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Imaging Macromolecular Interactions at an Interface

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Abstract

Important physiological, pathological, and technological processes occur at continuous and dispersed phase interfaces. Understanding these processes is limited by inability to quantitate molecular events occurring at the interface. To provide a model-independent measurement of protein concentration and mobility at the interface, we employed confocal laser scanning microscopy (CLSM). Fluorescently labeled albumin and fibrinogen were studied singly, pairwise, and with a surfactant, Pluronic F-127, in aqueous droplets. CLSM enables measurement of molecular behaviors manifest as surface inhomogeneity and of biophysical quantities including partitioning between the bulk and the gas-liquid (GL) interface. We conclude that albumin and fibrinogen behave substantially differently at the GL interface, that adsorption from multi-species solutions is fundamentally different than adsorption from solutions of single species, and surfactants can inhibit proteins from occupying the interface.

Keywords

Protein Adsorption; Competitive Adsorption; Confocal Microscopy; Surface Concentration; Surfactant; Protein; Gas-Liquid Interface; Air-Water Interface

Introduction

Understanding interactions between proteins and interfaces is critical to understanding the significant role of proteins in physiology, pathology and technological processes including food manufacturing, medical sample analysis, and sample loss in lab-on-a-chip or HPLC applications. Protein interactions can be classified as specific, i.e. receptor-ligand binding, or non-specific, i.e. protein adsorption. Interfaces are classified by their constituents (gas, liquid, solid), their geometry (shape and length scale) and their surface energy. Interest in gas embolism pathology motivates our focus on the non-specific interactions between blood-borne proteins and gas-liquid (GL) interfaces, with the ultimate purpose of developing methods for reducing protein adsorption to the GL interface, such as through the use of polymeric surfactants.

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In order to understand, and eventually demonstrate control over protein adsorption from multi-protein solutions, it is necessary to quantify accurately the surface concentration. Customarily, equilibrium surface concentration at the GL interface is estimated by fitting measurements of equilibrium surface tension to a surface equation of state (SEOS). A SEOS is derived through the combination of the Gibbs adsorption equation,

$$\Gamma = - \frac{1}{RT} \left(\frac{\partial \gamma}{\partial \ln C} \right), \quad (1)$$

which relates the surface excess, Γ , to the change in surface tension as a function of change in bulk concentration, $\left(\frac{\partial \gamma}{\partial \ln C} \right)$, using the universal gas constant, R , and the temperature, T , with an appropriate isotherm,

$$\Gamma = K f(C), \quad (2)$$

which relates the surface excess, Γ , to a function of concentration, $f(C)$, using the constant K . It is readily apparent from Eq. 1 and Eq. 2 that the system of equations can be solved without explicitly calculating Γ . Instead, a theoretical maximal surface excess value, $\Gamma_{\max}$, is calculated using the so-called maximal slope method or through a least squares fit of experimental data to the desired SEOS.

This methodology has been widely used with solutions composed of a single simple surfactant¹ when the bulk surfactant concentration is lower than the critical micelle concentration. Despite the widespread use of this technique, there are reasons to doubt assumptions required to implement the method.² The assumption that the interface is fully occupied before the majority of surface tension change occurs is particularly difficult to reconcile. Furthermore, this methodology is less successful with protein solutions because of protein structure complexity,³ the relatively weak surface activity of protein molecules,⁴ and variability in protein tertiary structure stability.^{5,6} Adsorption isotherms have been derived that treat surface active molecules as both thermodynamically ideal and non-ideal. The Henry isotherm treats surface-active molecules as ideal, while the Langmuir isotherm accounts for site occupancy, and the Frumkin isotherm accounts for site occupancy and non-idealities like electrostatic charge. However, the existing isotherms are widely acknowledged to be poor descriptors of single species protein adsorption. Attempts to use a SEOS to estimate surface concentrations for multi-species solutions comprised of multiple surfactants and/or proteins are even more questionable. The resulting surface concentration estimates are highly model-dependent and mathematically non-unique.

Current methods of surface concentration measurement or estimation (other than SEOS) have significant limitations. The adsorbed concentration on the GL interface has been measured using neutron reflectivity,⁶⁻⁸ ellipsometry,⁹⁻¹² and radiolabeling.¹³ Neutron reflectivity and ellipsometry are limited to single species experiments and data interpretation relies on a geometrical model. Radiolabeling provides model-independent concentration data, but only reports the concentration of a single species. Protein concentration at the solid-liquid interface can be measured using many techniques: bulk depletion,¹⁴⁻¹⁶ high performance liquid chromatography,¹⁷ total internal reflection fluorescence,¹⁸ and quartz crystal microbalance.¹⁹ However, the differences between a solid-liquid, liquid-liquid, or liquid-gas interface are likely to play a significant role in the protein adsorption process. Therefore, a need remains for

a generally applicable and model-independent surface concentration measurement that can be used in multi-species experiments at the gas-liquid or liquid-liquid interface.

