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ANALYSIS OF STRUCTURAL CHANGES CAUSED BY THE ASSOCIATION OF SMALL MOLECULES WITH GLOBULAR PROTEINS

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Jovany Jordan Betancourt

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To: Dr. Steven Oberbauer, Chairperson

Department of Biological Sciences

This Undergraduate Honors Thesis in Biological Sciences, written by Jovany Betancourt entitled "Analysis of Structural Changes Caused by the Association of Small Molecules with Globular Proteins", is submitted to you in partial fulfillment of the requirements for Undergraduate Honors in Biological Sciences. The Biological Sciences Undergraduate Honors Committee and the candidate's research supervisor have read this thesis. We recommend that it be approved.

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Dr. Jaroslava Miksovska,

Honors Research Supervisor

____

Dr. Walter M. Goldberg, Chairperson,

Undergraduate Honors Committee

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This thesis by Jovany Betancourt is approved.

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Dr. Steven Oberbauer, Chairperson

Department of Biological Sciences

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ABSTRACT

The biophysical study of proteins has allowed an improved understanding of the relationship between form and function. This relationship is the basis of allostery, the modulation of protein function by association of allosteric effectors that cause conformational changes. Protein interactions with electromagnetic fields often are used to experimentally measure and characterize structural changes caused by the binding of small molecules. One such molecule, the oxygen transporter hemoglobin (Hb) has been extensively studied since the 1960s but here is examined as a model system for structural dynamics. The functionality of Hb is naturally regulated by association of endogenously produced effectors such as 2,3-diphosphoglycerate (DPG). Quantification of effector binding strengths to Hb are useful for the development and comparison of compounds. Here, photoacoustic calorimetry (PAC) was employed in a novel way to quantify the dissociation constants (K_D) and thermodynamic profiles of two known Hb effectors, inositol hexakisphosphate (IHP) and 8-hydroxypyrene-1,3,6-trisulfonic acid (pyranine) binding to oxygenated Hb (oxyHb). The K_D values for IHP and pyranine were calculated at various temperatures and are consistent with previous studies. The thermodynamic profiles were also found and used to propose differences in the mechanisms of binding. Work must be done to optimize this technique to minimize variations in measurements, and to expand on the experimental potential of PAC. Environmental factors can also contribute to changes in protein shape. Perfluoroalkyl surfactants are a class of industrial compounds which have been studied recently due to their toxic properties and widespread contamination in foodstuffs and water sources. However, the effect of surfactants on protein structure is not well defined. Here, the effects of perfluorooctanoic acid (PFOA),

a member of the perfluoroalkyl surfactant family, were examined on Hb structure using emission spectroscopy, transient absorbance, and circular dichroism spectroscopy. It was found that PFOA had a substantial effect on Hb conformation as evidenced by blue-shifting of the emission spectra, differences in affinity of Hb for carbon monoxide (CO), and changes in Hb secondary structure. The results also show that changes in Hb structure were dependent on PFOA concentration, suggesting the presence of multiple binding sites of different affinities. These results indicate that amphipathic molecules have a profound effect on heterotetrametric globular proteins such as Hb. Future work should use docking simulations or crystallography to determine where PFOA binds, and the interactions of Hb with other amphipathic molecules.

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INTRODUCTION

Proteins are responsible for carrying out a majority of the most complex functions of living systems. Protein malfunction through genetic or environmental influences can lead to the onset of serious pathologies. Sickle cell disease is a human genetic disorder where a mutated version of hemoglobin (Hb) causes erythrocyte polymerization and deformation, leading to hypoxemia and death (Safo et al., 2014). Treatments for sickle cell disease include the use of synthetic allosteric effectors that promote structural transitions to prevent sickling (Beddell et al., 1984; Merrett et al., 1986; Safo et al., 2004; Abdulmalik et al., 2011). Allosteric effectors are compounds that alter the functionality or activity of a protein when binding to an allosteric site. Association of an effector to the allosteric site induces conformational changes that modulate the affinity of the protein to other ligands (Marden et al., 1990; Lal et al., 2017).

Using photoacoustic calorimetry to determine dissociation constants for the association of allosteric effectors to human hemoglobin

The naturally produced Hb effector 2,3-diphosphoglycerate (DPG) is well-studied due to its importance in regulating oxygen (O_2) affinity (Huehns, 1974; Nelson et al., 1974; Bordbar et al., 2006). Binding of DPG to Hb reduces O_2-affinity resulting in easier gas diffusion from erythrocytes to surrounding tissue. Diminution of DPG production is implicated in certain forms of anemia where increased O_2-affinity lowers tissue delivery and causes hypoxic conditions (Huehns, 1974). Loss of DPG due to long-term storage of donated blood is also a concern as transfusions are less effective (Scott, 2016). To treat

disorders that arise from the dysfunction of enzymes, it is necessary to restore physiologically optimal functionality through the use of exogenous effectors.

Like DPG, many organic phosphates are known for being allosteric effectors of Hb. Inositol hexakisphosphate (IHP) is a well-studied phosphate-containing cellular signaling molecule with a variety of functions in different organisms. Laser-scanning confocal microscopy studies in vascular plants by Lemtiri-Chlieh and colleagues (2000, 2003) have suggested that guard cells produce IHP as an important response factor to abscisic acid buildup by releasing Ca^{2+} from endomembrane stores. Mulugu et al. (2007) also suggested that IHP is crucial for yeast cell structure, and that inhibition of IHP kinase pathways leads to defects in cell morphology and functionality. In avian erythrocytes IHP is produced as an effector to modulate Hb activity (Messana et al., 1998). Studies of Hb protein dynamics often utilize IHP as the interactions are well established (Gray et al., 1971; Tomoda et al., 1977), where current models predict IHP binds to the DPG allosteric site and reduces O_2-affinity in a similar manner.

Biochemical studies of pH-sensitive protein conformational changes have used the fluorescent DPG analog 8-hydroxypyrene-1,3,6-trisulfonic acid (pyranine) as a fluorescent probe. First synthesized in 1939, pyranine has been used to study structural changes by measuring differences in fluorescence intensity caused by association and dissociation (Wolfbeis et al., 1983; Willoughby et al., 1998; Yessine et al., 2003; Roche et al., 2006; Shirron et al., 2011). Pyranine is believed to bind to Hb at the DPG allosteric site in the β-β terminus of the central cavity (MacQuarrie et al., 1972). Similar to IHP and DPG, pyranine decreases O_2-affinity upon binding to the DPG-site.

