PROTEIN ADSORPTION TO HYDROPHOBIC SURFACES

A Thesis in

Bioengineering

by

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ABSTRACT

Adsorption energetics of diverse purified proteins as well as whole-blood plasma and serum (aqueous-buffer) solutions were remarkably similar at two hydrophobic surfaces – water-air (liquid, vapor, LV) and solid-water (solid-liquid, SL). A ‘Traube-rule-like’ progression (molar concentration required to reach a specified spreading pressure decreases with increasing MW) was observed at both hydrophobic surfaces (LV and SL) for globular proteins spanning three-orders-of-magnitude in molecular weight (MW). Collective results from the observed ‘Traube-rule-like’ progression in interfacial-tension reduction, an invariant partition coefficient, and a constant Gibbs’ surface excess (as a measure of amount of protein adsorbed) all imply that water controls the energetics of the protein adsorption process. Hence, protein adsorption to hydrophobic surfaces has more to do with water than the proteins themselves. A relatively straightforward theory of protein adsorption predicated on the interfacial packing of hydrated spherical molecules with dimensions scaling as a function of MW accounts for the essential physical chemistry of protein adsorption and rationalizes significant experimental observations. From this theory, it is evident that displacement of interfacial water by hydrated proteins adsorbing from solution places an energetic cap on protein adsorption to hydrophobic surfaces. This phenomenon is generic to all proteins. As a consequence, protein adsorption is not found to vary significantly among diverse protein types. Variations from this general trend may reflect deviations in protein geometry from simple spheres and/or tendency of some proteins to adopt a more spread/compact configuration in the adsorbed state.
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Preface

Protein adsorption addresses an area of study that virtually defines the field of biomaterials surface science. The theme of proteins at interfaces has dominated the biomaterials literature for decades. Adsorption of plasma proteins is widely believed to be one of the first and instantaneous biological responses to a biomaterial surface in contact with blood that in turn dictates end-use biocompatibility. Exactly how these molecular-scale events transpiring at hydrated surfaces potentiate macroscopic outcomes such as blood coagulation, cell adhesion, or immune responses is generally understood only at the level of descriptive biochemistry. Work accomplished and outlined in this thesis focuses on a comprehensive understanding of the phenomenon of protein adsorption and is devoted to developing concise biophysical laws that guide design and synthesis of biomaterials widely used in medicine. Although these biophysical laws are most efficiently written in the stilted parlance of physical chemistry and tested using techniques of modern surface science/engineering, significant outcomes of this work hold considerable promise for widespread, practical bioengineering utility because of the fundamental nature of protein adsorption to medical-device performance.

Detailed results are presented in the following nine chapters, each of distinct journal article format. The underlying message from the work extends novel insights to biomaterials literature, challenging conventional wisdom about protein-surface selection and interfacial behavior. Primary purpose of this thesis has been to understand and interpret plasma-protein adsorption to hydrophobic surfaces in a systematic, organized fashion using principles of interfacial energetics (tensiometry and wettability).
The first chapter reviews current literature, including the fundamentals tenets of selectivity and specificity in protein adsorption to varied biomaterial surfaces; and introduces our results from time-and-concentration-dependent liquid-vapor (LV) interfacial tension $\gamma_{lv}$ of a variety of purified proteins ranging from albumin to ubiquitin spanning nearly three decades in molecular weight (MW), especially focusing on proteins of the blood-plasma coagulation cascade. The second develops a comprehensive theory of protein adsorption based on simple sphere-packing of protein molecules within a three-dimensional interphase and interprets scaling relationships introduced in Chapter 1. Though much has been accomplished in Chapters 1-2 for purified proteins at a model hydrophobic surface, adsorption of proteins from multi-protein mixtures is of more practical relevance to biomaterials. This is addressed in Chapters 3-4 using binary protein mixtures as well as whole-blood-plasma and serum at the LV surface. Chapter 3 also extends the theory of protein adsorption from purified proteins to protein mixtures, revealing mixing rules that describe how individual proteins comprising a mixture compete for space at a surface in the adsorption process. Chapter 4 applies these mixing rules of competitive protein adsorption to a systematic survey of concentration-dependent $\gamma_{lv}$ of blood plasma and serum derived from human and animal species. Results from chapters 1-4 probing molecular details of protein adsorption strongly implicate water as a moderating or mediating agent. Chapters 5-9 continue to pursue this water-oriented perspective of protein adsorption at a solid-water hydrophobic surface (solid-liquid, SL) for the same span of purified proteins and protein mixtures studied at the LV surface. Remarkably similar adsorption energetics is revealed at two hydrophobic surfaces for solutions of purified proteins as well as multi-protein mixtures.
Similarity in results between the two surfaces permits equal application of the theory of protein adsorption, thus accommodating significant experimental observations from interfacial energetics. Chapter 9 is unique in that it tests the utility of the sphere-packing model of protein adsorption (derived from interfacial energetics) as a tool in predicting adsorbed protein mass from a complementary experimental technique – quartz crystal microbalance.

Thus, the work is simply, a systematic investigation combining modern surface-science experimental methods and biophysical theory that reveals important biophysical rules of protein adsorption to material surfaces. This thesis constitutes first steps towards achieving a complete mass and energy inventory necessary for a comprehensive understanding of the protein adsorption process. In the course of the next nine chapters, I shall endeavor to give a detailed understanding of my doctoral research on protein adsorption to hydrophobic surfaces.
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This thesis reflects unwavering help and guidance from Dr Vogler. I am grateful to him for this opportunity, first handed to me when all I could offer was a simple sense of enthusiasm and commitment to education. Dr Vogler has carefully walked me through different facets of science and graduate school and life, for that matter – thus bringing me to this day – when I can say – thank you. The wholesome experience of graduate education at Penn State under Dr. Vogler’s tutelage has shaped the person I am today, and will remain the best years of my life.

I would also like to extend my gratitude to my thesis-committee members - Dr. Siedlecki, Dr. Hancock, Dr. Allara and Dr. Brown for helpful suggestions, criticisms and constant encouragement. I am equally thankful to Dr. Lipowsky for being available for me during many of my confused periods throughout graduate school. This work was initiated as an undergraduate research project by Jackie Sturgeon, whom I wish to thank for all her help with facilitating a smooth transition.

Very special thanks to Ivy Liu who readily made many of the thiol-self-assembled monolayer surfaces and quartz-crystal microbalance measurements crucial to this thesis. Thanks are also due to Karen Bussard who carefully prepared and shipped our human plasma and serum samples at short notice, from Dr. Siedlecki’s lab at Hershey Medical Center. I have been fortunate that my tensiometric instrument was always responsive to my demands and hence, I wish to thank Dr. Roger Woodward for instrument design and implementation. Paul Cha, my fellow researcher, with his sweet and sometimes, playful temper made long hours in the lab a
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There are a handful of people whose life’s sole ambition was to see me reach my doctoral degree. My father – who always stashed some money away so I could study in the United States, my mother – who constantly pushed me to look beyond the pressures of daily life and reach higher and vi – my husband – who stayed calm through days and nights of trouble and joy. No measure of thanks will cover what I owe them today.
Chapter 1

Scaled Interfacial Activity of Proteins at the Liquid-Vapor Interface

Abstract

A principal conclusion drawn from observations of time- and concentration-dependent liquid-vapor (LV) interfacial tension $\gamma_{lv}$ of a diverse selection of proteins ranging from albumin to ubiquitin spanning nearly three decades in molecular weight (MW) is that concentration scaling substantially alters perception of protein interfacial activity as measured by reduction in $\gamma_{lv}$. Proteins appear more similar than dissimilar on a weight/volume basis whereas molarity scaling reveals a “Traube-rule” ordering by MW, suggesting that adsorption is substantially driven by solution concentration rather than diversity in protein amphilicity. Scaling as a ratio-to-physiological-concentration demonstrates that certain proteins exhibit the full possible range of interfacial activity at-and-well-below physiological concentration whereas others are only weakly surface active within this range, requiring substantially higher solution concentration to achieve reduction in $\gamma_{lv}$. Important among this latter category of proteins are the blood factors XII and XIIa, assumed by the classical biochemical mechanism of plasma coagulation to be highly surface active, even in the presence of overwhelming concentrations of other blood constituents such as albumin and immunoglobulin that are shown by this work to be among the class of highly-surface-active proteins at physiologic concentration. A comparison of pendant drop and Wilhelmy balance tensiometry as tools for assessing protein interfacial activity shows that measurement conditions employed in the typical Wilhelmy plate approach fails to achieve the steady-state adsorption condition that is accessible to pendant drop tensiometry.
1.1. Introduction

A working hypothesis that has emerged as a fundamental biomaterials-surface-science tenet from more than three decades of research is that protein adsorption is the first step in the biological response to materials. With this simple yet powerful doctrine, the full panoply of macroscopic biological outcomes observed when a material is brought into contact with different biological milieu (the in vitro or in vivo biological response) is qualitatively explained as originating in preferential adsorption of protein(s) to a material surface, typically leading to the expression of epitope(s) that potentiate different biochemical reactions. The list of supporting examples is quite comprehensive;¹ and include blood coagulation (contact activation by blood factor XII and/or stimulation of platelets by adsorbed fibrinogen), cell adhesion (membrane-bound receptors to adsorbed adhesins), complement activation (contact activation of blood factor C3), haptotaxis (cell migration along an adsorbed gradient of cytostimulating agent); to name but a few from many. This overarching tenet asserts that the biological response to materials originates in molecular-scale processes occurring directly on, or at least vicinal to, hydrated material surfaces.

Protein adsorption can influence the biological response in at least two general but not entirely independent ways. First, as briefly mentioned above, adsorbed protein can expose receptors or ligands thereto and participate in one or more of the myriad “lock-and-key” biochemical reactions, especially those involved in signal transduction.² In these circumstances, a rather small amount of adsorbed protein can induce disproportionate effects, amplified by cascade-type reactions so familiar in modern molecular physiology (a little can do a lot). The second way has to do with interfacial energetics which is quite distinct from the aforementioned in that interfacial
energetics are not directly subject to any sort of biological amplification. Instead, protein-surface-water interactions are governed by purely kinetic/thermodynamic rules\textsuperscript{3-17} predicated on the basic principles of transport phenomena and energetics. Surfactant science fully elaborates these rules from which it is clear that, for a given surface chemistry/energy immersed in water, the free energy of adsorption is solely dependent on solute molecular characteristics rather than biological potential of that solute. Amphility (interaction energetics with water) is paramount among these solute characteristics, governing both sign and magnitude of the free energy change occurring upon adsorption. The linkage between these two different aspects of protein adsorption lies in the fact that interfacial energetics controls the amount of protein adsorbed and biochemistry influences activity of protein in the adsorbed state.