Confocal laser scanning microscopy (CLSM) has been used widely to measure protein concentration and localization. CLSM is a precise imaging technique with sub-micron axial and lateral spatial resolution, and high signal-to-noise ratio, even for low concentration fluorescent samples. The ability to record fluorescence data in contiguous optical thin sections makes CLSM well suited to generate three-dimensional images of biological processes in real time in cultured cells.²⁰ CLSM has been used to quantitate protein behavior in ligand-receptor interactions^{21,22} and within membranes,²⁰ as well as reaction rate kinetics²³ and in vivo gene expression.²⁴ CLSM has also been used to investigate bubble shape and size in microfluidic channels²⁵ and to investigate the rheology of immiscible fluid boundaries.²⁶ Very recently, confocal microscopy was used to measure absorbed BSA concentration on glass substrate (solid-liquid interface).²⁷

In the current study, we have developed quantitative CLSM fluorescence measurement techniques to probe protein behavior at the GL interface. Ratiometric measurements enable surface concentration measurement based on differences in the fluorescence intensity in the bulk and at the interface. CLSM enables kinetics analysis through real-time measurement of adsorption. Presented concentration measurements are benchmarked against published NR data, using the Henry isotherm. For clarity, those instances in which “measurement” is used involve model independent values, and wherever “estimate” appears is a reference to model dependent values. In addition, fluorescence recovery after photobleaching (FRAP) experiments provide insight into the reversibility of the adsorption process and the spatial stability of the adsorbed protein layer. Uniquely, CLSM provides a tool for studying the complex interfacial behavior of solutions containing multiple proteins or surfactants at the GL interface.

Materials, Methods, and Image Analysis

Protein and Surfactant Solution Materials

A PBS buffer solution was created from stock salts and MilliQ de-ionized water. The PBS buffer solution is 136 mM NaCl, 8.1 mM Na₂HPO₄, 2 mM KCl, 1.5 mM KH₂PO₄, and 31 mM NaN₃. These salts were used as purchased from Sigma without further purification. The PBS buffer was maintained at pH 7.4 and stored at room temperature.

Bovine serum albumin (BSA, Cat. No. A9647) and human fibrinogen (HF, Cat. No. F4129) were obtained from Sigma and used without further purification. BSA labeled with the fluorophore Texas Red (BSA-TR, Cat. No. A23017) and Human Fibrinogen (HF) labeled with Oregon Green (HF-OG, Cat. No. F7496) were purchased from Molecular Probes, a division of Invitrogen. The BSA conjugate has approximately 3 Texas Red fluorophores attached to the protein molecule. The HF conjugate has approximately 13 Oregon Green fluorophores attached to the protein molecule. A test drop of Texas Red in PBS buffer was imaged to ensure that Texas Red is not a surface active molecule.

Pluronic F-127 (Molecular Probes P-6866) was obtained as a 10% solution in DI water that had been 0.2 μ m filtered, and was used as purchased without further purification. More dilute solutions were created by mixing aliquots of the stock 10% PF-127 with the PBS buffer detailed above. This was done to provide uniformity in the buffer used in all experiments.

Surface Tension Measurements

Surface Pressure (Π) was measured using a KSV Minimicro System 2 with a Wilhelmy plate microbalance. Data were recorded by computer using proprietary software. The minitrough

was housed in a Plexiglas box, with openings appropriate for setup, cleaning, and data cables. The box contained several extra beakers of water to aid in humidity control. After an experiment was started, the box was sealed to allow for humidity stabilization to minimize evaporation. Experiments were run at room temperature.

Minitrough and Wilhelmy plate cleaning

The trough was emptied and cleaned after each experiment. The trough was then towel dried, wiped down with HPLC grade ethanol, rinsed thoroughly with distilled water, and then air dried. The Wilhelmy plate was cleaned in a Bunsen burner flame to remove any adsorbed protein.