To determine the strength of association between proteins and their effectors, researchers calculate either the association constant (K_A) or dissociation constant (K_D). These values are used to predict the behavior of competing substrates and their effect on a protein. *In silica* experiments are useful as they can quickly compute protein-effector values at different parameters without using physical reagents or requiring the design of hypothetical effectors (Lybrand et al., 1986; Åqvist et al., 1994; Ryde et al., 2016; Tobi, 2017; Chen et al., 2017). However, predictions made computationally must be translated into experimental methods to prove validity. Isothermal titration calorimetry (ITC) has been used as a "gold standard" for experimentally determining binding affinities of protein-ligand complexes. In ITC, a microcalorimeter measures the heat released by ligand binding to calculate thermodynamic values. A large range of biomolecular interactions has been characterized using ITC (Velazquez-Campoy et al., 2001; Leavitt et al., 2001; Rosenberry et al., 2017; Xia et al., 2018; Hu et al., 2018). There are limitations when using ITC for multi-site complexes (Zhao et al., 2015; Brautigam, 2015). Surface Plasmon Resonance (SPR) is a relatively new, label-free technique that measures rates for association and dissociation of protein-ligand complexes by observing changes in the resonance angle (Ahn et al., 2017; Burkhard et al., 2010). Still, proteins must be bound to the instrument surface which may disrupt protein structure or block binding sites.

Photoacoustic calorimetry (PAC) is a photothermal technique that utilize pulses of light generated by a laser to induce a photodissociative reaction; this results in an expansion of the solution that changes the pressure of the system. These measurements are collected using piezoelectric sensors and are digitized as signals using an oscilloscope. Rothberg and colleagues (1983) first developed the process to

experimentally determine the heat of reaction of radical pair formation by triplet benzophenone and aniline. Historically, PAC has been used to determine kinetic and thermodynamic parameters, such as dissociation enthalpies, for phototriggered reactions (Rothberg et al., 1983; Rudzki et al., 1985; Laarhoven et al., 1999). Photothermal techniques such as PAC are label-free, easily performed, and can be used to study a wide variety of systems. I will use PAC in a novel way to experimentally determine K_D of effector binding to Hb by plotting signal changes as a function of effector concentration.

Studying the effects of industrial surfactants on the structure of hemoglobin

Contamination of the environment with the byproducts of industrial operation continues to be a serious concern due to ecological and public health risks. Industrial surfactants have become the focus of intense research initiatives due to their extensive contamination of bodies of water and foodstuffs (Schwieger et al., 2013; Li et al., 2018; Wang et al., 2019). Surfactants are amphiphilic compounds used in the industrial production of polymers and other materials. These molecules are utilized to help dissolve molecules in solvents they normally would not be soluble in. Research into the environmental and health impacts of these industrial detergents has shown that they are toxic (Zhang et al., 2011) and a serious environmental concern (Ding et al., 2015).

In recent years perfluoroalkyl surfactants have been extensively studied due to their bioaccumulative properties and widespread use in industry (Fabrega et al., 2014). Perfluoroalkyl compounds have been found in human blood and breast milk (Wang et al., 2016), although their effects on the human body are not completely understood.

Perfluorooctane sulfontate (PFOS), a highly fluorinated eight carbon perfluoroalkyl surfactant, was shown to induce apoptosis in N9 microglial cells (Zeng et al., 2011). Sea urchins exposed to PFOS experienced changes in behavior and DNA methylation (Ding et al., 2015). A similar compound, perfluorooctanoic acid (PFOA), has been found in human blood, tissue, and breast milk (Olsen et al., 2007; Tao et al., 2008; Fabrega et al., 2014). Harada et al. (2005) studied the effects of PFOA on guinea pig-derived calcium channels and found that the surfactant shifted activation voltages and caused changes in polarization. Exposure of young mice to PFOA during critical points of neonatal growth led to significant changes in behavior (Johansson et al., 2008), suggesting that industrial surfactants can induce neurotoxic effects. Due to the wide distribution of PFOA in various tissues and the lack of knowledge on physiological effects, studies into the influence of this surfactant on important biochemical systems are warranted.

To understand the effects of surfactants on the structure of globular proteins, spectroscopic techniques will be used to measure conformational changes in Hb in the presence of PFOA. Circular dichroism (CD), transient absorbance spectroscopy, UV-visible absorbance spectroscopy, and emission spectroscopy will also be employed to study the structural changes caused by PFOA to this important transport protein.

MATERIALS AND METHODS

Using photoacoustic calorimetry to determine dissociation constants for effector binding to oxygenated hemoglobin

Preparation of Hb stock

Samples of Hb were prepared from methemoglobin (Sigma-Aldrich; St. Louis, MO). Concentration of Hb was determined using single-beam UV-Vis absorbance spectroscopy (Varian Cary 50 Bio, Agilent Technologies; Santa Clara, CA) with an extinction coefficient of $\varepsilon_{405\ nm} = 179\ mM^{-1}\ cm^{-1}$. The cuvette was sealed and flushed with argon gas for 15 minutes and the Hb solution was reduced to deoxygenated Hb (deoxyHb) using small amounts of fresh sodium dithionite (Fisher Scientific; Hampton, NH) dissolved in deionized water. The transition to deoxyHb was verified by observing the shift of the Soret band from 405 nm to 430 nm. The cuvette was unsealed and flushed with air to oxygenate the Hb (oxyHb), a transition reflected in the shift of the Soret band from 430 nm to 415 nm.

Preparation of allosteric effector stocks

Stock solutions of 85.3 mM IHP (Sigma-Aldrich; St. Louis, MO) and 50.0 mM pyranine (Life Technologies; Eugene, OR) were prepared and stored in a -20 °C freezer. Working stocks of 8.00 mM and 2.00 mM were prepared for both IHP and pyranine. Solutions were covered with aluminum foil to prevent photobleaching, kept at 4 °C when not in use, and replaced monthly.

