}{\tau_{1/2}} \quad (4)$$

To verify that the FRAP setup using Raman spectroscopy equipment provided accurate diffusion and mobile fraction data for supported lipids, the system was evaluated using a supported lipid bilayer (SLB) on SiO_2 formed using vesicle fusion. The average diffusion

coefficient and mobile fraction obtained for tagged lipids in a SLB on SiO₂ were $2.1 \pm 0.29 \mu\text{m}^2/\text{s}$ and 0.83 ± 0.06 respectively (Figure 2). These values agreed with literature values for SLBs on SiO₂ (diffusion coefficients of $1.2\text{--}2.5 \mu\text{m}^2/\text{s}$)^{31–33} and validated Raman-FRAP for accurate extraction of lipid fluidity parameters. Interestingly, both the average diffusion coefficient and mobile fraction for lipid formations on EG via vesicle fusion were considerably lower. Lipids in the supported structure on EG diffused at an average rate of $1.0 \pm 0.38 \mu\text{m}^2/\text{s}$ with a mobile fraction of 0.47 ± 0.09 (Figure 2). The drastically different diffusion coefficients and mobile fractions suggest that lipid interactions with graphene are considerably different from supported lipids on SiO₂.

3.2 Analysis of Graphene Quality and Layer Number and its Correlation to Lipid Diffusion

In addition to FRAP experiments, Raman spectra were evaluated at the same regions to determine graphene quality (Figure 1c), and photoluminescence (PL) maps were acquired to evaluate lipid homogeneity (Figure 1d). The analysis of Raman peaks for graphene at all FRAP sites confirmed the presence of graphene with negligible defects (detailed in supporting information) and allowed for extraction of the average number of graphene layers for that region. PL maps demonstrated that lipids had fairly uniform coverage on EG. A considerable advantage of performing Raman-FRAP experiments is that graphene quality can be assessed in the same location as lipid diffusion, allowing graphene features and lipid diffusion to be correlated. A few groups have proposed that the full-width-half-maximum (FWHM) of the 2D peak can be utilized to identify the number of layers in graphene.^{34–35} This theory was tested for our samples by correlating both FM-KPFM and Raman of the EG material, and we found that the FWHM cutoff for monolayer graphene is 42 cm^{-1} (Figure S6). In the range of $42\text{--}56 \text{ cm}^{-1}$ FWHM the graphene is a mixture of monolayer and multilayer material. Above a FWHM of 56 cm^{-1} the graphene forms multiple layers. The reason for this range of FWHM for the 2D band may be related to the limited spatial resolution of the micro-Raman setup ($\sim 1 \mu\text{m}$), which could capture monolayer EG in different strain states.³⁶ This FWHM 2D peak dependence is only true for graphene that is void of defects, which is represented by an absence of the D peak. If monolayer graphene is defective, the 2D peak FWHM can increase as the concentration of defects increase.³⁷ Pristine monolayer graphene that is charge screened through doping can also result in linewidth broadening, however this change seen in the overall FWHM of the 2D peak ($\sim 3 \text{ cm}^{-1}$) as a function of doping ($\sim 10^{11} \text{ cm}^{-2}$) is smaller than the difference between our measured monolayer material and multilayer material ($\sim 14 \text{ cm}^{-1}$).³⁸

By evaluating the FWHM and peak shape of the 2D peak using AFM and Raman spectroscopy (Figure S6), the average number of graphene layers was determined for each $10 \mu\text{m}$ FRAP site (Figure 3a-c). Each Raman-FRAP site from all EG samples were divided into three categories (monolayer, mixed monolayer and multilayer, and multilayer) depending on the FWHM for that specific site. Raman maps show the distribution of FWHM and Lorentzian RMS and demonstrate three distinct regimes of graphene layer number within the sample: monolayer, mixture of monolayer and multilayer, and multilayer (Figure 3a,b). We did not attempt to distinguish the exact graphene layer number beyond monolayer because we average the Raman response over the entire $10 \mu\text{m}$ diameter region.