Confocal Laser Scanning Microscopy

The inverted CLSM used in these experiments was manufactured by Olympus, model IX81 with Fluoview FV1000 controller. Fluoview version 1.6 was used to control the microscope, and a 40× water immersion objective (1.15 NA) was used to visualize the droplet interface. The HF-OG samples were excited by argon laser (488 nm, 30 mW), and BSA-TR samples were excited by HeNe laser (543 nm, 1 mW). Fluorescence was detected by photomultiplier detector. Scan speed ranged from 2.0 μs/pixel to 10.0 μs/pixel in measurements with different purposes with image sizes of 512×512 pixels under one way scan mode. The time consumed to take a single image was 0.5s the shortest and 2.7s the longest, which are small enough for us to detect the adsorption kinetics of protein adsorption.

The droplet holder, fabricated for this experiment, was comprised of a small (1.5" diameter) plastic Petri dish with a hole bored in the bottom. This hole was covered by a glass coverslip (standard thickness No. 1). The droplet of interest was deposited on the coverslip at the bottom of the Petri dish. The inverted microscope objective imaged the droplet from below the coverslip. The Petri dish lid was used to create a closed volume, and additional water droplets were placed in the Petri dish out of view to minimize evaporation. The Petri dish was cleaned with HPLC grade ethanol and water between each use.

Image Analysis

The fluorescence intensity emitted from the GL interface is calculated by subtracting the averaged bulk intensity from the total measured intensity, and then assigning the excess intensity to a single voxel. Fig. 1(A) shows an illustration of the experimental setup. To measure these quantities an x-y rectangular region of interest, ROI, Fig. 1(B), is defined with the long axis oriented radially relative to the droplet and crossing the GL interface. Measurements along voxel lines parallel to the length of the ROI were averaged over the width of the ROI. The output is the average intensity profile for a line oriented radially within the droplet depicted in Fig. 1(C). This averaging reduced the amount of noise in the intensity measurements. Then the average fluorescence intensity along the line drawn radially (and crossing the GL interface) was summed over a length known to contain the majority of light emitted from the interface.

$$I_{total} \sum_{j=1}^n I_j \quad (3)$$

where I_{total} is the summed intensity, n is the number of voxels in a line parallel to the length of the ROI depicted in Fig. 1(B), I_j is the average intensity of voxel j . For the data included in this paper, this sum extended from the first averaged intensity measurement on the air side of the interface that was greater than the average gas-side fluorescence intensity measurement, and was continued deep into the droplet. The contribution of the bulk fluorescence to the total

fluorescence was calculated by multiplying the average bulk intensity value by the number of voxels that resided in the bulk or at the interface, as shown in Eq. 4:

$$I_{bulk} = I_{avg\ bulk} * n \quad (4)$$

where I_{bulk} is the calculated bulk contribution, $I_{avgbulk}$ is the average bulk voxel intensity value, and n is the number of relevant voxels. The amount of fluorescence that is emitted from the interface can be calculated, $I_{excess} = I_{total} - I_{bulk}$, and then assigned to a single interfacial voxel using $I_{int} = I_{avgbulk} + I_{excess}$, as shown in Fig. 1(C).

To calculate the surface excess, fluorescence intensity has to be related to protein mass. The volume of each voxel in the image, the average intensity of the bulk volume, and the bulk concentration are all known. This information allows for the calculation of the protein mass from fluorescence intensity. This interfacial intensity calculation accurately measures the interfacial peak in spite of fluorescence lensing and diffraction effects.

Results and Discussion

Confocal laser scanning microscopy (CLSM) was used to observe the adsorption of fluorescently labeled proteins to the GL interface and measure surface concentration as a function of bulk concentration and time. Intensity data were quantified using ImageJ²⁸ and the method outlined above. This formulation requires that sources of error affect bulk and interfacial protein molecules similarly. Protein molecules adsorbed at the interface can be different from the protein molecules contained in the bulk. Proteins could change conformation or form intermolecular bonds at the interface. These potential conformational changes lead to concerns about the possibility of photobleaching or quenching having a greater effect on adsorbed proteins. Photobleaching during normal confocal imaging was found to be negligible for the proteins used in this study, both in the bulk and at the interface. To investigate the possible role of quenching, intensity measurements were made over several decades of concentration. These results indicate that the relationship between concentration and intensity is linear, that the linear fit passes through the origin, and the linear range is tunable by changing laser and photo-multiplier tube power settings. The results of these experiments are shown in Fig. 2. Microscope settings are shown in Table 1. The PMT gain and offset settings for high concentration HF-OG had to be changed to avoid saturation at the highest HF-OG concentration. To investigate the role the photomultiplier tube voltage on the partition coefficient, we calculated the partition coefficient for a droplet containing 75.3 μ M BSA-TR and 1.45 μ M HF-OG over a 250 volt range. The results demonstrate a constant calculated partition coefficient for both fluorophores between a power setting of 500 and 600 V as shown in Fig. 3. The evidence that intensity is a linear function of concentration and that the calculated partition coefficient is independent of PMT voltage reinforces the contention that CLSM can be used as a quantitative technique to measure labeled protein concentration.