Photoacoustic calorimetry

The PAC apparatus was set up as described by others (Larsen et al., 2007; Vetromile et al., 2011). A schematic of the instrument is shown in Figure 1. Photo-pulse intensity was measured using an average of 100 traces on an optical power meter (Model 1918-C, Newport; Irvine, CA). Honey was used as the coupling agent to attach the piezoelectric transducer (NDT V103, Olympus Panametrics; Tokyo, Japan) to the wall of a quartz cuvette. At each effector concentration, three measurements of 100 averaged traces were collected and averaged to construct titration curves.

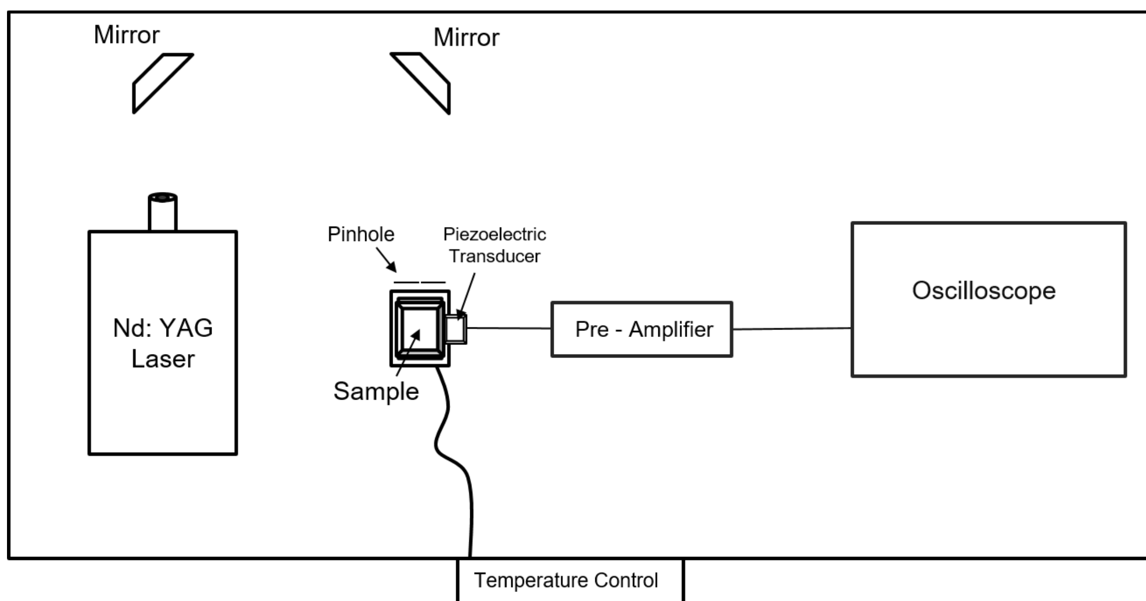


Figure 1: Schematic of PAC instrument setup utilizing a Neodymium-doped: yttrium aluminum garnet (Nd:YAG) crystal laser at 532 nm and a piezoelectric transducer coupled to a quartz cuvette.

Analysis of data

Titration curves were produced by plotting signal amplitude, the difference between the first positive and negative peak, as a function of effector concentration. The Hill equation was used to calculate K_D.

$$\theta = \frac{[S]^n}{[S]^n + K_D}, \quad (1)$$

In this equation Θ is the Hill coefficient, [S] is the concentration of substrate, and n is the cooperativity coefficient which represents the degree of cooperative binding of the substrate. The Van't Hoff relation states that changing temperature will cause the binding affinity to change proportionally.

$$ln(K_B) = \frac{-\Delta H}{RT} + \frac{\Delta S}{R}, \quad (2)$$

Here K_B is the binding constant which is equivalent to $1/K_D$, ΔH is reaction enthalpy ($kJ \cdot mol^{-1}$), ΔS is reaction entropy ($J \cdot K^{-1}$), R is the gas constant (8.3145 $J \cdot mol^{-1}$), and T is temperature (K). Reaction enthalpy and entropy were determined by linear regression, where $\frac{-\Delta H}{R}$ and $\frac{\Delta S}{R}$ are the slope and y-intercept, respectively.

Studying the effects of industrial surfactants on the structure of hemoglobin

Preparation of protein stocks

Stocks of Hb were prepared as previously described. Protein solutions of 10 µM were made fresh and stored at 4 °C.

Preparation of surfactant stocks

Perfluorooctanoic acid (PFOA) (Sigma Aldrich; St. Louis, MO) stock was made by dissolving solid PFOA in deionized water to produce an 8 mM solution. The stock was kept at room temperature in a sealed container.

Circular Dichroism Spectroscopy

Circular dichroism spectra of 5 µM Hb was recorded using a Jasco J-815 CD Spectrometer (Easton, MD) in the range 200 to 400 nm. The titrations were carried out by adding small aliquots of PFOA to a 250 µL sample of Hb in a 1 x 0.1 cm quartz cuvette. Each spectrum represents an average of three measurements.

Transient Absorbance Spectroscopy

Transient absorbance was used to study changes in the photodissociation of carbon monoxide (CO) from 10 µM CO-bound Hb (HbCO). A diode laser probe (MDL-III-447, Changehun New Industries Optoelectronics Tech; China) producing 447 nm light perpendicular to the excitation beam was passed through a Jobin-Yvon H20 series monochromator (HORIBA; Edison, NJ). Differences in probe beam intensity were detected using a PDA10A photodiode (Thorlabs; Newton, NJ). Traces consisted of an average of 100 sweeps and were displayed on a Wave Surfer 42Xs oscilloscope (Teledyne LeCroy; Chestnut Ridge, NY).

RESULTS

Calculating reaction parameters of IHP and pyranine to oxygenated hemoglobin using photoacoustic calorimetry

UV-vis absorbance spectroscopy was used to assess the effect of IHP and pyranine on the oxyHb microenvironment (Figure 2). Those spectra without an effector displayed the Soret band at 414 nm and two peaks at 541 and 576 nm, characteristic of the reduced oxygenated form of Hb. Spectra of oxyHb with IHP showed slight attenuation of the Soret band, (Figure 2a), while addition of pyranine exhibited an increase in the Soret band and presence of a new peak at 452 nm (Figure 2b). Peak intensities at 541 and 576 nm were unaffected by either of the effectors.