From our combined FRAP/Raman analysis it was identified that the lipid diffusion coefficient increases with graphene layer number (Figure 3d). This was an unexpected result, and the explanation for this correlation remains uncertain. However, we do know that the work function of the epitaxial graphene does decrease with layer number as demonstrated by the increase in the surface potential (contact potential difference) from our FM-KPFM scan (Figure S6) and several other groups analyses of epitaxial graphene.^{39–40} It is known that a change in the work function can result in a change of the surface dipole, therefore it is possible that the surface forces (polarization and electrostatic forces) may create additional resistance in the lipid diffusion when deposited on the monolayer epitaxial graphene. Interestingly, if an intercalating hydrogen layer is formed between the epitaxial graphene and the SiC substrate, the graphene work function layer dependence is inverted.⁴⁰ If these correlated FRAP/Raman measurements were performed on the quasi-free standing hydrogen intercalated epitaxial graphene, one should be able to confirm the proposed theory for this graphene layer dependence lipid diffusion. Regardless, this combined technique approach enabled the discovery of this substrate dependent diffusion, which could be useful for the analysis of lipids on other complex and inhomogeneous substrates. Additionally, this identified correlation offers researchers an approach to potentially tune the lipid diffusion on a single chip/substrate via control over the graphene layer.

Although there have been multiple studies of lipid structure on various types of graphene^{8–15}, there has been no previous work on determining mobile fractions of lipids on any kind of graphene. The mobile fraction for lipids on epitaxial graphene is considerably low at around 0.5, meaning that there is not full coverage of the lipids on EG and/or that some lipids are immobile. Two of the factors contributing to the coverage of supported lipids on graphene and their ability to diffuse are the heterogeneity and hydrophobicity of EG's surface.

One of the unique characteristics of EG that could influence lipid diffusion is the presence of step edges, which are steps of graphene formed by Si sublimation. These steps can vary in step width (1–100 μm), step height (0.1–10 nm), and step angle (30–40°), and are initiating sites for graphene nucleation.⁴¹ The EG samples obtained in this study were variable in all of these topographic features as confirmed by optical profilometry (Figure S7), resulting in a broad standard deviation for lipid diffusion. These carbon ramps can decrease lipid diffusion from the added inclines, cause variations in directional diffusion coefficients, and create differences in chemical potential from the multi-layers of graphene present at these terraces as supported with simulation work described later.

Another factor influencing lipid formations on EG is its wettability. Graphene has demonstrated wettability transparency to its transferred substrate, meaning that the contact angle of water on transferred graphene is more similar to its supporting substrate rather than graphene itself, although the degree of graphene's wettability transparency is uncertain.^{42–47} Since single or few layer graphene has shown wettability transparency, prior studies examining lipid formations on CVD-grown graphene likely observed typical supported lipid bilayer formation resulting from graphene's weak hydrophobicity on top of its supporting substrate. For epitaxial graphene grown on the Si-face of SiC, there is a buffer layer of sp^3 hybridized carbon separating synthesized graphene from the supporting SiC substrate.⁴⁸ The

measured contact angle of our EG samples were $80^\circ \pm 3$, demonstrating a greater degree of hydrophobicity compared to other substrates often used for supported lipid structures (Table S1).

Previous studies examining lipid formations on hydrophobic substrates observed that surfaces exhibiting water contact angles $\sim 80^\circ$ resulted in lipid monolayer formation.^{49–51} Additionally, our experiments utilized vesicle fusion conducted in a buffer with salt to facilitate vesicle rupture and formation of supported lipid structures.^{52–55} Prior studies demonstrated that introduction of salt during vesicle fusion can result in formation of stacked lipid structures.⁵⁶ Due to EG's hydrophobic surface and use of electrolytes during vesicle fusion, a more likely scenario for lipid structure on epitaxial graphene is a lipid sesquilayer (1.5 bilayer), where a lipid monolayer forms with hydrophobic lipid tails adjacent to graphene's surface and a lipid bilayer formed on top of the lipid monolayer. Although not a common lipid structure on supporting substrates, a lipid sesquilayer structure could be valuable for incorporation of membrane proteins into their biological environment because there is a hydrated interface (with the first half layer) and then a full lipid bilayer for membrane protein incorporation. To characterize supported lipid structure on EG, Langmuir-Blodgett and AFM experiments were used in conjunction with simulations.

3.3 Langmuir-Blodgett Technique for Lipid Monolayer Deposition on Epitaxial Graphene

To evaluate our hypothesis of supported lipid sesquilayer formation on EG, we constructed both a lipid monolayer and lipid sesquilayer on EG using Langmuir-Blodgett (LB) deposition. LB deposition was utilized to form a lipid monolayer on EG with the hydrocarbon chains oriented toward graphene's surface (Figure S2–3). To verify desired orientation of the lipid monolayer, contact angle measurements were acquired. The significant decrease in contact angle from $80^\circ \pm 3$ to $52^\circ \pm 8$ indicates lipid head groups in contact with water following LB deposition, thus confirming the lipid orientation (Figure S4 and Table S1). Further characterization using atomic force microscopy (AFM) was executed to provide information about the lipid monolayer coverage (Figure S8). Following monolayer formation and characterization of deposited lipids, a bilayer was formed on top of the lipid monolayer via vesicle fusion, using similar methods to previous lipid-functionalized EG samples. These EG samples were characterized using Raman-FRAP following deposition of the lipid monolayer and again after incubation with vesicles to form the lipid sesquilayer.