With all of this in mind, and focusing only on the interfacial energetics of protein adsorption, we are curious to know specifically how proteins, as a general class of (possibly glycosylated) polyamide biopolymers, can exhibit the widely-varying “biosurfactant”\textsuperscript{18} properties seemingly required to support the protein-adsorption tenet. That is to ask, how is it that particular proteins can adsorb to material surfaces from heterogeneous aqueous mixtures (such as blood) and evoke specifically-related biological responses when, in fact, protein amphility is limited to the permutations and combinations of (effectively) 20 different amino acids? True, it is well known that the primary amino-acid sequence folds into higher-order structure that distinguishes one protein type from any other and greatly influences bioactivity. But any such arrangement of primary structure and related aggregation into hydrophilic/hydrophobic domains still leads to net amphility that pales in comparison to the great range purposely built into synthetic surfactants drawn from anionic, cationic, non-ionic, zwitterionic, and perfluorinated categories.
So, bearing in mind that there is no known biological amplification of interfacial energetics, we are led to ask if there is sufficient “amphiphilic diversity” among proteins to account for the adsorption specificity implicit in the protein-adsorption tenet. All of this is to say, we seek to better understand the interfacial energetics behind the biological response to materials and the protein-adsorption tenet.

We report herein time-and-concentration-dependent liquid-vapor (LV) interfacial tension $\gamma_h$ of a variety of purified proteins ranging from albumin to ubiquitin spanning nearly three decades in molecular weight (MW), especially focusing on proteins of the blood-plasma coagulation cascade. Results are compared to that obtained for selected surfactant reference compounds. The liquid-vapor (LV) interface is a molecularly-smooth, model hydrophobic surface where interfacial energetics can be directly measured by tensiometric (surface thermodynamic) techniques.\textsuperscript{18} Scaling of interfacial tension measurements by weight, molarity, and especially as a ratio-to-physiologic-concentration reveals that (i) propensity to adsorb to the LV interface (a.k.a interfacial activity) of this diverse set of proteins is more alike than dissimilar and that (ii) concentration is a more important determinant of protein adsorption than is structure-related amphilicity. Outcome of experimental observations is interpreted in terms of the protein-adsorption tenet, suggesting that selective protein adsorption from heterogeneous mixtures may be over-emphasized in explication of diverse biological responses to materials. Quantitative application of this tenet in biomaterials science thus requires a more thorough accounting of interfacial energetics scaled to physiological-protein concentration.
1.2. Materials and Methods

Purified Proteins, Protein Mixtures, and Synthetic Surfactants: Table 1 compiles pertinent details on proteins, protein mixtures, and surfactants used in this work. Protein purity was certified by the vendor to be no less than the respective values listed in Column 5 of Table 1, as ascertained by electrophoresis (SDS-PAGE or immunoelectrophoresis). Mass, concentration, and molecular weights supplied with purified proteins were accepted without further confirmation. Human platelet poor plasma (HP, citrated) was prepared from outdated (within 2 days of expiration) lots obtained from the Hershey Medical Center Blood Bank. Human serum (HS) was prepared in 15 mL batches from HP by recalcification with 0.1 M CaCl$_2$ at 5:1 v/v plasma: calcium ratio and coagulation in clean glass-scintillation vials for about 15 min. The single value for physiological concentration of human proteins applied in this work was middle of the range listed by Putnam$^{19}$ as given in Table 1. Serial dilutions of protein stock solutions (usually 10 mg/mL) were performed in 96-well microtiter plates by (typically) 50:50 dilution in phosphate buffered saline solution (PBS) prepared from powder (Sigma Aldrich) in distilled-deionized (18 MΩ) water (interfacial tension of PBS and water was checked periodically by Wilhelmy-balance tensiometry). Between 24-30 dilutions were prepared in this manner, covering a dynamic range between $10^{-10}$ to 1 % (w/v), taking care to mix each dilution by repeated pipette aspiration and avoiding foaming of concentrated solutions. The first 12 dilutions (through about $10^{-5}$ %) were prepared one-at-a-time from the preceding solution in the series just before interfacial tension measurements (as a means of conserving concentrated-protein volume) whereas more dilute solutions were prepared all at once. Microtiter plates were covered by commercial plate-sealing film to retard evaporation during the course of analysis and, in those events when protein dilutions were not to be analyzed within two contiguous days,
plates were stored at 0 °C. Plates were equilibrated with ambient laboratory temperature (approximately 23 °C) and solutions older than about 5 days were discarded. Surfactants were stored under ambient conditions in clean-glass bottles sealed with polyethylene-lined screw-type caps. Surfactants were used as received from the vendor without further purification except in the case of sodium dodecyl sulfate that was twice recrystallized from absolute ethanol (see Table 1). Unless otherwise specified, surfactant solutions were prepared in PBS.

**Liquid-Vapor Interfacial Tension Measurements:** LV interfacial tensions $\gamma_{lv}$ reported in this work were measured by either Wilhelmy-balance tensiometry (WBT) or pendant-drop tensiometry (PDT). Unless specifically indicated otherwise, results refer to PDT performed on a commercial automated tensiometer (First Ten Angstroms Inc., Portsmouth VA). The tensiometer employed a Tecan liquid-handling robot to aspirate between 10-12 µL of solutions contained in a 96-well microtiter plate prepared by the serial dilution protocol described above. Actual pick-up volume was adjusted to accommodate desired dispense volume (as a means of conserving protein, especially at the highest concentrations) and a fresh disposable-pipette tip was used for each solution. We found that dip-coating of disposable polypropylene tips in a commercial perfluorocarbon hydrophobizing agent (NYEBAR, NYE Lubricants Inc.; Bedford, MA) followed by water wash and air drying greatly aided formation and hanging of symmetric pendant drops, especially at the highest protein concentrations (lowest $\gamma_{lv}$). This coating procedure was shown not to measurably affect interfacial tensions. The robot was used to reproducibly transfer the tip with fluid contents into a humidified (99+ % RH) analysis chamber and dispense between 6-11 µL pendant drops (smaller drop volume required for lower interfacial tensions) within the focal plane of a magnifying camera. These and all other aspects of pendant
drop analysis were performed under computer control. Proprietary algorithms supplied by the vendor were used to deduce interfacial tensions from drop images captured at a programmed rate by a frame grabber. Typically, 600 images were captured at a rate of 1 image every 6 sec following 0.25 sec delay to permit vibrations of the expelled drop to dampen. Drop evaporation rates within the humidified chamber deduced from computed-drop volumes (based on image analysis) were observed to vary with solute concentration, generally ranging from approximately 25 nL/min for pure water to 10 nL/min for solute solutions > 0.1% w/v. The impact of this evaporation rate over the 60 min time frame of the experiment was apparently negligible, as gauged from the behavior of purified surfactants discussed in the results section. Precision of $\gamma_{lv}$ was about 0.5 dyne/cm based on repeated measurement of the same pendant drop. The instrument was calibrated against pure water interfacial tension and further confirmed on occasion against Wilhelmy-balance tensiometry. The analysis chamber was thermostated to a lower-limit of 25±1 °C by means of a computer-controlled resistive heater. Upper-temperature limit was not controlled but rather floated with laboratory temperature, which occasionally drifted as high as 29 °C during summer months. Thus, reported $\gamma_{lv}$ values were probably not more accurate than about 1 dyne/cm on an inter-sample basis considering the small, but measurable, variation of water interfacial tension with temperature. This range of accuracy was deemed adequate to the conclusions of this report which do not strongly depend on more highly accurate $\gamma_{lv}$ that is difficult to achieve on a routine basis. Instead, veracity of arguments raised herein depend more on a breadth of reliable measurements made across the general family of human proteins.
Wilhelmy-balance tensiometry was performed using a commercial computer-controlled instrument (Camtel CDCA 100, Royston UK) using solvent-and-plasma-discharge-cleaned glass coverslips as the plate (Fisher brand 22X30 mm #1; three sequential rinses of as-received coverslips in each of water, isopropanol, and chloroform; plasma-discharge-treated for ~ 5 min at 100 mtorr air in a Harrick plasma cleaner, Ossining, New York). The balance was calibrated with standard weights thereby accounting for local variation in the force of gravity. No attempt was made to thermostat the balance and all reported measurements were made at ambient laboratory temperature. Also, no attempt was made to correct for the (presumably small but not measured) variation in the perimeter of glass coverslips used as plates in the Wilhelmy method. Solutions (approximately 10 mL) were contained in disposable polystyrene beakers (Fisher) previously determined not to measurably affect interfacial tension of water contained therein.

**Computation and Data Representation:** Computational, statistical, and theoretical methods used in this work have been discussed in detail elsewhere.\textsuperscript{18,20,21} Briefly, time-dependent $\gamma_\nu$ data corresponding to different solutions were recovered from PDT files and correlated with concentrations, leading to a matrix of results with row values representing concentration and time (in sec) as column values. It was generally observed that $\gamma_\nu$ data takes on a sigmoidal shape when plotted on logarithmic-concentration axes,\textsuperscript{18,20} with well-defined low-concentration asymptote $\gamma_{\nu}^-$ and high-concentration asymptote $\gamma_{\nu}^+$. Successive non-linear least-squares fitting of a four-parameter logistic equation $\gamma_\nu = \{[(\gamma_\nu^- - \gamma_\nu^+)/((\ln C_\nu + 2)/(\ln C_\nu)^4)] + \gamma_\nu^+\}$ to concentration-dependent $\gamma_\nu$ data for each time within the observation interval quantified $\gamma_{\nu}^-$ and $\gamma_{\nu}^+$ parameters with a measure of statistical uncertainty.
Fitting also recovered a parameter measuring concentration-at-half-maximal-change in interfacial activity, $\ln C_{\Pi}^{1/2}$ (where $\Pi^1 = \frac{1}{2} \Pi^{\text{max}}$ and $\Pi^{\text{max}} \equiv \gamma^* - \gamma'$), as well as a parameter $M$ that measured steepness of the sigmoidal curve. This multi-parameter fitting to concentration-dependent $\gamma^*$ data was a purely pragmatic strategy that permitted quantification of best-fit protein and surfactant characteristics but is not a theory-based analysis.\textsuperscript{18,20,21} Three-dimensional (3D) representations of time-and-concentration $\gamma^*$ data were created in Sigma Plot (v8) from the data matrix discussed above and overlaid onto fitted-mesh data computed from least-squares fitting. Two-dimensional (2D) representations were created from the same data matrices at selected observation times. Concentration-dependent Wilhelmy-balance data was treated similarly except time dependence in $\gamma^*$ was not a controlled variable.