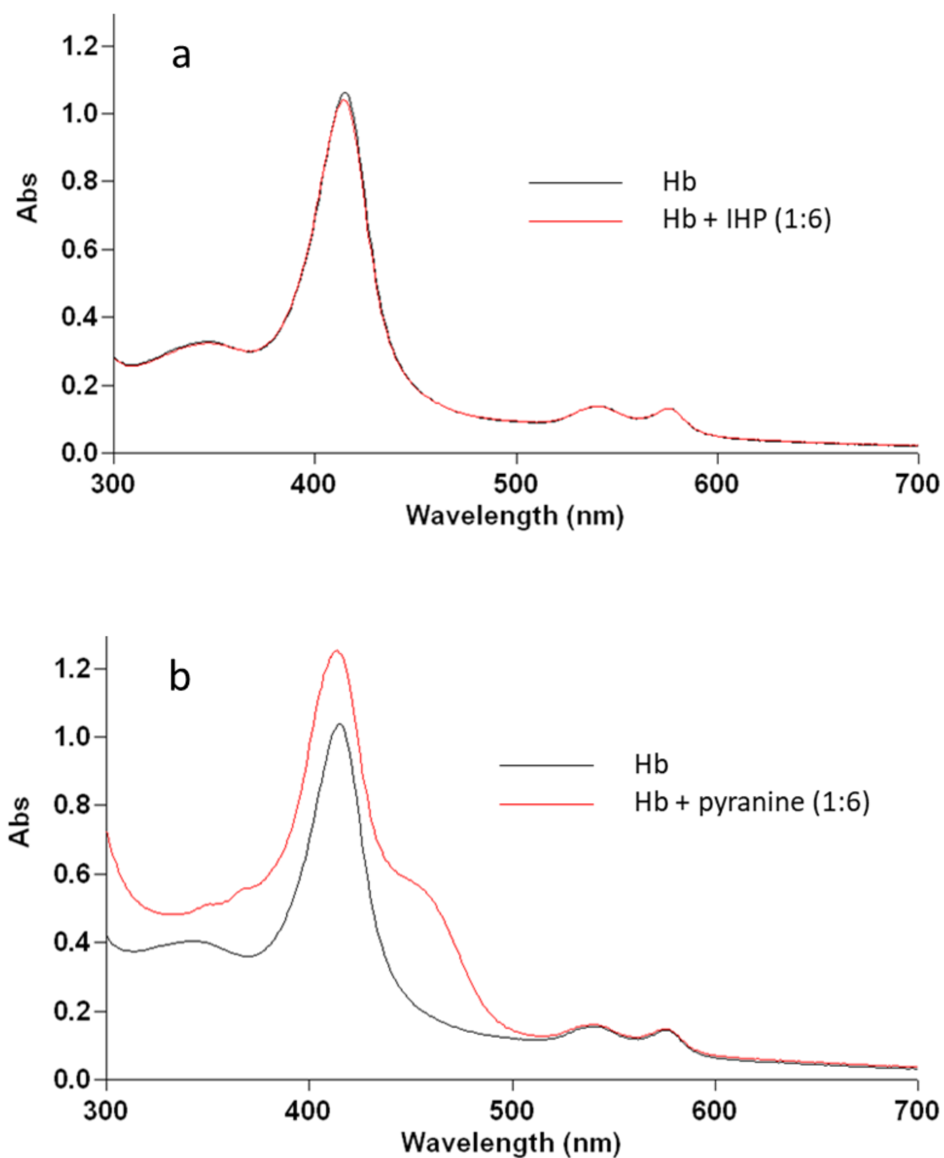


Figure 2: UV-vis absorbance spectra of oxyHb in the presence of IHP (Figure 2a) and pyranine (Figure 2b) at a 1:6 molar ratio. Peaks at 414, 541, and 576 nm are associated with the reduced oxygenated form of Hb. Addition of IHP had no effect on absorbance peak intensity, while pyranine increased peak intensity at 414 nm and introduced a new peak at 452 nm.

Changes in Hb oxygen affinity were determined by PAC measurements in the presence of varying concentrations of effector. Figure 2 shows a titration with IHP and its corresponding effect on the PAC signal. The signal represents the total change in volume associated with the photocleavage of oxygen-heme complexes and subsequent oxygen escape from the heme-pocket. Both effectors caused a measureable drop in amplitude intensity until the Hb was fully saturated.

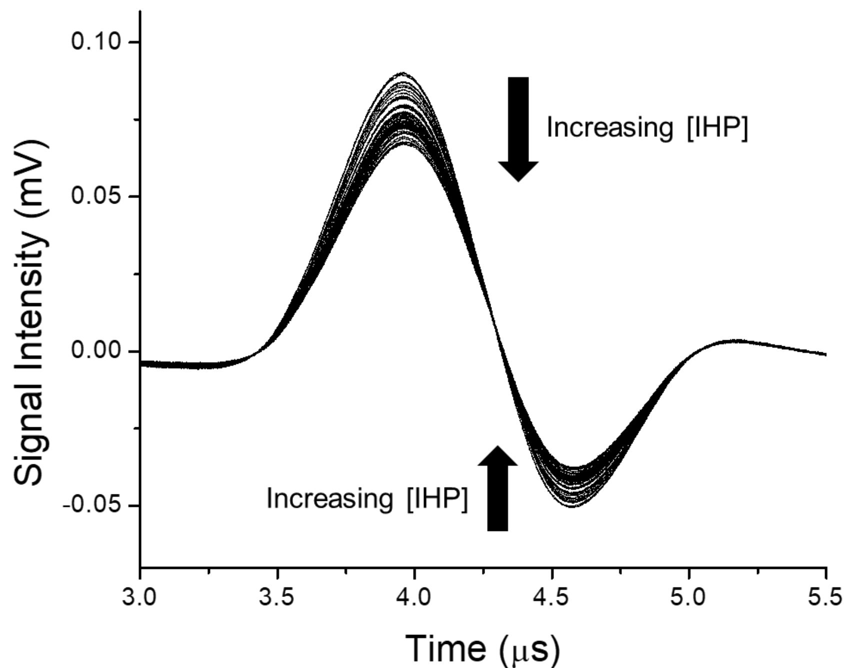


Figure 3: Changes in PAC signal for oxyHb in the presence of increasing concentrations of IHP (0 to 400 μM) at 28 °C. Arrows indicate the direction the signal shifts due to addition of IHP. The signal is produced by detection of acoustic waves generated by the photodissociation of oxygen from Hb. Differences in signal intensity are due to IHP-induced changes in Hb oxygen affinity.