The effect of fluorophore labeling on the surface activity of the proteins was investigated using a Langmuir trough and Wilhelmy plate. Solutions of 150 pM protein were made for BSA and BSA-TR and solutions of 29 pM protein were made for HF and HF-OG. Surface tension was measured as a function of time for more than 20 h. The labeling of BSA had no observable effect on surface activity and the labeling of HF reduced the surface activity of the protein marginally, although the reduction of surface activity is within the range of error for published protein surface tension data,⁴ as shown in Fig. 4. It should be noted that the maintenance of surface activity is not sufficient to demonstrate that physiological protein function has not been altered by fluorescent labeling.

Assigning all fluorescence intensity to a single interfacial voxel likely underestimates the actual surface concentration (in true concentration units of mole/m³) by overestimating the interfacial volume. However, following the work of Guggenheim,²⁹ the adsorbed surface excess mass can be measured from an arbitrarily large interfacial volume, provided the concentration difference between the interfacial and bulk volumes can be measured. An additional benefit of these ratiometric comparisons between the bulk and the interface is that each intensity measurement is scaled to a known bulk concentration, intrinsically compensating for inter-measurement or inter-experiment fluorescence intensity variability due to laser settings, microscope settings or photobleaching. To this end, following the work of Krishnan et al.,⁴ a partition coefficient may be defined as

$$P = \frac{I_{\text{int}}}{I_{\text{avgbulk}}} = \frac{C_{\text{int}}}{C_{\text{bulk}}} = \frac{M_{\text{int}}/V_{\text{voxel}}}{M_{\text{bulk}}/V_{\text{voxel}}} = \frac{M_{\text{int}}}{M_{\text{bulk}}} \quad (5)$$

where P is the partition coefficient, C is the concentration in standard units of both the interface and the bulk, I represents the measured intensity in arbitrary units from both the interface and the bulk, M represents the mass in one voxel at the interface or the bulk, and V represents the volume imaged in a single voxel. The surface excess mass calculation is straightforward.

Partition coefficient data for BSA-TR and HF-OG are presented in Fig. 5. The existence of a CMC-like critical concentration is evident for the two proteins. The solid black symbols are for concentrations below the critical concentration, and the gray symbols with black outlines are for concentrations above the critical concentration. While the data below the critical concentration show some variation, the error bars on the data overlap despite a 10-fold increase in bulk concentration. In addition, the change in the mean value over this single order of magnitude increase in bulk concentration is ~ 5 , which is less than the spread in the data at each concentration. Therefore, we treat the partition coefficient as a constant below the CMC-like concentration.

Using the total intensity calculated from Eq. 3 and the known size of the voxel, the surface excess (mole/m²) as well as the adsorbed footprint per molecule in a complete monolayer may be calculated. In protein surface tension experiments, there is a protein-specific bulk concentration that is sufficient to ‘fill’ an interfacial monolayer, similar to a critical micelle concentration for surfactants. Treating the surface layer as ideal, e.g. the Henry isotherm or the Langmuir isotherm at low concentrations, the partition coefficient defined in Eq. 5 is expected to be constant for a given protein when the bulk concentration is lower than this critical concentration. This assumption ignores potential non-idealities such as steric interference (e.g. the Langmuir isotherm at high concentrations), electrostatic charge, hydrophobic cooperation (e.g. the Frumkin isotherm) or post adsorption conformation change that could exist during protein adsorption. The use of a different model would require the partition coefficient to be a function of the bulk concentration and possibly time. Because it is unclear that there exists an isotherm appropriate to describe protein adsorption, and our goal herein is to demonstrate the applied utility of the measurement technique, this idealized model is used solely for method benchmarking and to enable interpretation of single protein and multi-protein experiment comparison.