1.3. Results and Discussion

Results and discussion are combined into one section because it is efficient to introduce and discuss the different facets of the work in the sequence that follows and then combine separate observations in the Conclusions section. The presentation is organized in the following order. First, general characteristics of the quantitative data are described, comparing proteins and surfactants and placing data into an overall context for interpretation. Second, these results are interpreted graphically by scaling concentration-dependent $\gamma^*$ in three different ways (termed herein “interfacial tension curves” or simply “$\gamma^*$ curves”) that give different insights into protein interfacial activity and provoke interpretation in terms of the protein-adsorption tenet briefly discussed in the Introduction. Third, a brief comparison of Wilhelmy-balance and pendant-drop tensiometry of protein mixtures and surfactant reference compounds is given which, although
somewhat tangential to the main theme of this paper, is an important analytical consideration that might affect future attempts to reproduce or extend results presented herein. Finally, within this context, we focus on results obtained for blood factors XII and XIIa because these proteins turn out to be relatively strong biosurfactants, at least as rated against other proteins, but yet are only weakly surface active at physiological concentrations.

**General Aspects of the Data:** Tables 1-3 compile qualitative and quantitative results of this work. Interfacial tension parameters measured by PDT listed in Tables 2 and 3 are the average fitted values corresponding to final 25 $\gamma$ curves recorded within the 60-minute time frame of the PDT experiment and listed error is standard deviation of this mean. Interfacial tension parameters measured by WBT result from best fit to a single concentration-dependent $\gamma$ curve (no time dependence was recorded for WBT) and listed uncertainty is standard-error-of-the-fit to the four-parameter logistic equation discussed in Methods and Materials. Secure interpretation of concentration-dependent $\gamma$ is highly dependent on the purity of solute from which solutions were prepared. There are two considerations of particular importance in this regard for protein solutes. The first is that purified proteins should be substantially free of all other proteins or protein fragments and ideally should retain native conformation. Protein purity has been quantified by electrophoresis provided by the vendor as part of product certification (column 5 of Table 1) but protein conformation remains an unknown/uncontrolled variable. Activity is of particular concern in the case of enzymes of Table I but of course activity is not a direct measure of purity. The second important consideration is that of surfactant contamination possibly occurring in the various fractionation/purification steps employed to separate single proteins from complex mixtures such as blood. Indeed, surfactant contamination can significantly affect
\( \gamma \), even at trace concentrations (e.g. <0.01%) that may not be easily resolved by modern bulk analytical techniques; especially for proteins available only in small quantities/concentrations.

As it turns out, PDT techniques employed herein are among the most sensitive techniques available to detect surfactant contamination and can distinguish between surface activity of proteins and surfactants, as well surfactant mixtures. From these measurements, it can be safely concluded that purified proteins studied herein were not overwhelmingly contaminated with synthetic surfactants, especially methyl silicones widely used in separation media, because these detergents typically reduce \( \gamma \) to much lower levels than observed for protein solutions.

Nevertheless, this fact alone does not guarantee that proteins were not very slightly contaminated with strong surfactants or even significantly contaminated with hydrocarbon surfactants (that have generally low surfactant strength) at the precise solution concentrations required to mask protein surface activity. It is noteworthy in this regard that interfacial activity among the wide array of proteins derived from a variety of sources studied herein was quite conserved (relative to synthetic surfactants) and happens to be quite similar to human plasma/serum that was not subjected to any fractionation/purification steps (compare Table 2 and 3). Thus, we conclude that if proteins were in fact contaminated with surfactants, then this contamination must have occurred in an unlikely manner leading to similar \( \gamma \) for all proteins and in proportions that just happen to cause solution \( \gamma \) to overlap with that of unpurified plasma/serum. We add further that protein \( \gamma \) results reported herein were not substantially different from previous similar studies (see for examples refs. 20,21,23 and citations therein) and that we were able to detect cases of protein contamination, as described below in specific reference to FXIIa, as well as inadvertent contamination induced by contact with plate sealing film (we have found that Nalge-
Nunc plate-sealing film can be used as described in Methods and Materials in that it does not
detectably alter water interfacial tension whereas $\gamma_w$ of water in 96-well plates covered with
Corning film was reduced to $\sim 65$ dyne/cm and exhibit time-dependence consistent with
surfactant contamination). Thus, we conclude that concentration-dependent $\gamma_w$ of proteins listed
in Table 1 was dominated by the natural polypeptide amphilicity and cannot be attributed to
surfactant contamination.

As further noted in Table 1, replicate protein preparations were studied for human albumin (FV
and FAF HSA), IgG, IgM and blood factors FII, FXII, FXIIa. Occasionally different vendors
were used as a means of controlling for discrepancies that might arise from sourcing. In
consideration of all experimental variables ranging from sourcing to data reduction, we conclude
that variation associated with manipulation and dilution of protein concentrates ($\sim 10$ mg/mL;
includes transfer losses by adsorption to pipettes, vials, and wells; slight but inevitable foaming
of proteins brought into solution from powder; etc.) overwhelms $\gamma_w$ measurement error
discussed in the Materials and Methods and that differences between preparations or vendors
were insufficient to affect basic conclusions of this work. We call particular attention to the case
of FXIIa (preparations 1 and 2) for which as-received proteins were apparently contaminated
with an unidentified surface-active compound, leading to $\gamma_w$ curves that were distinctly not
sigmoidal with a mid-range-concentration “step” that precluded analysis in the manner described
in the Materials and Methods section. We speculate that FXIIa was contaminated during
preparation (FXII $\rightarrow$ FXIIa) procedures because FXII from the same vendors did not exhibit this
behavior which was unlike that observed for all other proteins, protein mixtures (blood plasma
and serum), and surfactant reference compounds. Only results obtained with preparation 2 of FXIIa are provided herein although both preparations behaved similarly.

**Quantitative Comparison of Proteins and Surfactants:** Protein adsorption fell into three broad categories identified herein as Type 1, 2 and 3. Column 2 of Table 2 assigns adsorption type observed for the listed proteins. Type 1 $\gamma_v$ curves were clearly sigmoidal with distinct low- and high-concentration plateaus measured by $\gamma_v^\prime$ and $\gamma_v^\prime\prime$ parameters listed in columns 3 and 4 (see, as examples, Figs. 1 A and B). Steady-state was apparently achieved in Type 1 adsorption at or before 3600 sec drop age whereupon kinetic effects in $\gamma_v^\prime$ were no longer resolvable at any concentration (less than about 1 dyne/cm). No semblance of a $\gamma_v^\prime\prime$ plateau was reached at the highest concentrations studied for Type 2 proteins (see, for examples, results for ubiquitin in Fig. 2 and FXII in Fig. 3). It is not evident from this work if these proteins would exhibit Type 1 behavior if yet-higher concentrations were available for study. We note, however, that kinetic effects had fully dampened for Type 2 adsorption within the interval $3500 < t < 3600$ sec of the PDT experiment. This strongly suggests that steady-state had in fact been achieved, but that higher-solution concentrations were required to saturate the surface and achieve a verifiable $\gamma_v^\prime\prime$ plateau. Moreover, we note that large proteins such as IgM and $\alpha_2$-macroglobulin achieved steady state over the 60 min time frame (see Figs. 1, 2), confirming that mass transport and adsorption kinetics could indeed be completed within the time frame of the PDT experiment. Thus, we conclude that Type 2 adsorption was concentration rather than time limited. Only graphical estimates for Type 2 parameters are provided in Table 2 because firm values could not
be ascertained by statistical fitting procedures described in the Materials and Methods section. However, experimental experience suggests that $\gamma^*_w$ and $\Pi^{\text{max}}$ were within 10 dyne/cm of the stated value and $\ln C_w^{1/2}$ within about 2 units.

We made no attempt to estimate the exponential parameter $M$ for Type 2 proteins. Steady-state adsorption was not achieved for Type 3 and no well-defined $\gamma^*_w$ plateau was observed. Instead, $\gamma^*_w$ drifted continuously lower with increasing concentration, giving the appearance that a high-concentration limit was just on the horizon but was never quite realized. In this work, Type 3 behavior was observed only as a transient state ultimately leading to steady state. However, Type 3 adsorption has been observed at all realizable concentrations up to the solubility limit for certain animal proteins (such as bovine serum, plasma, albumin and IgG) as will be reported elsewhere. Results for $\alpha_2$-macroglobulin (Fig. 1C) are especially interesting with regard to adsorption types, illustrating kinetic effects that arguably transition from Type 2 behavior at early drop age, and ending with (poorly-defined) Type 1 adsorption behavior.

Examining data of Table 2 in detail now, note that $\gamma^*_w$ data (column 3) fall between 70 and 72 dyne/cm, consistent with the interfacial tension of water near 25 °C. Variations within this range can be traced to uncertainty in the statistical fit to concentration-dependent $\gamma^*_w$ data (listed error) and variation in ambient temperature. Moving to column 4 of Table 2, it is apparent that proteins generally exhibited a lower interfacial tension $\gamma^*_w > 30$ dyne/cm, with FXII yielding the lowest value among those listed in Table 2. This is of special interest to blood plasma coagulation
because FXII adsorption from whole blood or plasma to procoagulant materials is thought to potentiate the intrinsic pathway of coagulation and enhanced “biosurfactancy” is consistent with this view. However, as will be discussed subsequently, FXII surface activity is substantially mitigated by a low-physiologic concentration that cannot sustain a high level of interfacial activity.

Column 5 gives values for the half-maximum of interfacial tension reduction \( \ln C_{1/2}^{\Pi} \) that fall within a broad range of about 10 to 22 natural- (base e) log units (for concentration expressed in pM). Steepness of the sigmoidally-shaped \( \gamma_{b}^{\nu} \) curve measured by \( M \) is given in column 6 (with larger negative numbers consistent with steeper \( \gamma_{b}^{\nu} \) curve) likewise covers a rather broad range. The significance of \( \ln C_{1/2}^{\Pi} \) and \( M \) parameters is best gauged graphically from Figs. 1-3. The maximum reduction in interfacial tension \( \Pi^{\max} \equiv \gamma_{b}^{\nu} - \gamma_{b}^{\nu} \) observed for the proteins listed in column 7 was calculated assuming \( \gamma_{b}^{\nu} = 71.97 \) in all cases, for the purpose of internal consistency. Error in \( \Pi^{\max} \) is therefore identical to error in \( \gamma_{b}^{\nu} \). Table 3 construction is similar to that of Table 2 using the same parameters that quantify \( \gamma_{b}^{\nu} \) curves. The purpose of Table 3 is to compare results of pendant-drop tensiometry (PDT) and Wilhelmy-balance tensiometry (WBT) applied to protein mixtures and surfactant reference compounds that were available in sufficient volume to satisfy demands of the Wilhelmy method (about 10 mL, see Materials and Methods).
In general, it was noted that \( \gamma'_{l_v} \) and \( \gamma''_{l_v} \) parameters from PDT and WBT were in reasonable agreement (and hence \( \Gamma^{\text{max}} \)) for all solutes but \( \ln C_{B}^{\frac{\Pi}{2}} \) and \( M \) parameters were in substantial disagreement, especially for proteins and protein mixtures. Our interpretation is that WBT, as applied in this work, does not measure adsorption kinetics in the same way as PDT due to quite different mass-transfer and adsorption processes occurring at the moving plate-meniscus region than at the stagnant-drop interface. These differences were negligible at low concentrations and overwhelmed by adsorption from high-concentration solutions, leading to differences in measured \( \gamma_{l_v} \) for only intervening concentrations that, in turn, led to differences in \( \ln C_{B}^{\frac{\Pi}{2}} \) and \( M \).