Plotting amplitude intensity versus effector concentration of effector produced saturation curves that could be fitted using Eq. 1 to calculate effector K_D values for binding to oxyHb (Figure 4). The average K_D at a given temperature was found by averaging values from three different saturation plots. The K_D values for IHP and pyranine at 28 °C are 86.8 ± 28.6 μM and 55.7 ± 16.6 μM, respectively. Values were determined at 16, 20, 28, and 35 °C for both effectors.

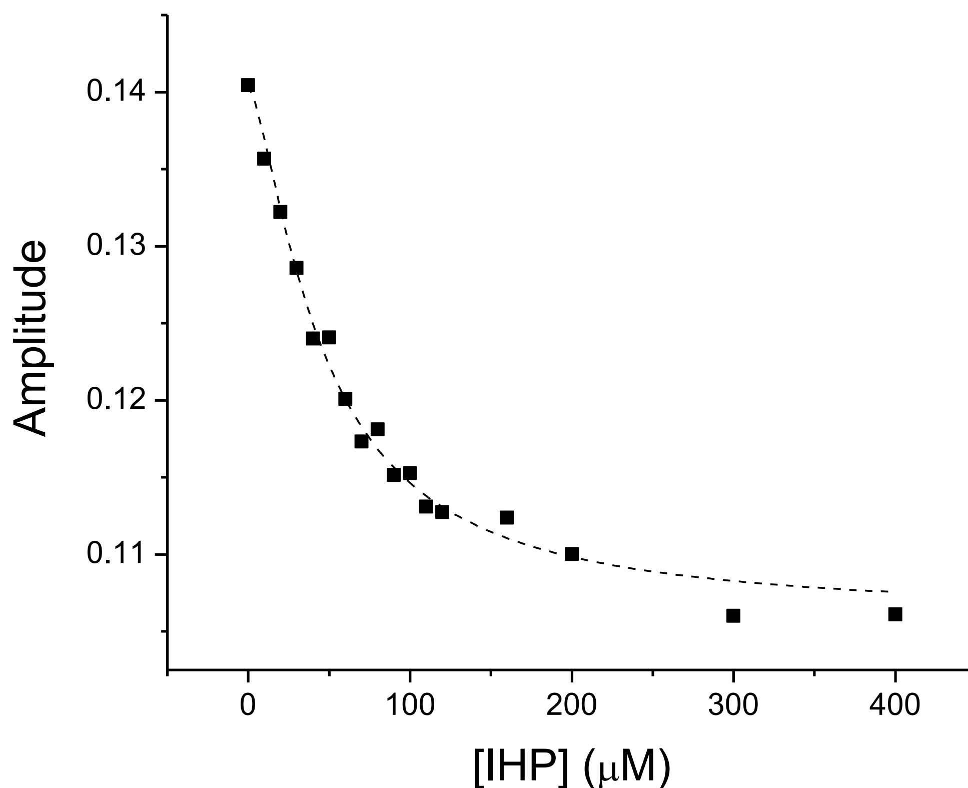


Figure 4: A saturation curve made by plotting PAC signal amplitude against IHP concentration (0 to 400 μM) at 28 °C and fitted with the Hill equation (Eq. 1). Dissociation constants (K_D) were calculated based on the fit of Eq. 1.

The Van't Hoff equation (Eq. 2) relates binding affinity to temperature and was used to determine values for enthalpy (ΔH) and entropy (ΔS) (Fig. 5). Values for K_B were found by taking the reciprocal of K_D. A qualitative benefit of using Van't Hoff plots is the ability to visualize whether reactions are exothermic or endothermic based on if the slope is positive or negative, respectively. The thermodynamic parameters for IHP and pyranine were calculated to be $\Delta H = -21.4 \pm 3.49$ kJ·mol^{-1}, $\Delta S = 7.22 \pm 11.7$ J·K^{-1} and $\Delta H = -13.3 \pm 1.08$ kJ·mol^{-1}, $\Delta S = 37.7 \pm 3.62$ J·K^{-1}, respectively.