Using previously reported modeling,⁴ the bulk concentrations required to ‘fill’ the interface are estimated to be 10.5 μM for BSA-TR and 1.2 μM for HF-OG, which is in agreement with the data in Fig. 5. The partition coefficient for BSA-TR at or below a concentration of 0.07% was found to be 17.9 ± 7 . Using the voxel volume, the partition coefficient estimate, the bulk concentration and the interfacial area the surface excess, Γ in units of mass/area, can be

calculated and compared with published NR results. The CLSM surface excess estimate is highly dependent on the bulk concentration value, so the method was benchmarked against NR data for a 5 mg/L BSA solution, a concentration that guarantees incomplete monolayer adsorption. Using the mean calculated partition coefficient of 17.9, a pixel volume of $0.81 \mu\text{m}^3$ and an interfacial area of $0.9 \mu\text{m}^2$, the estimated surface excess concentration is $0.81 \pm 0.32 \text{ mg/m}^2$ with an estimated molecular footprint of $13,612 \pm 8663 \text{ \AA}^2$. These values compare remarkably well with published NR data⁷ which estimate the surface excess concentration to be 1.0 mg/m^2 with an interfacial area of $11,000 \text{ \AA}^2$ for the same bulk BSA concentration. The partition coefficient for HF-OG at or below a concentration of $1.2 \mu\text{M}$ was found to be 42.8 ± 4 . Using the same voxel volume and GL surface area, the calculated surface excess for a $1.2 \mu\text{M}$ HF-OG solution is in the range of 14.1 to 16.7 mg/m^2 with an estimated footprint per adsorbed molecule ranging from 3409 to $4047 \text{ \AA}^2/\text{molecule}$. Similar benchmarking data are not available for HF. There is evidence that HF absorbs in multilayers,^{4,16} so the 2-D footprint estimation is likely to underestimate the actual footprint per molecule.

These data follow the expected trends that larger molecules will fill a monolayer at lower concentrations, and that larger molecules will have lower interfacial concentrations.^{4,14} Additionally, the partition coefficients indicate that for a given bulk molar concentration, twice as many HF-OG molecules will occupy the interface as BSA-TR, for this experimental system. This corresponds to $\sim 20\%$ increase in free energy gain due to adsorption per molecule, based on $\Delta G = -RT \ln P$,¹⁶ where P is the partition coefficient. It is important to note that the error in the concentration measurement does not impact the partition coefficient calculation, as the volumes of the voxels cancel out in the calculation.

It does not appear that fluorophore quenching at the interface poses any experimental difficulty for the labeled proteins studied here. Quenching effectively changes the number of active fluorophores per labeled protein molecule. It is expected that quenching phenomena would be concentration dependent, thus rendering the partition coefficient a function of bulk concentration. Although the measured error in the BSA-TR partition coefficient is large ($\sim 42\%$), the overall trend was for the partition coefficient to increase with concentration, rather than decrease as would be anticipated with quenching. The consistency of the BSA-TR surface excess measured by confocal microscopy with the BSA surface excess measured by NR further suggests that quenching is not significant for BSA. There are no NR data to which we can compare our HF-OG results. However, the error in the partition coefficient is both relatively small ($\sim 16\%$) and consistent with the error reported for fibrinogen using bulk depletion measurements.¹⁶ In general, there is considerable variability in surface science experimental results, and the errors reported here are consistent with those in the literature. The magnitude of quenching experienced by the adsorbed protein molecules in this study is smaller than the inherent noise in the experimental system.

Proteins do not always organize homogeneously at the GL interface. Different interfacial phases have been observed for lysozyme and β -casein.³⁰⁻³² Interfacial inhomogeneity has also been suggested to play a role in the observed lag time between surface excess formation and surface tension change for low concentration protein solutions.³³ Fig. 6 shows the existence of two adsorbed phases of HF-OG at the interface of a 29 pM HF-OG droplet. This interface was much more mobile than the more homogeneous layers formed by HF-OG at higher concentrations. The expected surface tension for this HF solution at the time of this image is $\sim 60 \text{ mN/m}$.³⁴ This image implies that HF aggregates at the interface, and results in a series of islands of aggregated HF surrounded by areas of non-aggregated HF. The resolution provided by CLSM ($\sim 0.5 \mu\text{m}$ in the x-y plane) supports the aggregation hypothesis of Rao et al.³³ in the case of fibrinogen.

The lack of surface concentration data during competitive adsorption experiments has posed a significant challenge to competitive adsorption modeling efforts. Competitive adsorption can be divided into three different regimes based on bulk concentration conditions:¹⁴ [1] total bulk protein concentration is insufficient to fill a monolayer, [2] total bulk protein concentration is sufficient to fill a monolayer, but none of the single protein concentrations is sufficient to fill the interface independently, [3] one or all of the bulk protein species is present at a concentration sufficient to fill the interface independently. The third regime is the most physiologically relevant, and may provide more insight into the nature of the interfacial layer.