**Graphical Interpretation of Results:** Time-and-concentration-dependent LV interfacial tension \( \gamma_{l_v} \) profiles were obtained for the purified proteins, protein mixtures, and surfactants listed in Table 1. Representative results for the anionic surfactant SDS, purified protein HSA (fatty acid free, FAF), and \( \alpha_2 \)-macroglobulin are compared in Fig. 1 in both three-dimensional (3D, \( \gamma_{l_v} \) as a function of time and concentration) and two-dimensional (2D, \( \gamma_{l_v} \) as a function of concentration at specified times) representations. Note that the logarithmic-solute-concentration ordinate \( \ln C_{B} \) in Fig. 1 is expressed in picomolarity units (pM, \( 10^{-12} \) moles solute/L solution; see following and Materials and Methods section for computational and data representation details). Examining first 3D and 2D representations of SDS interfacial activity (Fig. 1A) which serves as a reference compound, it was observed that the \( \gamma_{l_v} \) curve was strictly sigmoidal in shape, with a well-defined low-concentration asymptote \( \gamma^*_{l_v} \) and a high-concentration asymptote \( \gamma''_{l_v} \) characteristic of Type 1 adsorption. In this latter regard, SDS and HSA exhibited similar concentration-limiting behavior (compare Fig. 1 A and B) that is typically interpreted as
formation of a critical micelle concentration (CMC), at least for surfactants. This paper provides no evidence of micelles, for either proteins or surfactants, and so only acknowledges a limiting behavior at which further increase in solute concentration did not measurably change $\gamma_v$. Unlike SDS, however, adsorption/mass transfer kinetics significantly affected early-time $\gamma_v$ measurements of HSA solutions, although steady state was achieved well before the final observation time at 3600 sec. As mentioned in the preceding section, kinetic effects are even more pronounced for large proteins such as $\alpha_2$-macroglobulin for which it is observed that adsorption behavior transitions from Type 2 at the earliest observations, to Type 1 as steady state is achieved near 3600 sec. Thus Fig. 1 captures the significant adsorption trends quantified in Tables 2 and 3.

**Scaled Interfacial Activity:** Figs. 2 A, B, and C compare $\gamma_v$ curves for proteins selected from Table 1 to cover the full range of molecular weight (MW) studied in this work, scaled in three different ways for direct comparison; by weight, molarity, and ratio-to-physiological concentration. Smooth curves represent a “slice” taken from 3D curves like those of Fig. 1 at or near 3600 sec. Supporting data are not shown with smooth curves of Fig. 2 for the sake of clarity, but representative $\gamma_v$ curves with authentic data are amply illustrated in Figs. 1 and 3. The following discusses results of each scaling method in order of appearance on Fig. 2. These different scaling methods sharpen general impressions about the energetics of protein adsorption that are particularly pertinent to the protein-adsorption tenet discussed in the Introduction.
Fig. 2A compares $\gamma_{hv}$ curves with $C_B$ expressed in parts-per-trillion (i.e. PPT, grams solute/10$^{12}$ g solvent, so that the $\ln C_B$ scale has positive values at all concentrations). Weight scaling is applicable to purified proteins, protein mixtures, and surfactants alike, permitting comparison of interfacial activity on a single concentration axis.$^{18,20,21}$ Two features are readily apparent from Fig. 2A. First, it is noted that the dynamic range of $\Pi_{\text{max}} = \gamma_{hv}^* - \gamma_{hv} < 40$ dyne/cm. This is to be interpreted in light of the $\Pi_{\text{max}}$ range observed for synthetic surfactants that can exceed 60 dyne/cm, with $25 < \Pi_{\text{max}} < 50$ dyne/cm for ordinary hydrocarbon soaps and surfactants.$^{24,25}$ Thus, it would appear that proteins and protein mixtures are relatively weak surfactant systems with $\Pi_{\text{max}}$ only slightly overlapping with the weakest of surfactant systems. Second, it is somewhat surprising that $\gamma_{hv}^*$ data for this diverse set of proteins (with MW spanning nearly three orders of magnitude; see Table 1) lies within a relatively narrow 20 dyne/cm band. Again, this should be interpreted in light of the full range observed for synthetic surfactants wherein $\gamma_{hv}^*$ varies substantially over the entire measurable range. Thus, it is evident from Fig. 2A that, on a weight basis, proteins exhibit quite similar LV interfacial activity. These results corroborate and extend similar previous investigations with focus on a narrower range of protein MW.$^{18,20,21}$ Bearing in mind the great range in MW spanned by proteins in Fig. 2, it is reasonable to conclude that commensurate variability in protein structure does not confer widely varying LV interfacial activity; at least not in comparison to the full range available to ordinary surfactants.

However convenient weight scaling may be, it is nevertheless true that free-energy and stoichiometry scale on a molar basis. Thus, for the purpose of better understanding interfacial energetics, it is therefore useful to express $C_B$ in molar units. We have chosen to express solute
dilution $C_B$ in picomoles/L (i.e. picomolarity, pM, $10^{-12}$ moles solute/L solution so that the \( \ln C_B \) scale has positive values for all dilutions) for solutes with a known (nominal) MW. This approach is applicable to purified proteins and surfactants but not to chemically-undefined protein mixtures such as plasma and serum. Variability in interfacial activity among the diverse purified proteins reported in Fig. 2 is much more evident on a molar rather than weight basis (compare Fig. 2A to Fig. 2B). Of course, molar scaling does not alter observations regarding the range of $\Pi_{max}$ mentioned above, but it does effectively expand the concentration axis by moving higher-MW proteins (such as $\alpha_2$-macroglobulin and IgM) to the left and lower-MW proteins (such as albumin and ubiquitin) to the right. It is apparent from Fig. 2B that high-MW proteins reduce $\gamma_{lv}$ at lower molarity than low-MW proteins. This progression in MW is suggestive of the Traube-rule for linear hydrocarbon surfactants in which it is observed that the concentration required to reduce $\gamma_{lv}$ to a specified value decreases in a regular progression with each \(-\text{CH}_2\)-unit in a homologous series.\textsuperscript{26-29} Of course, proteins are hardly simple hydrocarbon molecules and have many more degrees of orientational freedom at the interface.\textsuperscript{3,30}

Furthermore, proteins are not interrelated in any known homologous series (other than perhaps being comprised of a limited selection of amino acids). Still, the inference taken from Fig. 2B is that protein concentration required to reduce $\gamma_{lv}$ to a specified value decreases with MW in a manner loosely consistent with the addition of a generic amino-acid-building-block having an “average amphilicity” that increases MW but does not radically change protein interfacial activity. Otherwise, if MW increased by addition of amino-acid-building-blocks with highly-variable amphilicity, then $\Pi_{max}$ would be expected to be a much stronger function of protein
MW than is observed in Fig. 2B. Thus, it appears that molar variability in $\gamma_v$ is achieved by aggregating greater mass of similar amphiphilic character, as opposed to accumulating greater amphilicity with increasing MW.

Weight and molar scaling are very useful experimental and conceptual constructs that may turn out to have little direct relevance to the in vivo biological response to materials because these scales do not account for the widely-varying natural abundance of the many different proteins comprising the mammalian proteome. Thus, for the purpose of better understanding protein interfacial activity within a physiologic context, we have found it useful to ratio $C_B$ to nominal (mean) physiological concentration $C_P$ and express $C_B / C_P$ on a (base 10) logarithmic scale $(\log C_B / C_P)$. In this way, the physiologic condition can be readily identified at $(\log C_B / C_P) = 0$ with more-dilute-protein solutions lying to the left (negative $(\log C_B / C_P)$ values) and more-concentrated solutions lying to the right (positive $(\log C_B / C_P)$ values; see dashed vertical line on Fig. 2C). This approach is applicable to purified proteins for which humoral or cellular concentrations are known. Thus, Fig. 2C reveals a different kind of diversity in protein interfacial activity than discussed above in reference to weight or molar scales.

Certain proteins, notably albumin, IgG and IgM, produced the full $\Pi^{\text{max}}$ range well below physiologic concentration ($\gamma_v$ data lies substantially to the left of $(\log C_B / C_P) = 0$). By contrast, C1q, prothrombin (FII), and ubiquitin did not express $\Pi^{\text{max}}$ at physiologic concentration ($\gamma_v$ curves cross the $(\log C_B / C_P) = 0$ line) but were still observed to be substantially surface active at physiologic concentrations. Interestingly, only a limited portion of the available $\Pi^{\text{max}}$ range
was exhibited by blood factor XII and the enzyme form XIIa ($\gamma_{\text{v}}$ data lie substantially to the right of $(\log C_B / C_p) = 0$), as will be discussed separately below in reference to Fig. 3 because this has special significance in blood coagulation. It appears that $(\log C_B / C_p)$ is a pragmatic scaling of interfacial activity with relevance to biomedical materials that reveals concentration-driven diversity in interfacial activity observed among humoral and cellular proteins.

Fig. 3 focuses on results obtained for blood factors FXII and FXIIa. Activation of FXII by adsorption to procoagulant surfaces is thought to potentiate the intrinsic pathway of blood coagulation (see refs. 32,33 and citations therein). Thus, understanding the interfacial activity of FXII and FXIIa is important toward a full appreciation of the contact activation mechanism and anticoagulation as well. Fig. 3 is prepared in basically the same format as Fig. 2C except that smooth curves here correspond to different observation times up to 3594 sec. Data points for the 3594 sec drop age are included for preparation 2 of FXIIa verifying that, although less-concentrated solutions of FXIIa indicated presence of an unidentified surface-active contaminant, higher-protein concentrations were not measurably different than that obtained for FXII that presented no such difficulties (data corresponding to preparation 1 not shown). This suggests that, at these higher concentrations, FXIIa surfactancy overwhelmed that of the putative contaminant and that FXIIa interfacial activity is not too different than FXII.