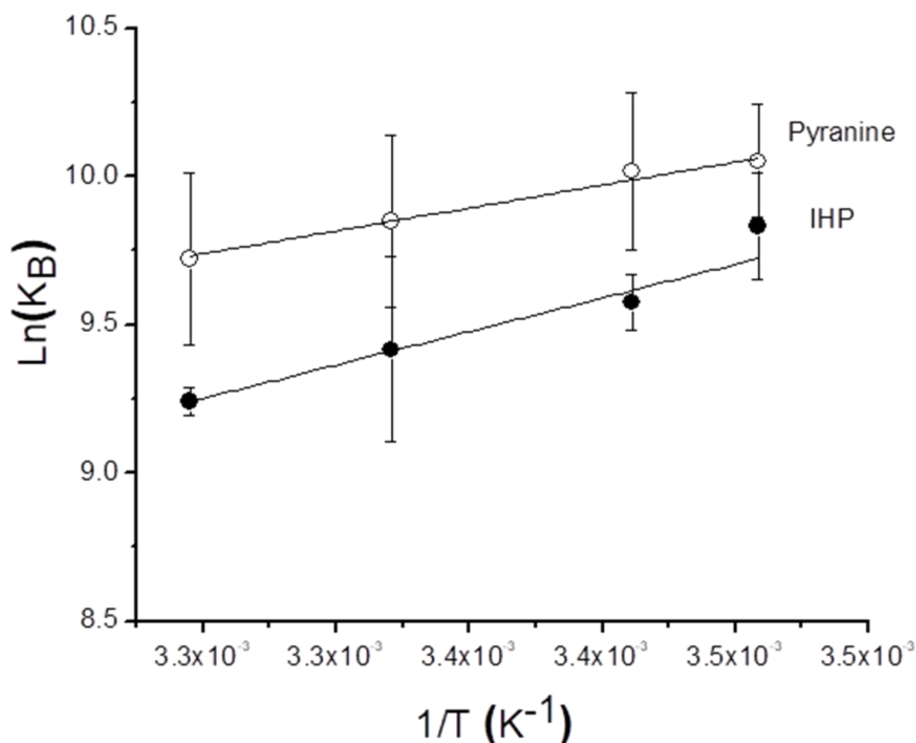
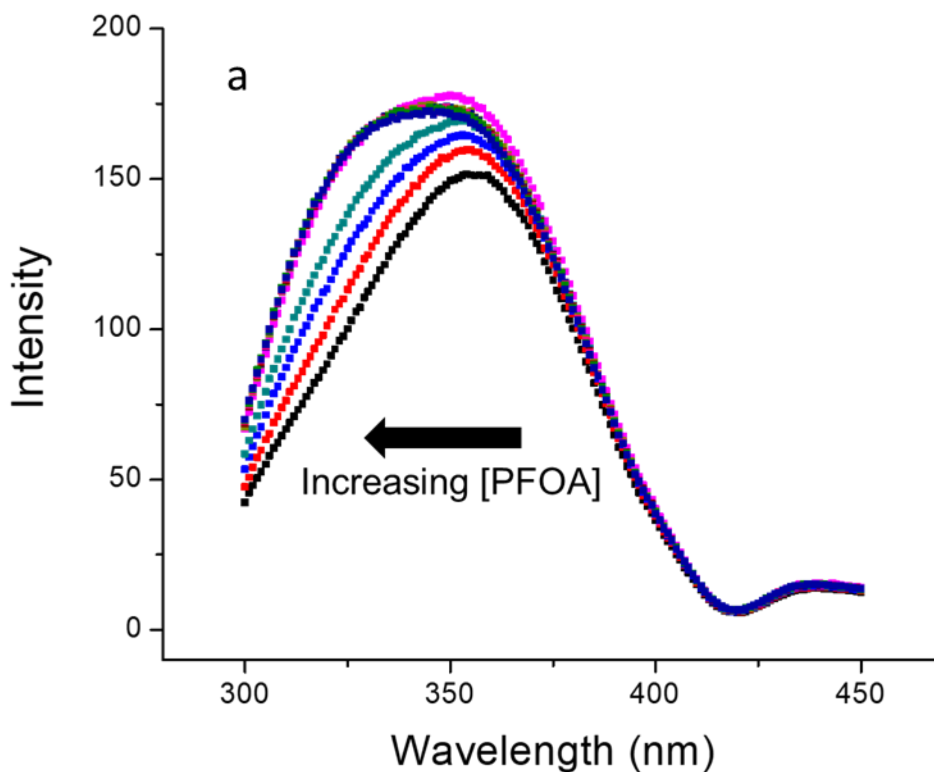


Figure 5. Van't Hoff plots of IHP (●) and pyranine (○) binding affinities over the temperature range 16 to 35 °C. Values for enthalpy (ΔH) and entropy (ΔS) were calculated using the slope and y-intercept, respectively (Eq. 2). Error bars represent the standard deviation of three calculations.

Detecting PFOA-induced structural changes to hemoglobin

Emission spectroscopy was utilized to observe PFOA-induced changes to the Hb microenvironment (Figure 5). These changes caused blue-shifting and broadening of the emission spectra (Figure 5a) and an increase in emission intensity until saturation (Figure 5b). At 120 μM there is a substantial change in the emission spectra where the peak begins to broaden, possibly from the presence of an additional peak. This is also reflected

in the intensity where there is a spike at 120 μM followed by saturation at a lower intensity. The saturation plot was used to calculate the cooperativity constant (n) and K_D of PFOA binding to HbCO by fitting Eq. 1 ($K_D = 52.8 \pm 4.76$ μM, n = 2.03). Values of n greater than one suggest cooperative binding.



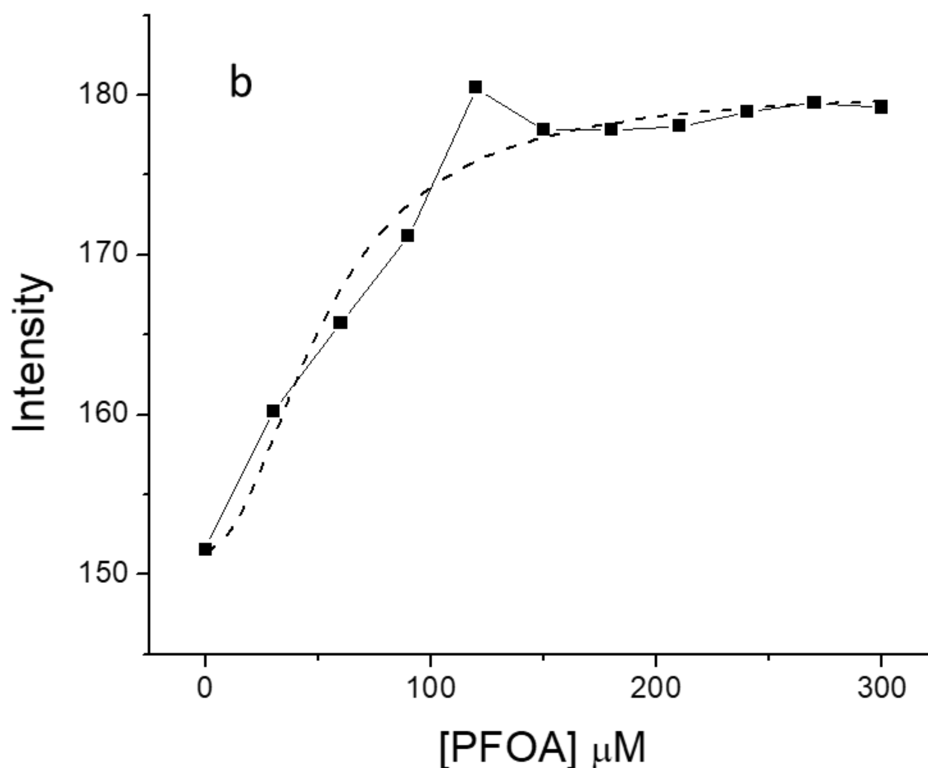


Figure 5a: Emission spectra of 10 μM HbCO titrated with an increasing concentration of PFOA (0 to 300 μM PFOA) (Figure 5a). Excitation was done at 280 nm with 10 mm wide excitation and emission slits. Each trace represents an average of three traces. The data shows a blue-shifting of the emission spectra with increasing PFOA concentration and a broadening of the emission peak at 120 μM PFOA. Figure 5b: The K_D was 52.8 ± 4.76 μM with a cooperativity constant (n) equal to 2.03. There is a substantial jump in intensity at 120 μM PFOA before saturating at a lower intensity at 150 μM PFOA.