Binary solutions of BSA-TR and HF-OG were used to investigate competition between two blood borne proteins. Table 2 shows the concentrations used in these experiments. The concentration ratio 75.3 μ M BSA-TR:1.4 μ M HF-OG was chosen because both concentrations are sufficient to fill the monolayer. Interestingly, the measured interfacial concentration ratio was in the range 1.40-1.73 for all solutions with bulk molar concentration ratio of 1, as shown in Table 2. Also, for the solution containing 75.3 μ M BSA-TR:1.4 μ M HF-OG, the partition coefficient ratio (P_{BSA-TR}/P_{HF-OG}) of 1.6 falls within that range, although the interface contains more BSA in molar units because the bulk molar concentration ratio (BSA-TR:HF-OG) is $\sim$ 51. Based on the single species partition coefficient estimates, the partition coefficient ratio is $\sim$ 0.42, where the single species data suggest that HF-OG is significantly more surface active than BSA-TR. However, when the two proteins are mixed at a variety of equal and unequal bulk molar concentrations, BSA was found to have preferentially adsorbed to the GL interface within the time frame of 1 h. These CLSM results run contrary to previous surface tension modeling,³⁵ and indicate that multiple species adsorption cannot be modeled as a linear combination of single species adsorption. This also demonstrates that further research into multi-species adsorption should use the complete fluid, i.e. whole blood or serum, as the test liquid. The entire blood proteome will not respond as a sum of single well-defined components. This CLSM method will make it possible to develop physical models of competitive protein adsorption that consider the effects of isoelectric point, hydrophobicity, molecular size and tertiary structure flexibility on surface concentration.

We used fluorescence recovery after photobleaching (FRAP) experiments to investigate the nature of the adsorbed protein layer. The potential repopulation of a photobleached region of interest (ROI) provides insight into interfacial mobility, protein adsorption kinetics, and the reversibility of the protein adsorption process. ROIs were photobleached before the interface and the bulk had reached equilibrium. Therefore the fluorescence intensity of the interface increased as a function of time, even in the unbleached regions, because proteins continued to partition at the interface throughout the experiment. Fluorescence recovery can occur through diffusion along the interface of adsorbed proteins, or through the continued adsorption of new protein molecules to the GL interface.

Four ROIs were bleached on the GL interface in 15-min intervals starting at time 0, ($\tau < 1$ sec). ROIs were bleached using 365 and 351 nm lasers for 10 sec. In these experiments the ROI was microns wide, and centered on the interface to minimize photobleaching in the bulk.

BSA droplets, at all concentrations and times, recovered fully from photobleaching in less than 0.5 s, the timescale required to complete a single image. In contrast, HF-OG recovery was much slower. An image of a photobleached HF-OG droplet is shown in Fig. 1(E). Fig. 7 shows the percent fluorescence recovery at 1 h for three concentrations of HF-OG. One hour was chosen as the final time point because the interfacial intensity value was reaching its asymptotic value. Ordinate values in Fig. 7 represent the age of the interface when the ROI was photobleached. The ROI photobleached at time 0 on a 2.96 μ M HF-OG droplet does not fully recover from photobleaching. The ROIs photobleached at times 0 and 15 min on a 290 pM HF-OG droplet fully recover within 1 h, while the ROI photobleached at 30 min does not. The

29 pM HF-OG interface recovered in less time than the image acquisition timescale, similarly to the BSA-TR solutions.

These experiments demonstrate the significant differences between the adsorbed layers of BSA-TR and HF-OG. The very rapid fluorescence recovery of the BSA interface demonstrates that the adsorbed layer is unstructured and highly mobile for a minimum of 1 h, even at physiological concentrations $O(100 \mu\text{M})$. In contrast, droplets of HF-OG, at varying concentrations, establish immobile and irreversibly adsorbed protein layers. The time required to establish an immobile and irreversibly adsorbed layer is concentration dependent. The immobile layer is established in less than one second for a HF-OG concentration of $2.9 \mu\text{M}$. The same process takes longer than 15 min for a droplet containing 290 pM HF-OG, and takes longer than 1 h for a droplet containing 29 pM HF-OG. Furthermore, the speed at which fluorescence recovery occurs was different for the two proteins. For $1.5 \mu\text{M}$ BSA-TR, fluorescence recovery occurred too quickly to be observed even after 45 min, while for 290 pM HF-OG at time zero fluorescence recovery took minutes to occur (data not shown).