For lipid monolayer samples previously described, Raman-FRAP experiments demonstrated that the fluorescently tagged lipid monolayer on EG had low fluorescence intensity, even with the sensitive detector used for Raman spectroscopy. Due to graphene's strong distance dependent quenching, FRAP experiments were unable to be executed because of the minimal change in fluorescence intensity before and after photobleaching (Figure S8).²³ From considerably higher fluorescence intensity previously observed with vesicle fusion samples and ability to perform Raman-FRAP experiments, we concluded that the lipid structure on EG was not a lipid monolayer and must consist of more than one lipid layer.

For the Raman-FRAP experiments of LB-constructed lipid sesquilayer samples, similar fluorescence intensities were observed compared to previous vesicle fusion experiments

conducted. Resulting diffusion coefficient was $1.2 \pm 0.51 \mu\text{m}^2/\text{s}$, similar to the diffusion coefficient obtained for vesicle fusion-EG samples (Figure 2). The considerably higher mobile fraction for monolayer deposition created lipid sesquilayer, 0.76 ± 0.06 , demonstrated that the controlled deposition of LB facilitates formation of a supported lipid structure with less defects as observed from prior studies.^{57–58} The similar values for fluorescence intensities and nearly identical results for lipid diffusion suggest that a lipid sesquilayer is the structure that forms on EG for vesicle fusion samples. Since FRAP relies on fluorescence intensities and photobleaching to extract parameters for lipid diffusion, we propose that the lipid monolayer on EG was not directly involved in the diffusion coefficient and mobile fraction previously obtained from Raman-FRAP experiments of vesicle fusion samples. Instead, only the two leaflets in the overlying lipid bilayer contribute in the calculation of diffusion parameters.

3.4 Atomic Force Microscopy and Simulations for Determining Lipid Structure on Epitaxial Graphene

Atomic force microscopy (AFM) and simulations were performed to confirm the supported lipid structure forming on EG. From AFM measurements, it was observed that the thickness of lipids on EG was $8.3 \text{ nm} \pm 0.44$ (Figure 4a). Lipid thickness of 8 nm is too high to be a supported lipid bilayer, with previous AFM studies of lipid bilayers resulting in a 4–6 nm thickness.^{59–62} A thickness of 8 nm is indicative of a lipid sesquilayer consisting of a lipid monolayer about 2 nm in height, a 2 nm water layer between the lipid monolayer and bilayer, and 4 nm thickness of the lipid bilayer. To validate this thickness as a 1.5 lipid bilayer, simulations were utilized.

We performed all-atom molecular dynamics simulations of the lipid sesquilayer on graphene to examine the height of the lipid structure. A carbon nanotube was incorporated into the lipid bilayer to allow for the passage of water for determination of the water layer thickness at equilibrium. From simulations, we show that 8 nm is representative of a lipid sesquilayer and agrees with our experimentally determined value for this structure (Figure 4 b, c). In addition to thickness of the supported lipid structure, AFM also shows the poor coverage of the lipid sesquilayer, explaining the low mobile fraction previously observed from Raman-FRAP experiments.

A previous computational study examined supported lipid structure formation on graphene using lipid dip-pen nanolithography.¹² Interestingly, these simulations demonstrated that lipids deposited on graphene in air, the standard approach for L-DPN, form an inverted lipid monolayer and transition to a lipid monolayer when introduced to an aqueous environment. The results from this computational study agreed with prior experimental results using L-DPN.^{13–14} This previous work and the findings in our study highlight the differences in supported lipid structure formed on graphene depending on the technique performed (L-DPN vs. vesicle fusion).

3.5 Simulations of Lipid Sesquilayer on Graphene

To further investigate diffusion of lipids on epitaxial graphene, a 170 ns MD simulation on the lipid sesquilayer was carried out for both monolayer graphene and mixed monolayer and

multilayer (mixed) graphene, the two primary structural features of EG observed experimentally (simulations detailed further in supporting information). To normalize lipid diffusion coefficients on graphene from simulations, the diffusion coefficient from an SLB on SiO₂ was used from previous simulations.⁶³ To extract a diffusion value for lipids on SiO₂ from this prior simulation study, the water layer between SiO₂ and the lower bilayer leaflet was estimated to be 1 nm, as observed from previous experimental studies investigating thickness of the water layer between SLBs on silica.^{64–66} For the comparison of computational and experimental results, the experimental value for lipid diffusion on SiO₂ from this study was used to normalize lipid diffusion coefficients determined by Raman-FRAP for EG.