Similarity between FXII and FXIIa is consistent with the fact that FXIIa differs from FXII only by cleavage of a disulfide bridge and not wholesale molecular alteration. This latter feature of zymogen $\rightarrow$ enzyme conversion is substantially different from the prothrombin $\rightarrow$ thrombin (FII $\rightarrow$ FIIa) reaction that cleaves a much smaller protein with quite different biosurfactant properties
Fig. 3 thus demonstrates that FXII and FXIIa were only weakly surface active at physiologic concentrations and that $\Pi^{\text{max}}$ was not achieved unless solutions were nearly two orders-of-magnitude more concentrated. Moreover, maximum reduction in $\gamma_\nu$ was achieved only at substantially long equilibration times ($> 1800$ sec). These adsorption kinetics must be interpreted in terms of the blood-plasma-coagulation process that can be complete within 300 sec or so when blood plasma is saturated with high-surface-area procoagulants. Results obtained with purified FXII and FXIIa are seemingly inconsistent with the traditional biochemical mechanism of contact activation of blood plasma coagulation that asserts rapid FXII adsorption onto hydrophilic procoagulant surfaces (see refs. 32,33 and citations therein). FXII adsorption, and FXIIa desorption for that matter, must occur in the presence of overwhelming concentrations of other blood proteins, notably albumin and IgG that this work demonstrates to be much more surface active on both molar and physiological-concentration scales (Fig. 2). Furthermore, we note that FXII adsorption to the LV interface is not rapid relative to other proteins such as albumin, as might be expected for a protein with putatively enhanced interfacial activity. Although mechanisms of adsorption to hydrophilic (efficient procoagulant) surfaces are not necessarily the same as those leading to adsorption to hydrophobic (inefficient procoagulant) surfaces, it is the general experience that much more protein is adsorbed to hydrophobic surfaces.

On this basis, we expect that FXII and FXIIa would exhibit even less adsorption to hydrophilic procoagulants than we observe at the hydrophobic LV surface within a time-frame relevant to coagulation. All taken together, these studies of FXII and FXIIa adsorption to the hydrophobic LV interface support our contention that FXII does not adsorb directly onto hydrophilic
procoagulant surfaces in a manner that displaces water (surface dehydration) and that contact activation of FXII → FXIIa occurs by some process other than suggested by the traditional mechanism.34-36

1.4. Conclusions

Time-and-concentration-dependent liquid-vapor (LV) interfacial tension \( \gamma_{lv} \) has been measured for a diverse set of human proteins ranging from albumin to ubiquitin, with special focus on blood-plasma proteins. Three different methods of scaling concentration dependence were explored: weight, molarity, and ratio-to-physiologic concentration. On a weight basis, proteins among the group appeared more similar than dissimilar, especially when viewed from the perspective of the full range available to synthetic-surfactant reference compounds, reducing \( \gamma_{lv} \) by no more than about 40 dyne/cm (\( \Pi^{\text{max}} \equiv \gamma_{lv}^\prime - \gamma_{lv}^\prime < 40 \) dyne/cm) and all proteins falling within a relatively narrow 20 dyne/cm band (\( 55 < \gamma_{lv}^\prime < 30 \) dyne/cm). We interpret this observation to mean that there is insufficient amphiphilic diversity among the proteins studied to support wide-ranging interfacial activity at the LV interface, even though molecular weight (MW) varied over nearly three orders-of-magnitude. Molar scaling revealed that interfacial activity followed a progression in MW, with the concentration required to reach a specified \( \gamma_{lv} \) value decreasing with increasing MW.

This progression in MW is reminiscent of the Traube-rule for linear hydrocarbon surfactants in which it is observed that the concentration required to reduce \( \gamma_{lv} \) to a specified value decreases
in a regular progression with each CH$_2$ unit (i.e. MW) in a homologous series.$^{26-29}$ The parallel interpretation for proteins is that molar variability in $\gamma'$ is achieved by aggregating greater mass of similar amphiphilic character (blocks of amino acids), as opposed to accumulating greater amphilicity with MW. The significance of this observation is that it suggests that the structural variability that confers vastly different bioactivity does not greatly affect interaction energetics in water that drive adsorption to the LV interface. Scaling interfacial activity to physiological concentration revealed that certain proteins, such as albumin and IgG, produced the full $\Pi^{\text{max}}$ range at-and-well below physiologic concentration whereas others, notably blood factor XII (Hageman factor) required concentration by more than two-orders-of-magnitude above the nominal-physiologic concentration to express the full range of $\Pi^{\text{max}}$ characteristic of this protein.

In summary, results reported herein support the overall conclusion that combinations and permutations of the 20 naturally-occurring amino acids comprising the primary sequence of mammalian proteins is insufficient to support widely-varying LV interfacial activity, no matter how these sequences happen to fold into higher-order structure. Furthermore, we contend that protein concentration, not diversity in molecular structure, is the significant energetic driver of adsorption to the LV interface. Interpreted in terms of the protein-adsorption tenet discussed in the Introduction, these results suggest that assertion of differential protein adsorption to biomaterial surfaces from heterogeneous mixtures such as blood requires careful justification in terms of both concentration and kinetics.
In this regard, blood factor XII is especially relevant to the development of hemocompatible 
materials because adsorption of FXII from blood onto procoagulant surfaces is generally 
accepted to be the first step in potentiation of the intrinsic pathway of plasma coagulation. A 
significant question that arises particular to FXII and FXIIa asks how specific adsorption to 
procoagulant surfaces can occur when FXII is so weakly surface active at physiologic 
concentrations, especially in the presence of overwhelming concentrations of proteins such as 
albumin or IgG that are shown by this work to be among the class of highly-surface-active 
proteins at physiologic concentrations. Perhaps specific interactions with solid surfaces and 
(rapid) Vroman-effect interchange of adsorbed proteins not explored in this research can 
rationalize all observations and theories. Even so, such descriptive biochemical explanations 
will have much more authority when shown to be consistent with the interfacial energetics of 
adsorption.
Citations


(3) Ramsden, J. J. Puzzles and Paradoxes in Protein Adsorption, Chemical Society Reviews 1995, 24, 73.


(19) Putnam, F. W. Alpha, Beta, Gamma, Omega - The Roster of the Plasma Proteins.

(20) Vogler, E. A. Practical Use of Concentration-Dependent Contact Angles as a

(21) Vogler, E. A. Practical Use of Concentration-Dependent Contact Angles as a

(22) Feri, J. K.; Stebe, K. J. Which Surfactants Reduce Surface Tension Faster? A

(23) Tripp, B. C.; Magda, J. J.; Andrade, J. D. Adsorption of Globular Proteins at the
Air/Water Interface as Measured via Dynamic Surface Tension: Concentration Dependence,

(24) Schwartz, A. M.; Perry, J. W. *Surface Active Agents*; Interscience Publishers:


(26) Traube, J. Ueber die Capillaritatscontanten Organischer Stoffe in Wassergen

(27) Tanford, C. *The Hydrophobic Effect: Formation of Micelles and Biological

(28) Adamson, A. W. *Physical Chemistry of Surfaces*, 2 ed.; Interscience Publishers:
(29) Tanford, C. How Protein Chemists Learned about the Hydrophobic Factor, 


(30) Andrade, J. D.; Hlady, V. Protein Adsorption and Materials Biocompatibility: A 


(31) Anderson, N. L.; Anderson, N. G. The Human Plasma Proteome: History, 


(32) Mitropoulos, K. A. The Levels of FXIIa Generated in Hyman Plasma on an 

Electronegative Surface are Insensitive to Wide Variation in the Concentration of FXII, 


(33) Mitropoulos, K. A. High Affinity Binding of Factor FXIIa to an Electronegative 

Surface Controls the Rates of Factor XII and Prekallirien Activation in vitro, *Thrombosis 


Activation of the Plasma Coagulation Cascade. 1. Procoagulant Surface Energy and Chemistry, *J. 


Activation of the Plasma Coagulation Cascade. 2. Protein Adsorption on Procoagulant Surfaces, 


(36) Vogler, E. A.; Nadeau, J. G.; Graper, J. C. Contact Activation of the Plasma 


(37) Colman, R. W. Contact Activation Pathway: Inflammatory Fibrinolytic, 

Anticoagulant, Antiadhesive, and Antiangiogenic Activities. In *Hemostasis and Thrombosis:*


<table>
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<tr>
<th>CLASS</th>
<th>NAME (acronym)</th>
<th>MOLECULAR WEIGHT g/mol</th>
<th>AS-RECEIVED FORM (mg/ml)</th>
<th>PURITY (electrophoresis or ACTIVITY)</th>
<th>PHYSIOLOGIC CONCENTRATION mg/100ml (nominal value)</th>
<th>METHOD (Table #)</th>
<th>VENDOR</th>
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<td>HUMAN SERUM ALBUMIN</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>(FAF HSA)</td>
<td>Prep 1</td>
<td>66,300</td>
<td>Powder</td>
<td>73%</td>
<td>3500-5500 (4500) (Also applied to FAF HSA)</td>
<td>PDT/WBT (2,3)</td>
<td>ICN Biomedicals</td>
</tr>
<tr>
<td></td>
<td>Prep 2</td>
<td>73%</td>
<td></td>
<td></td>
<td></td>
<td>PDT (2)</td>
<td>ICN Biomedicals</td>
</tr>
<tr>
<td></td>
<td>Prep 3</td>
<td>96-99%</td>
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<td></td>
<td>PDT (2)</td>
<td>Sigma Aldrich</td>
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<tr>
<td></td>
<td>Fraction V (FV HSA)</td>
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<td>Powder</td>
<td>96-99%</td>
<td></td>
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<td>4</td>
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<td>Solution (1.4)</td>
<td>121 PEU/mg</td>
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<td>Solution (0.8)</td>
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<td>COMPLEMENT COMPONENT C1q (C1q)</td>
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<td>Single band by immuno electrophoresis</td>
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<td>N/A</td>
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<td>N/A</td>
<td>PDT/ WBT (3)</td>
<td>Blood Bank</td>
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<tr>
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<td>Prep 3</td>
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<td>CETYL BROMIDE (CDAB)</td>
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<td>PERFLUOROOCtANOIC ACID (PFOA)</td>
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<td>N/A</td>
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Table 2: Pendant-Drop Tensiometry (PDT) Liquid Vapor (LV) Interfacial Activity Of Purified Protein Solutions