The results of the single species FRAP experiments provide significant insight into protein adsorption at the GL interface. While it is clear that intermolecular interactions can play a significant role at the interface, as is true for HF-OG, the BSA-TR data suggest that these interactions are not significant for all protein solutions. The lack of a coherent and widely accepted model of protein adsorption could in part be explained by these observations. Additionally, the essential absence of interfacial mobility in the adsorbed HF-OG layers is striking. The photobleached ROIs were roughly $10 \mu\text{m}$ long $\times$ $3 \mu\text{m}$ wide $\times$ $1 \mu\text{m}$ deep, corresponding to the confocal plane. Because the ROI photobleached at time 0 min on the $2.9 \mu\text{M}$ HF-OG drop did not leave the $1\text{-}\mu\text{m}$ thick focal plane, or translate in the focal plane, we can conclude that the ROI did not move more than $1 \mu\text{m}$ in 60 min.

In the experiments where the ROI does not diffuse out of the focal plane, FRAP measurements can be used to measure both adsorption and interfacial diffusion kinetics. In the $2.9 \mu\text{M}$ and the 290 pM HF-OG FRAP experiments the fluorescence recovery rate is almost identical to the fluorescence increase rate of the unbleached interface at times greater than 30 min (data not shown). The similarity of these two rates suggests that adsorbed HF-OG cannot diffuse along the interface, and that continued adsorption is primarily responsible for the fluorescence recovery. If protein adsorption is irreversible, then interfacial diffusion kinetics could be analyzed by comparing the fluorescence recovery rate of the ROI to the fluorescence increase rate of the unbleached interface.

FRAP experiments were also conducted to investigate competitive adsorption. In this experiment a droplet containing $7.5 \mu\text{M}$ BSA-TR and 145 pM HF-OG was used. Several characteristics of the interface could be observed. First, adsorbed BSA-TR and HF-OG were homogeneously distributed along the GL interface, indicating that BSA-TR is trapped in, or a member of, an HF-OG interfacial layer. Second, photobleached BSA-TR was evident in this experiment, although it was never observed in single species BSA-TR droplets (see Fig. 1(D) and Fig. 8). This confirms that the single species BSA-TR interface is extremely mobile. Finally, the ROI bleached at time zero in the droplet containing both BSA-TR and HF-OG did not exhibit total fluorescence recovery at 1 h, while the ROI bleached at time zero in the single species HF-OG droplet containing a higher HF-OG concentration (290 pM) did exhibit full recovery. This suggests that the HF-OG is able to form a stable interfacial network that contains a significant amount of non-HF (BSA) proteins, even at low concentrations. These results convincingly prove that adsorbed layers of multiple proteins cannot be described by linear combinations of the adsorbed single protein layers.

Research from our lab has previously demonstrated that non-toxic surfactants confer protection during gas embolism by preserving endothelial cell function^{36,37} and inhibiting bubble induced thrombogenesis.^{38,39} To determine if the mechanisms of surfactant protection during gas embolism were related to protein adsorption inhibition, as opposed to surface tension reduction, intensity data were collected as a function of HF-OG concentration with a PF-127 concentration of 80 μ M. Pluronic F-127 is a polyethylene oxide and polypropylene oxide block copolymer that is FDA approved and commonly used in drug compounding. As shown in Fig. 9, no HF-OG intensity peaks were detected at the interface. Clearly, PF-127 is dominating the interface, effectively inhibiting fibrinogen absorption. This finding has profound implications for the mechanism through which PF-127 is able to preserve endothelial function during gas embolism.³⁷ In addition to reducing the contact line tension of an occluding micro-bubble, PF-127 prevents significant protein adsorption, which explains the observed attenuation of the physiological reaction to the bubble interface.^{36,38,39}

Conclusions

CLSM promises to impact greatly our understanding of molecular adsorption to GL interfaces, with extensions to other interfaces comprised of solids, liquid, or gases. Although the method was benchmarked against published data using the Henry Isotherm, the imaging technique allows for the measurement of protein surface excess without the need for an isotherm, knowledge of protein conformation or orientation, or the use of surface tension data. Surface excess measurements will prove particularly useful for competitive adsorption between multiple proteins or between proteins and surfactants. Images of 29 pM HF-OG suggest that HF-OG forms a heterogeneous interfacial layer, at least at low concentrations. This CLSM technique has shown that BSA-TR was preferentially adsorbed onto the interface in place of HF-OG with the two proteins mixed both at equal and unequal bulk concentrations, indicating that interfacial concentration ratios are not equivalent to the bulk concentration ratios. This technique has also confirmed that the surfactant PF-127 successfully outcompetes the protein for interfacial area. This phenomenon has significant application in a number of physiological, pathological, and technological processes. The FRAP experiments performed here prove that adsorbed layers of BSA-TR and HF-OG are fundamentally different, and prove that adsorbed layers containing more than one surface active species cannot be modeled as a linear combination of the two constituents. In short, this CLSM method provides model-independent data on surface concentration, protein adsorption rate, and interfacial mobility, diffusion, and homogeneity as a function of time, concentration, and number of proteins. Additionally, CLSM makes it possible to investigate some of the complex macromolecular adsorption processes such as changes in conformation, orientation, and polymerization through imaging techniques such as fluorescence lifetime imaging or resonance energy transfer. While this technique has provided insight into the physiological interaction between the GL interface and its environment, it is broadly applicable beyond our specific interest.