The simulations demonstrated that mixed monolayer and multilayer graphene with a step height of about 2.2 nm caused lipids in the bilayer to have 25% lower diffusion coefficient compared to that on planar graphene (Figure 5b, c). Simulations further showed that there was a much larger difference in diffusion between the upper two leaflets on mixed graphene compared to monolayer regions, indicating that step edges significantly impact diffusion of the two lipid leaflets proximal to its surface making it different from the top most leaflet in the sesquilayer system (Figure 5c). For additional insight into lipid diffusion on EG, directional diffusion coefficients were calculated for mixed graphene (Figure 5d). For the flat region of mixed graphene, isotropic lipid diffusion was observed. On the other hand, the stepped section of mixed graphene demonstrated anisotropic diffusion, with preference for diffusion across steps compared to moving parallel to the carbon steps. By normalizing data to the corresponding value for lipid diffusion on SiO₂, trends in the experimental and simulation results were compared (Figure 5e). Since previous experiments in this investigation demonstrated that fluorescence from a tagged lipid monolayer on EG was quenched and therefore did not contribute to diffusion calculations from Raman-FRAP, only diffusion coefficients of upper leaflet a and b were used for the comparison of simulated and experimental results of lipid diffusion on graphene. Comparing lipid diffusion coefficients obtained for lipids on SiO₂ and EG between experimental and computational work, the general trend of lipid diffusion coefficient variation was similar. Both sets of results demonstrate that lipid diffusion on SiO₂ is higher compared to lipid diffusion on EG, with the reduction in lipid diffusion coefficient on EG being more pronounced in experimental work. The simulations evaluated lipid sesquilayer diffusion on graphene in two distinct cases, monolayer and mixed, and they did generally explain the differences and trends seen with lipids on EG and SiO₂. However, experimental diffusion coefficients had broader variations due to sample variability in step size, height, and angle.

Conclusions

From this study, we have devised an imaging and spectroscopic technique to examine lipid and graphene features simultaneously using Raman spectroscopy. Using Raman-FRAP experiments, structure and fluidity of lipids on epitaxial graphene were evaluated, and the number of graphene layers was correlated to lipid diffusion. The significantly different diffusion coefficient and mobile fraction compared to lipid diffusion on standard SiO₂ substrates indicated that the substrate properties of epitaxial graphene have a large impact on lipid dynamics. We demonstrate through experimental and computational techniques that a

lipid sesquilayer forms on epitaxial graphene. Additionally, simulations demonstrated that the step edges of EG result in distinct lipid diffusion trends and are a major influencing factor for the slower lipid diffusion observed. The lipid-functionalized epitaxial graphene developed and the characterization technique utilized in this study provides an improved understanding of lipid interactions with graphene, produces a unique platform for enzymatic devices based on membrane proteins where the presence of lipid bilayers provide a stabilizing influence⁶⁷, and generates a foundation to gain better understanding of cellular interactions with pristine graphene surfaces.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Raman spectroscopy setup utilized in this study for characterization of lipid and graphene features.

Fluorescence Recovery after Photobleaching (FRAP) and Raman measurements were acquired using a Horiba LabRam HR Evolution system with a 488nm Ar ion Melles Griot laser. **a)** The system was modified to allow for continuous FRAP experiments by adding a neutral density (ND) filter in front of the laser. For the initial and post-photobleach fluorescence intensities of the supported lipid structure on epitaxial graphene, the ND filter was inserted to reduce laser power from 25 mW to 25 μW. The ND filter was removed for the photobleaching step to increase laser power back to 25 mW. The addition of the ND filter allowed the fluorescence intensity of the tagged lipids to be continuously monitored to ensure all fluorescence recovery was acquired after photobleaching. Using the slightly modified Raman spectroscopy setup, graphene quality could be obtained at each

FRAP site to analyze its degree of defects, strain, and layer thickness and its correlation to lipid dynamics. Raman-FRAP experiments provided information of lipid homogeneity on graphene's surface through photoluminescence maps and lipid diffusion coefficient and mobile fraction values were extracted from FRAP curves. **b)** Resulting FRAP curves from the Raman-FRAP setup for lipid diffusion on SiO₂ and epitaxial graphene samples from vesicle fusion and Langmuir-Blodgett experiments. From these curves, the lipid diffusion coefficient and mobile fraction can be extracted. **c)** An example Raman spectrum, after SiC subtraction (see Supporting Information) acquired for a site on epitaxial graphene to determine graphene quality. From the spectrum, the quality of the graphene can be analyzed from its characteristic peaks (D, G, and 2D). **d)** A photoluminescence map showing the lipid coverage on epitaxial graphene's surface after FRAP experiment. A lower fluorescence intensity is observed for the photobleached region due to low mobile fraction of lipids.