Transient absorbance spectroscopy was used to measure time-dependent rebinding of CO to Hb in the presence of PFOA (Figure 6). Changes in the transient absorbance signal indicate structural differences that influence the CO affinity of Hb. Initially, increasing PFOA concentration only sped up the rebinding of CO. After PFOA concentration

surpassed 150 μM, there is a slowing of the rebinding at longer timescales while shorter timescales continued to speed up. This suggests that PFOA-induced changes in CO affinity are concentration-dependent.

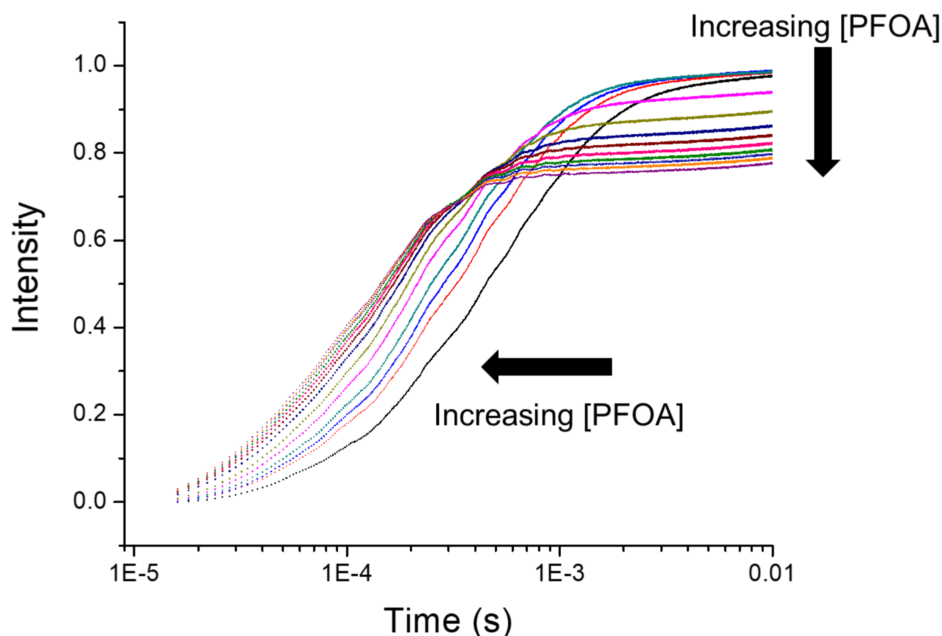


Figure 6: Changes in the transient absorbance of HbCO at increasing concentrations of PFOA (0 to 300 μM). The transient absorbance signal is produced by photodissociation and reassociation of CO to Hb causing differences in the intensity of a continuous photo-probe. Addition of PFOA caused speeding up at shorter timescales and slowing down at longer timescales that was concentration-dependent.

Circular dichroism (CD) spectroscopy was used to assess PFOA-induced changes in the secondary structures of Hb (Figure 7). Secondary structures such as α-helices absorb circularly polarized light in a consistent way. Differences in absorption indicate changes to Hb α-helical content. The CD spectra of Hb alone shows detection of light rotated by -20° at 222 nm, which is associated with large α-helical content. After addition

of 250 μM PFOA there is no longer detection of rotation, indicating complete unfolding of Hb. This suggests that at a molar ratio of 1:50, PFOA completely unfolds Hb secondary structures.

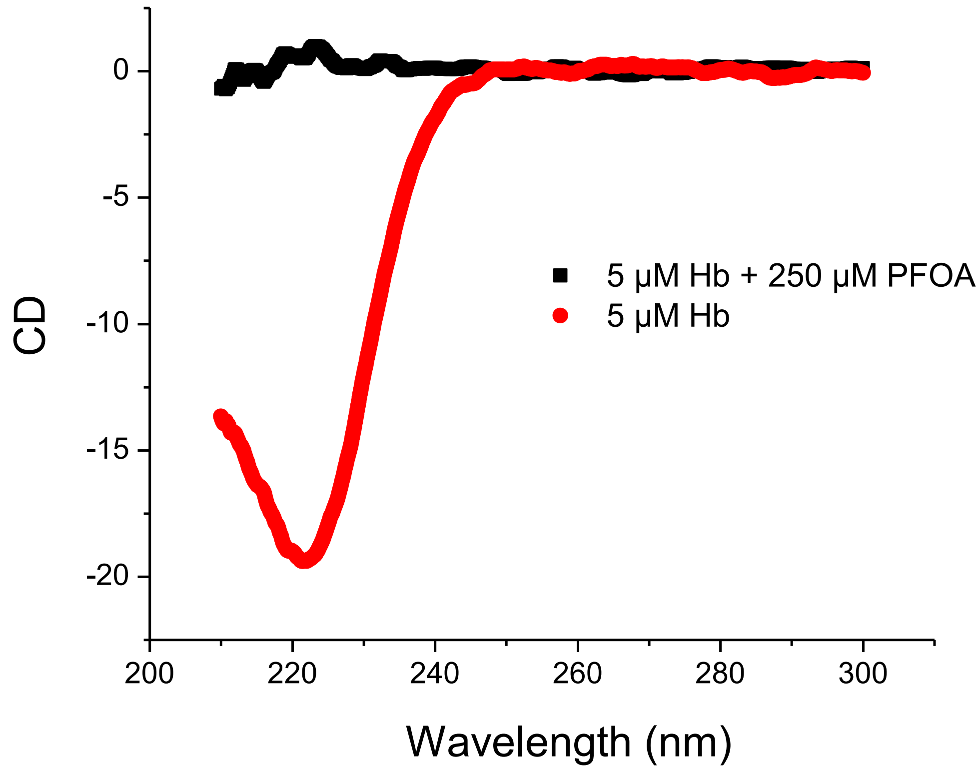


Figure 7: Continuous CD spectra of 5 μM Hb in the presence of 250 μM PFOA showing changes to the secondary structure. Values on the y-axis represents rotation of polarized light in degrees. The trough at 222 nm is associated with α-helical content and is completely absent after addition of PFOA which indicates protein unfolding.