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Figure 1.

Intensity measurement and calculation using ImageJ, (A) illustration of inverted microscope setup, (B) measuring box over a sample BSA-TR image, (C) a schematic for the calculation of the surface intensity with the measurement shown on the left, and the calculation result shown on the right. The confocal image plane (x-y plane) is represented by the dashed line and water is depicted between the objective and the coverslip. Sample images from the photobleaching experiments for (D) BSA-TR and (E) HF-OG. Scale bar is 10 μm .

Figure 2.

Measured fluorescence intensity is a linear function of protein concentration. The linear range can be chosen by changing laser power and PMT voltage settings. BSA-TR is represented by the circles and HF-OG is represented by the triangles. The open symbols represent high power settings and the closed symbols represent low power settings. CLSM settings are given in Table 1.

Figure 3. Effect of PMT voltage on measured partition coefficient. The solid line represents the HF-OG results, and the dashed line represents the BSA-TR results.

Figure 4.

Fluorescent labeling of protein molecules has a negligible effect on surface activity. HF-OG is represented by the solid line, HF is represented by the dotted line (••), BSA-TR is represented by the dashed line (- -), and BSA is represented by the dash-dot-dot (-••) line.

Figure 5.

Plot of measured GL interface partition coefficients for BSA-TR and HF-OG. The HF-OG partition coefficient is represented by squares (■), and the BSA-TR partition coefficient is represented by circles (●). Black symbols represent data included in the partition coefficient average. Gray symbols with black outlines were not included in the average because the bulk concentration was higher than the concentration required to 'fill' the interfacial layer.

Figure 6. HF-OG creates a highly mobile, inhomogeneous interfacial layer. Confocal image of the GL interface for a HF-OG droplet with a bulk protein concentration of 29 pM in a PBS buffer. Scale bar is 10 μ m.

Figure 7.

FRAP results for three concentrations of HF-OG, black represents 2.9 μM HF-OG, light gray represents 290 pM HF-OG, and dark gray represents 29 pM HF-OG. Unbleached-area-interfacial intensity increases as a function of time during the experiments. Error bars represent the 90% confidence interval on the mean.

Figure 8.

FRAP data for a two species droplet containing 7.5 μM BSA-TR and 753 pM HF-OG.

Fluorescence recovery for BSA-TR is represented by the black bars and fluorescence recovery for HF-OG is represented by the gray bars. Unbleached-area-interfacial intensity increases as a function of time during the experiments. Error bars represent the 90% confidence interval on the mean.

Figure 9.

Pluronic F-127 blocks protein adsorption. Plot shows four average intensity profiles measured at 1 h near the droplet edge. The ImageJ ROI, referred to in the abscissa, is shown in Fig. 1(a). The thick solid line is the intensity profile for a droplet containing 2.9 μM HF-OG and 80 μM PF-127. The thick dashed line is the intensity profile for a droplet containing 29 μM HF-OG and 80 μM PF-127. The thin solid line is the intensity profile for a droplet containing only 2.9 μM HF-OG, and the thin dashed line is the intensity profile for a droplet containing only 29 μM HF-OG.

Table 1

CLSM settings used to investigate fluorescence intensity

Protein (settings)	Laser	Laser power	PMT voltage	PMT offset	PMT gain
BSA-TR (low)	543 nm	15%	499 V	3	8
BSA-TR (high)	543 nm	55%	571V	3	8
HF-OG (low)	488 nm	20%	321 V	1	7
HF-OG (high)	488 nm	40%	571V	3	8

Table 2

BSA-TR:HF-OG competition results

[BSA-TR]:[HF-OG]	$P_{\text{BSA-TR}}$	$P_{\text{HF-OG}}$	$\left(\frac{P_{\text{BSA-TR}}}{P_{\text{HF-OG}}}\right)$
75.3 μM:1.45 μM	11.3±5.82	7.12±4.38	1.60
1.5 μM:1.5 μM	7.2±0.51	5.18±0.45	1.40
753 pM:753 pM	24.71±1.87	15.85±2.38	1.57
300 pM:300 pM	77.9±8.09	44.98±2.55	1.73