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<th>NAME (acronym)</th>
<th>TYPE</th>
<th>$\gamma_v$ (dynes/cm)</th>
<th>$\gamma_v'$ (dynes/cm)</th>
<th>$\ln C_{PB}^{\Pi}$ (PPT (pM))</th>
<th>$M$ (dimensionless)</th>
<th>$\Pi_{max}$ (dynes/cm)</th>
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<td>Prep 1</td>
<td>70.74±0.32</td>
<td>51.96±0.44</td>
<td>17.23±0.11 (13.04±0.11)</td>
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<td>Prep 2</td>
<td>71.39±0.59</td>
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<td>16.34±0.14 (12.15±0.15)</td>
<td>-17.3±3.0</td>
<td>19.79±0.61</td>
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<td>70.83±0.30</td>
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<td>16.25±0.16 (12.06±0.16)</td>
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<td>18.33±0.47</td>
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<td><strong>Fraction V (FV HSA)</strong></td>
<td>Prep 1</td>
<td>72.4±1.2</td>
<td>50.3±1.2</td>
<td>16.32±0.28 (12.14±0.28)</td>
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<td>70.8±1.1</td>
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<td>16.61±0.51 (12.44±0.53)</td>
<td>-7.3±2.2</td>
<td>25.5±2.5</td>
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<td><strong>PROTHROMBIN (FII)</strong></td>
<td>Prep 1</td>
<td>70.50±0.73</td>
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<td>17.55±0.45 (12.72±0.35)</td>
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<td><strong>THROMBIN (FIIa)</strong></td>
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<td><strong>FACTOR XII (FXII) †</strong></td>
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<td>29</td>
<td>19 (15)</td>
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<td>37</td>
<td>19 (15)</td>
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<td>14.47±0.57</td>
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<td><strong>HEMOGLOBIN (Hb)</strong></td>
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<tr>
<td><strong>UBIQUITIN (Ub) †</strong></td>
<td>72</td>
<td>46</td>
<td>17 (15)</td>
<td>-</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td><strong>COMPLEMENT COMPONENT C1q (C1q)</strong></td>
<td>Prep 1</td>
<td>71.59±0.54</td>
<td>54.2±1.6</td>
<td>17.27±0.36 (11.27±0.38)</td>
<td>-14.2±4.1</td>
<td>17.5±1.6</td>
</tr>
<tr>
<td></td>
<td>Prep 2</td>
<td>71.65±0.55</td>
<td>50.2±3.1</td>
<td>18.52±0.32 (11.64±0.35)</td>
<td>-14.2±4.2</td>
<td>21.5±3.1</td>
</tr>
<tr>
<td></td>
<td>Prep 3</td>
<td>71.16±0.53</td>
<td>51.6±1.3</td>
<td>17.59±0.12 (10.69±0.12)</td>
<td>-21.0±4.3</td>
<td>20.1±1.3</td>
</tr>
</tbody>
</table>

* pM = picomoles/L (10^{-12} moles/L); Listed data result from statistical fit (See Materials & Methods).
† Parameters are estimates for Type 2 (See Materials & Methods).
Table 3: Pendant-Drop Tensiometry (PDT) and Wilhelmy Balance Tensiometry (WBT) of Proteins, Protein Mixtures and Synthetic Surfactants

<table>
<thead>
<tr>
<th>NAME*</th>
<th>PDT TYPE</th>
<th>γlv (dynes/cm)</th>
<th>γlv' (dynes/cm)</th>
<th>lnC_B ΠΠΠΠ² PPT (pM)**</th>
<th>M (dimensionless)</th>
<th>ΠΠΠΠmax (dynes/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP</td>
<td>1</td>
<td>71.70±0.62</td>
<td>48.55±0.71</td>
<td>19.56±0.19</td>
<td>23.1±1.3</td>
<td>-23.3±3.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72.29±0.46</td>
<td>51.7±5.6</td>
<td>23.1±1.3</td>
<td>-13.5±3.6</td>
<td>23.15±0.71</td>
</tr>
<tr>
<td>HS</td>
<td>1</td>
<td>70.54±0.46</td>
<td>47.61±0.62</td>
<td>19.91±0.17</td>
<td>24.1±1.1</td>
<td>-23.1±3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>73.08±0.61</td>
<td>43.0±6.1</td>
<td>24.1±1.1</td>
<td>-10.3±3.8</td>
<td>24.08±0.62</td>
</tr>
<tr>
<td>FAF HSA †</td>
<td>1</td>
<td>70.74±0.32</td>
<td>51.96±0.44</td>
<td>17.23±0.11</td>
<td>15.90±0.46</td>
<td>-23.1±3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72.82±0.51</td>
<td>59.5±1.5</td>
<td>13.04±0.11</td>
<td>11.71±0.46</td>
<td>-13.4±5.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.04±0.11</td>
<td>11.71±0.46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFOA †</td>
<td>2</td>
<td>71</td>
<td>15</td>
<td>20 (21)</td>
<td>20.22±0.11 (21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>73.00±0.29</td>
<td>9.2±2.0</td>
<td>-20.6±1.6</td>
<td>57</td>
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</tr>
<tr>
<td></td>
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<td>-20.6±1.6</td>
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<tr>
<td>CDAB</td>
<td>1</td>
<td>72.74±0.63</td>
<td>35.54±0.50</td>
<td>16.70±0.07 (17.67±0.07)</td>
<td>16.64±0.40 (17.60±0.35)</td>
<td>-35.8±4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72.7±1.3</td>
<td>36.4±2.1</td>
<td>16.70±0.07 (17.67±0.07)</td>
<td>16.64±0.40 (17.60±0.35)</td>
<td>-35.8±4.3</td>
</tr>
<tr>
<td>TWN80</td>
<td>1</td>
<td>72.8±2.3</td>
<td>36.0±1.6</td>
<td>15.27±0.32 (15.05±0.33)</td>
<td>14.65±0.14 (14.38±0.14)</td>
<td>-14.8±3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72.75±0.31</td>
<td>37.63±0.67</td>
<td>15.27±0.32 (15.05±0.33)</td>
<td>14.65±0.14 (14.38±0.14)</td>
<td>-7.30±0.47</td>
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<tr>
<td>AOT</td>
<td>1</td>
<td>71.89±0.35</td>
<td>23.86±0.39</td>
<td>15.08±0.08 (15.89±0.08)</td>
<td>18.75±0.28 (19.66±0.22)</td>
<td>-17.6±1.7</td>
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<tr>
<td></td>
<td></td>
<td>72.03±0.58</td>
<td>20.61±0.58</td>
<td>15.08±0.08 (15.89±0.08)</td>
<td>18.75±0.28 (19.66±0.22)</td>
<td>-13.2±1.6</td>
</tr>
<tr>
<td>SDS</td>
<td>1</td>
<td>71.15±0.20</td>
<td>33.30±0.58</td>
<td>18.79±0.07 (20.04±0.07)</td>
<td>-20.5±1.3</td>
<td>38.29±0.58</td>
</tr>
</tbody>
</table>

Notes:
* See Table 1 for acronym definition.
** pM = picomoles/L (10^-12 moles/L); Listed data result from statistical fit (See Materials & Methods)
† Parameters are estimates for Type 2 (See Materials & Method).
‡ Repeated from Table 2 for ease of comparison.
Figure 1: Interfacial tension profiles in 3D ($\gamma_v$ as a function of analysis time (drop age) and logarithmic (natural) solution concentration $C_B$) and 2D ($\gamma_v$ as a function of logarithmic solution concentration $C_B$ at selected times) formats comparing sodium dodecyl sulfate (SDS, panel A), human serum albumin (FAF HSA, panel B, preparation 1 Table 1), and $\alpha_2$-macroglobulin (panel C). In each case, solute concentration $C_B$ is expressed in picomoles/L (pM) (natural) on a logarithmic scale. Symbols in 2D panels represent time slices through 3D representations (filled circle = 0.25 sec, open circle = 900 sec, filled triangles = 1800 sec, and open triangles = 3594 sec). Notice that adsorption kinetics dominate $\alpha_2$-macroglobulin adsorption whereas steady-state is achieved within about 1000 sec for HSA, and no adsorption kinetics is detected for SDS.
Figure 2: Concentration dependence of protein interfacial tension $\gamma_v$ scaled in three different ways: by logarithmic (natural) weight-volume (panel A), molarity (panel B), and logarithmic (base 10) ratio-to-physiological concentration (panel C), for selected proteins spanning an order-of-magnitude of molecular weight. Only statistically-fit smooth curves are shown for clarity (see Figs. 1 and 3 for similar plots including authentic data and Table 2 for statistics of fit). Interfacial activity among proteins appears more similar than dissimilar when viewed on a weight basis but diversity among proteins becomes more apparent when scaled on a molar or ratio-to-physiological concentration. Physiologic scaling (panel C) shows that FAF HSA, IgG, and IgM (preparations 1, Table 1) are fully surface active at-and-below physiological concentrations whereas C1q, FII (preparation 1), and Ub require concentration by as much as two orders-of-magnitude to achieve maximum reduction of $\gamma_v$. 
**Figure 3:** Interfacial activity of blood factors FXII (symbols, preparation 1 Table 1) and FXIIa (cross, preparation 2) solutions with concentration scaled as a ratio-to-physiological concentration. Data points for FXIIa (3594 sec drop age) suggest that adsorption of these two proteins to the LV interface was not substantially different. Statistically-fit smooth curves drawn for FXII at 0.25, 900, 1800, and 3594 sec drop age illustrate FXII adsorption kinetics.
$\gamma_v$ (dynes/cm)

$\log_{10}([\text{Solution Concentration, } C_B]/[\text{Physiologic Concentration, } P])$
Chapter 2

Traube-Rule Interpretation of Protein Adsorption at the Liquid-Vapor Interface

Abstract

Pendant-drop tensiometry of aqueous-buffer solutions of purified human proteins spanning nearly three orders-of-magnitude in molecular weight (MW) reveals that reduction in liquid-vapor (LV) interfacial tension $\gamma_{lv}$ followed a systematic progression in MW with the molar concentration required to reach a specified $\gamma_{lv}$ value decreasing with increasing MW in a manner reminiscent of the Traube-rule for linear hydrocarbon surfactants. Furthermore, concentration-dependence of interfacial tension ($d\gamma_{lv}/d\ln C_B$, where $C_B$ is bulk-solution concentration) is observed to be surprisingly invariant among this disparate group of proteins (i.e. approximately constant apparent Gibbs’ surface excess $\Gamma = -1/RT d\gamma_{lv}/d\ln C_B$). These findings are interpreted through a model of protein adsorption predicated on the interfacial packing of spherical molecules with dimensions scaling as a function of MW. The Traube-rule-like ordering is rationalized as a natural outcome of an invariant partition coefficient that entrains a fixed fraction of bulk-solution molecules within a LV interphase which thickens with increasing protein size (MW). Thus, protein adsorption follows a homology in molecular size rather than composition. Calibration of the sphere-packing model to previously-reported neutron reflectometry (NR) of albumin adsorption permitted interpretation of tensiometric results in terms of interphase thickness and multilayering, predicting that relatively small proteins with MW < 125 kDa (e.g. albumin) fill a single layer whereas larger proteins with MW ~ 1000 kDa (e.g. IgM) require up to five molecular layers to satisfy a constant partition coefficient.
2.1. Introduction