DISCUSSION

Utilizing photoacoustic calorimetry as a technique for determining binding parameters

Table 1. Summary of reaction parameters computed for IHP and pyranine binding to oxyHb.

Effector	*Temperature (°C)*	K_D (μM)	ΔH ($kJ \cdot mol^{-1}$)	ΔS ($J \cdot K^{-1}$)
IHP	16	54.8 ± 9.70	-21.4 ± 3.49	7.22 ± 11.7
	20	69.8 ± 6.66		
	28	86.8 ± 28.6		
	35	97.0 ± 4.30		
Pyranine	16	44.2 ± 8.35	-13.3 ± 1.08	37.7 ± 3.62
	20	46.7 ± 12.3		
	28	55.7 ± 16.6		
	35	63.3 ± 16.9		

Dissociation constant (K_D) values determined by saturation curves from PAC titrations using the Hill equation (Eq. 1). Reaction enthalpy (ΔH) and entropy (ΔS) values calculated using plots modeled after the Van't Hoff relation (Eq. 2). Error determined by standard deviation.

The results presented here have produced K_D values for IHP and pyranine determined by using PAC in a novel manner (Table 1). The K_D values for IHP binding to oxyHb are in agreement with previous measurements by Tomoda et al. in 1977 (K_D = 69.0 μM at room temperature). Thermodynamic values for IHP also fall within the range described by Messana et al. (1998) given differences in ionic strength and pH of the solutions.

Interestingly, the PAC determinations of pyranine show a lower K_D than IHP, suggesting stronger binding. Previous displacement studies have found that pyranine binding is weaker than IHP in CO-bound Hb (HbCO) at pH 6.35 (Gottfried et al., 1997).

However, these researchers found that HbCO exhibits a different conformational state than oxyHb. Additionally, binding affinity is significantly affected by solution conditions (Messana et al., 1998), and Gottfried et al. (1999) showed that pyranine binding is affected by changes in pH. Therefore, it is possible that a combination of higher pH and oxygenation of Hb produced an environment where pyranine can bind more strongly than IHP. This prediction can be tested using a system that measures changes in fluorescence intensity caused by displacement of pyranine as suggested by Gottfried et al.

The thermodynamic values of IHP and pyranine calculated here can be compared to predict binding mechanisms since both effectors bind to the DPG site (Tomoda et al., 1977; Gottfried et al., 1997; Messana et al., 1998; Gottfried et al. 1999). The more negative ΔH value for IHP binding to Hb indicates that the association is more energetically favorable compared with pyranine. This result is expected considering IHP is composed of six negatively-charged phosphate groups which binds electrostatically to the positive lysine residues in the $\beta 1$-$\beta 2$ binding site (Klotz et al., 1985). The larger ΔS value for pyranine binding may be a result of its larger and more hydrophobic physical properties which promote water displacement from the binding site. This shows that PAC can be used to probe differences in binding mechanisms for small molecules that share binding sites based on thermodynamic profiles.

Allosteric effectors are important for regulating enzymatic activity in response to changing environments. The development of synthetic effectors for use in treating malfunctioning Hb, such as in sickle cell disease, outlines the clinical relevance and potential assigned to them (Beddell et al., 1984; Merrett et al., 1986; Safo et al., 2004).

Determinations of K_D allow researchers to assess the strength and specificity of novel effectors binding to protein targets. Current techniques such as surface plasmon resonance and fluorescence spectroscopy are useful but require manipulation of the protein through surface-binding or labeling which may change substrate binding.

Past studies using PAC have utilized the changes in signal amplitude to study the thermodynamics of short-lived reaction intemediates (Rothberg at al., 1983), the structural reorganization of bacterial cytochrome proteins (Miksvoska et al. 2005), and differences in thermodynamic profiles of proteins based on the solvent environment (Butcher et al., 2017). These studies used PAC to determine structural changes based on differences in thermodynamic values.

The work I presented here has expanded on the uses of PAC to include the determination of binding affinities that can provide information on the binding mechanisms and characteristics of protein-substrate complexes. More work should be done to optimize this technique to reduce variability in measurements and to further explore the systems to which it can be applied.

Changes in hemoglobin structure caused by PFOA

Contamination of the perfluoroalkyl surfactants in the environment has prompted the study of their effects on biological systems such as neurodevelopment (Johansson et al., 2008) and liver function (Darrow et al., 2016). The work presented here has shown that

PFOA has a distinct effect on the structure and functionality of Hb, an important transport protein for the circulatory system.

Shifts in the emission spectra of Hb shown in Figure 5 shows that PFOA causes alterations in the microenvironment that is indicative of a conformational change (Hirsch et al., 1980). This is supported by the transient absorbance data (Figure 6) that shows an asymmetric PFOA-dependent change in the CO affinity of Hb. These changes in CO rebinding and broadening of the emission peak both occur at 120 or 150 μM PFOA, suggesting that there are multiple binding sites that differ in affinity, perhaps with one site being inaccessible until surfactant concentration reaches the appropriate value. When the molar ratio of Hb to PFOA is at least 1:50, the protein is completely unfolded as evidenced by the CD data (Figure 7). Therefore, it is possible that the changes in emission and transient absorbance are either caused by the unfolding process or some other mechanism that cannot be determined here. To determine the physical phenomenon responsible for these changes in light interaction, crystallographic experiments or docking simulations can be conducted to ascertain the exact location of PFOA binding (Henry et al., 2015).

Despite being banned from manufacture in the United States, perfluoroalkyl surfactants still pose a health concern as their toxicological effects and the extent of environmental contamination are not fully understood (Fabrega et al., 2014). The work I have presented here has shown a previously unknown effect of one of these surfactants on the structure and functionality of the main oxygen transport protein of the human circulatory system. While it is unlikely that perfluoroalkyl surfactants may contribute to

anemia by disruption of Hb function, the physical explanation for PFOA-induced changes to Hb may be important for better understanding the interactions of globular proteins with amphipathic molecules.

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