Figure 2. Experimental results for lipid diffusion on epitaxial graphene (EG) and silica using Raman-FRAP.

Experimental diffusion coefficient and mobile fraction data for supported lipids on EG and SiO_2 . Vesicle fusion and 1.5 lipid bilayer (constructed using the Langmuir-Blodgett technique) samples demonstrate nearly identical diffusion coefficients, indicating similarity in structure. Lipid diffusion on EG is observed to be considerably lower than diffusion of a supported lipid bilayer on SiO_2 . For the vesicle fusion samples, the diffusion parameters were calculated from five supported lipids on EG samples with a total of 21 Raman-FRAP sites. For the 1.5 lipid bilayer samples (constructed from Langmuir-Blodgett deposition), three LB-constructed lipid sesquilayer samples were used for Raman-FRAP experiments for a total of 14 Raman-FRAP sites. The diffusion coefficient and mobile fraction values were averaged from each of the site values for all samples.

Figure 3. Analysis of average graphene layer number and experimental correlation of number of graphene layers to lipid diffusion coefficient on epitaxial graphene from Raman spectroscopy.

a) Analysis of the FWHM of graphene's 2D peak from a Raman map was conducted to determine the average number of graphene layers for each FRAP site. This Raman map shows the distribution of monolayer, mixed, and multilayer graphene for this specific site. **b)** Analysis of the figure of merit (RMS) for a Lorentzian fit to the 2D peak is used to confirm the graphene layer designation from the FWHM map. **c)** 2D peaks observed for EG depending on the average layer number of graphene. The FWHM increases as graphene layer number increases and monolayer graphene, with minimal defects, should fit to a Lorentzian function. **d)** For each Raman-FRAP site, lipid diffusion and graphene layer number were calculated for both vesicle fusion and 1.5 lipid bilayer experiments (35 sites total). The lipid diffusion of each FRAP site was divided into the three graphene layer number categories (monolayer, mixed monolayer and multilayer, and multilayer) based on the FWHM for that site. For each division of graphene layer number, the average diffusion coefficient was calculated. Monolayer graphene resulted in lower lipid diffusion compared to pure multilayer regions.

Figure 4. Experimental and computational results for height of the lipid sesquilayer on EG at 8 nm agree on the structure of lipid on epitaxial graphene to be a sesquilayer.

a) AFM height sensor image of the supported lipids using liquid AFM with an inset of an example step height from the supported lipid structure. Step heights across the image were averaged to obtain a lipid thickness of $8.3 \text{ nm} \pm 0.44$. **b)** Schematic depicting the simulated lipid sesquilayer with a carbon nanotube incorporated to allow for calculation of the water layer between the lipid monolayer and bilayer at equilibrium. **c)** Plot showing the thicknesses for each layer of the lipid sesquilayer, including the water layer. The height of the lipid sesquilayer from simulation is 8 nm, agreeing with the height of lipids determined experimentally.

Figure 5. Molecular dynamics simulations of lipid sesquilayer (1.5 lipid bilayer) on epitaxial graphene.

a) All-atom model of a lipid sesquilayer on monolayer graphene. A lipid monolayer is placed with its lipid tails oriented towards the graphene's surface (referred to as lower leaflet); a lipid bilayer is placed on top of the lipid monolayer (comprised of upper leaflet a and b). **b)** A mixed monolayer/multilayer graphene membrane. The graphene steps range in length from 0.14 to 0.28 nm; the overall step's height is 2.2 nm. **c)** Simulated diffusion coefficients of lipids in the three leaflets of the sesquilayer structure on monolayer and mixed graphene. **d)** Simulated directional diffusion of lipids on the flat and stepped regions of the mixed graphene system. Coordinate system is displayed in Figure 4b. **e)** Simulated and experimentally measured diffusion coefficients for monolayer and mixed graphene and SiO₂. Both experimental and simulation results show that lipid diffusion is higher on SiO₂ compared to EG. Experimental data for diffusion coefficient on EG is closer to diffusion of mixed graphene compared to the monolayer graphene model. In panels c-e, diffusion coefficients are normalized by the corresponding SiO₂ value. Error bars in panels c-d correspond to the standard deviation.