Isidor Traube’s 1891 investigation of the liquid-vapor (LV) interfacial tension $\gamma_v$ of hydrocarbon acid, alcohol, ester, and ketone homologs dissolved in water\(^1\) may well be the first systematic observation of what is now commonly known as the hydrophobic effect.\(^2\)-\(^4\) Traube observed that the molar concentration required to reduce $\gamma_v$ to an arbitrary value decreased in regular progression with each added methylene unit within a particular homologous series. This pattern eluded Traube’s predecessor Emile Duclaux (1840-1904) who, working exclusively in weight/volume (w/v) dilutions, failed to scale concentration-dependent $\gamma_v$ by solution molarity and discern the pattern that later became evident to Traube.\(^5\) Hence history-of-science celebrates the ‘Traube rule’ and remembers Duclaux better by his work with Pasteur and leadership of the Pasteur Institute than his efforts in physical chemistry. Nearly a quarter century later in 1917, Irving Langmuir applied a straightforward thermodynamic interpretation of the Traube rule\(^6\) that systematized existing surfactancy data by assuming that surfactant adsorbed to a planar LV interface as a single molecular layer, with hydrocarbon ‘tails’ protruding through the plane. This insight, which we take quite for granted today, effectively allowed him to the estimate the work required to expel a methylene group from aqueous solution (approx. 640 cal/mole).\(^7\)

Accordingly, the Traube rule results from the fact that neither “amphilicity” (interaction energetics with water) nor adsorbed ‘foot print’ of an extended methylene chain (the hydrophobic moiety of a hydrocarbon surfactant) changes significantly within a homologous series and, as a consequence, $\gamma_v$ scales in a regular and predictable pattern with solution molarity. Thus it is now understood that adsorption of these simple surfactants to the LV interface is effectively dictated by the energetics of hydrophobic hydration.\(^8\)
These water-orchestrated effects were not entirely lost on natural scientists contemporaneous with Langmuir who were just beginning to piece together the now-familiar biochemistry of life (see, as examples refs. 9,10 and especially 11,12 for a history-of-science perspective). Not fully appreciated then but commonly acknowledged today is that the hydrophobic effect underlies many important biological functions such as formation/stability of lipid bilayers, folding of proteins into higher-order structure, and the “biosurfactancy” of proteins that controls adsorption to medical-device surfaces. This latter subject is extremely important in the field of biomaterials, the primary motivation of this work, because it is generally agreed within the biomaterials community that protein adsorption mediates and directs the observed biological response to artificial materials (see refs. 15, 16 and citations therein). Exactly how this occurs at the molecular level is not so clear however, and protein adsorption remains one of the most controversial and recondite topics in contemporary biomaterials surface science. Failure to come to grips with the protein-adsorption problem is as curious as it is vexing, for one might have otherwise anticipated that water, the universal biological solvent system, would impart more obvious regularity in protein adsorption than is readily discernable from the intensive research effort invested in this subject. That is to say, no Traube-like rule for protein adsorption is apparent from decades of focused research, especially as it relates to biocompatibility of materials.

With all of the aforementioned in mind, we have undertaken an extensive study of time-and-concentration-dependent $\gamma_v$ of purified human-protein solutions. A significant motivation of this work was to broaden the scope of previous investigation to include proteins with molecular weight (MW) spanning three orders-of-magnitude in search of a pattern missed in similar
previous studies with narrower focus (see especially ref. 19 and citations therein). As in these
earlier studies, the LV interface was chosen as a molecularly-smooth, model hydrophobic surface
where interfacial energetics are directly accessible to tensiometric (surface thermodynamic)
techniques and adsorbed-protein concentrations $\Gamma$ can be deduced by application of Gibbs’
 isotherm. In a manner evocative of the brief history reviewed above, we observe that
concentration-dependent $\gamma_{lv}$ among these diverse proteins is more similar than dissimilar when
scaled on a w/v basis whereas molarity scaling reveals a Traube-rule-like ordering by MW at
nearly constant $\Gamma$.

These results are herein interpreted through a model predicated on the packing of globular-
protein molecules having nearly spherical dimensions within the LV surface region. This model
explains the Traube-rule-like progression in MW as resulting from adsorption of progressively-
larger spheres within a commensurately-thickening surface region, leading to a homology in
protein size (rather than methylene units in linear hydrocarbon surfactants). Unlike Langmuir’s
two-dimensional (2D) interpretation of the interface, which was a good approximation for small-
molecule surfactants, the model explicitly treats the surface as a 3D interphase with finite
volume that accommodates larger protein molecules. Just as in Langmuir’s analysis of
hydrocarbon surfactants, however, it is concluded that protein amphilicity does not significantly
change within the homologous series in molecular size and, as a consequence, it is possible to
rationalize how it happens that $\gamma_{lv}$ scales with solution molarity at nearly constant $\Gamma$. Hence, we
find that water does indeed impose discernable regularity in protein adsorption; a factor that may
help better understand the energetics behind the biological response to materials.
2.2. Materials and Methods

**Purified Proteins:** Table 1 compiles pertinent details on proteins and surfactants used as received without further purification. Protein purity was certified by the vendor to be no less than the respective values listed in Column 4 of Table 1, as ascertained by electrophoresis (SDS-PAGE or immunoelectrophoresis). Mass, concentration, and molecular weights supplied with purified proteins were accepted without further confirmation. Ref. 15 discloses all details related to protein solution preparation including serial dilutions of protein stock solutions (usually 10 mg/mL) that were performed in 96-well microtiter plates by (typically) 50:50 dilution in phosphate buffered saline solution (PBS) prepared from powder (Sigma Aldrich) in distilled-deionized (18 MΩ) water (interfacial tension of PBS and water was checked periodically by Wilhelmy-balance tensiometry).

**Liquid-Vapor Interfacial Tension Measurements:** LV interfacial tensions \( \gamma_{lv} \) reported in this work were measured by pendant-drop tensiometry using a commercial automated tensiometer (First Ten Angstroms Inc., Portsmouth VA) applying techniques discussed in detail elsewhere. Ref. 15 Briefly, the tensiometer employed a Tecan liquid-handling robot to aspirate between 10-12 µL of solutions contained in a 96-well microtiter plate prepared by the serial-dilution protocol mentioned above. The robot was used to reproducibly transfer the tip with fluid contents into a humidified (99+ % RH) analysis chamber and dispense between 6-11 µL pendant drops (smaller drop volume required for lower interfacial tensions) within the focal plane of a magnifying camera. These and all other aspects of pendant drop analysis were performed under computer control. Precision of \( \gamma_{lv} \) was about 0.5 dyne/cm based on repeated measurement of the same pendant drop. The instrument was calibrated against pure water interfacial tension and further
confirmed on occasion against Wilhelmy-balance tensiometry. The analysis chamber was thermostated to a lower-limit of 25±1 °C by means of a computer-controlled resistive heater. Upper-temperature limit was, however, not controlled but rather floated with laboratory temperature, which occasionally drifted as high as 29 °C during summer months. Thus, reported $\gamma_{lv}$ values are probably not more accurate than about 1 dyne/cm on an inter-sample basis considering the small but measurable variation of water interfacial tension with temperature. This range of accuracy is deemed adequate to the conclusions of this report which do not strongly depend on more highly-accurate $\gamma_{lv}$ that is difficult to achieve on a routine basis. Instead, veracity of arguments raised herein depend more on a breadth of reliable measurements made across the broad selection of human proteins listed in Table 1. Data analysis and statistical methods are fully described in ref. 15.

2.3. Theory

**General Features of the Protein-Adsorption Model:** The protein-adsorption model disclosed below is based on two related experimental observations and implications thereof; namely, (i) the surprisingly slight variation in the concentration dependence of liquid-vapor interfacial tension $\gamma_{lv}$ among the diverse globular proteins studied herein spanning nearly three decades of molecular weight MW (see Tables 2.1-2.2) and (ii) the substantially constant value of the apparent Gibbs’ surface excess $I$ for these proteins. The model asserts that these are outcomes of a relatively constant partition coefficient $P$ that entrains protein within an interphase region separating bulk-solution from bulk-vapor phases. The interphase thickens with increasing protein size because volume occupied by adsorbed-protein molecules is proportional to MW according to the well-known relationships among MW, solvent-exposed area, volume, and
packing density.\textsuperscript{20} As a consequence, interphase concentrations $C_I$ of larger proteins are lower than that of smaller proteins at constant $P \equiv C_I/C_B$. This latter effect leads directly to a Traube-rule-like ordering for proteins. These principal assertions coupled with a packing model for adsorbed protein provide analytical relationships that, when fitted to experimental data, yield scaling relationships for protein adsorption relating molecular size (MW) to interfacial energetics.

**Liquid-Vapor (LV) Interphase:** The LV surface region is modeled as a layer with finite thickness bounded on one side by the bulk-vapor phase and bulk-solution phase on the other. This “3D” surface-region paradigm is consistent with Gibbs or Guggenheim constructions and certain venerable adsorption-kinetics models including a sub-surface surface region (such as Ward and Tordai),\textsuperscript{21} but is not necessarily consistent with a strictly “2D” concept in which all adsorbate is constrained to a single interfacial layer (see refs. 3,14 and citations therein for more discussion relevant to protein adsorption). More specifically, the interphase is modeled (following Schaaf and Dejardin for example)\textsuperscript{22} as consisting of $N$ slabs with thickness $\delta$, the characteristic size of the protein molecule under study (in cm), such that the total interphase thickness (in cm) $\Omega = N\delta$ and volume $V_I = A\Omega = AN\delta$ (in cm$^3$); where $A$ is the interfacial area (cm$^2$) and $N$ is an integer number of slabs that may be fully or partially filled. The interphase solute concentration $C_I$ (in moles/cm$^3$) is related to these variables through Eq. 1:

$$C_I = n_I / V_I = \left(\frac{n_g + n_b}{AN\delta}\right) = \left(\frac{\Gamma_k}{N\delta}\right) + C_B = \frac{\Gamma_N}{\Omega} + \frac{C_I}{P} = \frac{\Gamma_N}{\Omega} \left(\frac{P}{P - 1}\right) \approx \frac{\Gamma_N}{\Omega} \quad \text{...Eq. 1}$$
where $n_I$ is the total number of solute moles (comprised of adsorbate $n_a$ over-and-above $n_B$ contributed by the bulk phase) that reside within $V_I$ and $\Gamma_{lv}$ is the Gibbs’ surface excess (moles/cm$^2$), respectively. The approximation is specific to the case that adsorption is energetically favorable for which the (dimensionless) partition coefficient $P \equiv C_I/C_B >> 1$; as generally anticipated for protein adsorption to a hydrophobic interface,$^3$ so that bulk-phase contribution to $n_I$ is negligible relative to that adsorbed from solution at steady-state (i.e. $n_B < n_a$; $C_I$ is dominantly adsorbate). As will be discussed in greater detail below, $\Gamma_{lv}$ generally differs from the apparent $\Gamma$ deduced directly from experimental measurements.

**Proteins and Protein Packing within the LV Interphase:** Oblate-spheroid, globular-protein molecules are approximately spherical in aqueous solution$^{4,23}$ and are consequently modeled as spheres with radius $r_v = 6.72 \times 10^{-8} M W^{1/3}$ (packing-volume radius in cm for MW expressed in kDa; see ref. 20 for a review of literature up to 1977 and refs. 24-29 for subsequent work regarding spherical dimensions and molecular packing proteins). This strategy is similar to that adopted by Ostuni *et al.*$^{30}$ in which sphere packing is taken to be the simplest physically-relevant model intended to yield semiquantitative description of general trends in protein adsorption. This model is not expected to account for the myriad complexity and variations among proteins that no doubt invalidate spheric assumptions at a detailed level of investigation. Thus, we assume that spherical dimensions apply approximately to proteins with $10 < MW < 1000$ kDa, although we have no specific evidence for this other than the above-cited literature and the extent to which the derived model simulates or fits experimental data. With these molecular dimensions, the highest conceivable solution concentration of protein corresponds to face-centered-cubic (FCC, or equivalently hexagonal) close packing of spheres with radius $r_v$. Such a
close-packed unit cell contains eight \( \frac{1}{8} \) spheres and six \( \frac{1}{2} \) spheres (total of 4 spheres) residing within a cubic volume \((2r_v\sqrt{2})^3\) and hence the FCC protein concentration \(C_{FCC} = 4\) molecules/(\(2r_v\sqrt{2})^3\). Using HSA as an example protein with MW = 66.3 kDa and calculated \(r_v = 2.7\) nm, \(C_{FCC} = 14.6\) mM (or about 24X physiological concentration;\(^{31,32}\) \(\ln C_{FCC} = 23.4\) in pM). Such high concentrations are not at all likely in either bulk solution or within the interphase. However, it seems reasonable to propose that the maximal interphase concentration \(C_{I\text{max}}\) is proportional to \(C_{FCC}\) through a packing-efficiency parameter \(\varepsilon\) that effectively measures how close protein spheres can pack, such that \(C_{I\text{max}} = \varepsilon C_{FCC}\). We assume that packing within an aqueous interphase is limited by repulsion of hydrated spheres\(^{33}\) and, as a consequence, regard \(\varepsilon\) as a generic factor independent of protein (sphere) size that is approximately constant for all globular proteins discussed herein. If sphere repulsion is uniform and symmetric, then \(C_{I\text{max}}\) effectively results from packing spheres with radius \(R = \chi r_v\); where \(\chi\) is a factor measuring the excluded volume surrounding each molecule. Given that \(C_{FCC} = 4\) molecules/(\(2r_v\sqrt{2})^3\) and \(C_{I\text{max}} = 4\) molecules/(\(2R\sqrt{2})^3\) = \(\varepsilon C_{FCC}\), it follows that \(\chi = \varepsilon^{-\frac{1}{6}}\). Hence, molecular dimensions \((2r_v)\) differ from the characteristic dimensions \(\delta = 2r_v\chi = 2r_v\varepsilon^{-\frac{1}{6}}\).

It is of interest to express the protein-packing concepts discussed above in terms of volume fractions as a means of probing further the physical meaning of \(\varepsilon\). The volume fraction occupied by protein \(\Phi_p = n_pV_p/V_I = C_I V_p\); where the protein molar volume \(V_p = \frac{4}{3}\pi r_v^3\) \(N_A\) if \(N_A\) is the Avogadro number. At interphase saturation, \(C_I = C_{I\text{max}}\) so that \(\Phi_p^{\text{max}} = 0.74\varepsilon\); revealing that \(\Phi_p^{\text{max}}\) is equivalent to FCC packing at \(\varepsilon = 1\) (by model construction) and decreases linearly with (fractional) \(\varepsilon\). It is noteworthy that \(\Phi_p^{\text{max}}\) is independent of MW because efficiency of packing spheres does not depend on size, even though the volume occupied by large spheres is greater...
than that of small spheres. Thus, the protein-packing model views adsorption as a process leading to displacement of a fixed amount of water that is controlled by $\varepsilon$ and that interphase capacity for protein is controlled by the energetics of what amounts to be interphase dehydration.$^{34}$

**A Traube-Like Rule for Protein Adsorption:** Eq. 2 combines the notion of $C_{I}^{\max}$ with the MW-dependent radius of spherical proteins, where experimentally-convenient dimensions of picomolarity (10$^{-12}$ moles/L) have been introduced:

$$C_{I}^{\max} = \frac{\varepsilon(4 \text{ molecules})}{(2r_{v}\sqrt{2})^{3}} = \frac{0.177\varepsilon \text{ molecules}}{(6.72 \times 10^{-8} MW^{1/3})^{3}} = \frac{9.68 \times 10^{11}\varepsilon}{MW} \left(\text{picomole/L}\right) \quad \text{...Eq. 2}$$

It is apparent from Eq. 2 that maximal interphase protein concentration varies inversely with protein size (MW), with higher concentrations for low-MW proteins and sharply-lower concentrations for higher-MW proteins. Interphase saturation occurs at $C_{I}^{\max}$ and must therefore correspond to the bulk concentration $C_{B}^{\max}$ at which the limiting interfacial tension $\gamma_{lv}^{'}$ is achieved (i.e. the concentration at maximum spreading pressure $\Pi^{\max} = \gamma_{lv}^{'} - \gamma_{lv}^{'}$). $C_{B}^{\max}$ can be estimated from concentration-dependent $\gamma_{lv}$ curves (see Appendix, 2.6.1) and is related to $C_{I}^{\max}$ through the partition coefficient $P = C_{I}^{\max}/C_{B}^{\max}$. Eq. 3 states this relationship as a logarithmic expression that is convenient to apply to (steady-state) concentration-dependent $\gamma_{lv}$ data:

$$\ln C_{B}^{\max} = \ln \left(\frac{C_{I}^{\max}}{P}\right) = \ln(9.68 \times 10^{11}) - \ln MW + \ln(\varepsilon / P) = -\ln MW + [27.6 + \ln(\varepsilon / P)] \quad \text{... Eq. 3}$$
Assuming that $\varepsilon/P$ is constant for all proteins within this study (as discussed above for $\varepsilon$ and below for $P$), Eq. 3 predicts a linear relationship between $\ln C_B^{\text{max}}$ and $\ln MW$ with a slope of $-1$. A value for the unknown ratio $\varepsilon/P$ can be extracted from the intercept (see Results section).

Traube’s rule for hydrocarbon surfactants stipulates that the concentration required to reduce $\gamma_{lv}$ to an arbitrary value $\gamma_{lv}^*$ decreases in a regular progression with each -CH$_2$- unit (i.e. MW) in a homologous series. Gibbs’ adsorption isotherm can be combined with Eq. 3 to derive a Traube-like rule for proteins by noting that the apparent Gibbs’ surface excess $\Gamma$ is approximated by slope of the linear-like region of experimental concentration-dependent $\gamma_{lv}$ curves (i.e. the ‘surface excess region’ falling between $\gamma_{lv}^o$ and $\gamma_{lv}^*$ centered at $\ln C_B^{\text{IP}}$; see Fig.2.1A annotations):

$$\Delta \gamma_{lv} \approx \frac{\Delta \ln C_B}{\Delta \ln C_B^{\text{max}}} \ln C_B = \frac{\left(\gamma_{lv}^* - \gamma_{lv}^o\right)}{\ln C_B^{\text{max}} - \ln C_B^*} \quad \text{Eq. 4}$$

$$\gamma_{lv}^* = \gamma_{lv}^o + RTT \left[27.6 + \ln \left(\varepsilon/P\right) - \ln C_B^* - \ln MW\right] \quad \text{Eq. 4a}$$
where $C_B^*$ is the concentration required to achieve any arbitrary $\gamma^*_{lv}$ within the surface-excess region. Eq. 4a follows directly from Eq. 4 by insertion of Eq. 3 and is a Traube-like rule for globular proteins where the homology is in protein size rather than methylene units in linear surfactants. This size homology results directly from the proportional increase in protein volume with MW.20

**Protein Partition Constant:** A central assumption of this work is that the partition coefficient $P$ is approximately constant for all proteins. The Guggenheim interphase construction37,38 can be used to explore this notion more explicitly which, for a two-component solution (surface-active solute ‘2’ and solvent ‘1’), the concentration dependence of liquid-vapor interfacial tension is given by Eq. 5:

$$\frac{\delta \gamma_{lv}}{\delta \ln x_2} = \frac{-RT}{A} \left[ n_1^I - \left( \frac{x_2}{x_1} \right) n_1^I \right] \approx \frac{-RT}{A} \left[ n_2^I - \left( \frac{n_1^I}{n_1} \right) n_2^I \right] \quad \text{.................Eq. 5}$$

where the superscript “I” differentiates the interphase region from the bulk phase (with no superscript), $n$ is number of moles of 1 or 2, and $x$ is mole fraction. The approximation applies to dilute-solute solutions where $x_2 = n_2/(n_1 + n_2) \sim n_2/n_1$ and $x_1 \sim 1$. Eq. 5 can be written in terms of solvent and solute partition coefficients $P_1 = C_{l1}/C_{B1} = (n_1^I/n_1)(V/V_I)$ and $P_2 = C_{l2}/C_{B2} = (n_2^I/n_2)(V/V_I)$, respectively:

$$\frac{\delta \gamma_{lv}}{\delta \ln x_2} = \frac{-RTC_{B2} V_I}{A} [P_2 - P_1] = \frac{-RTC_{B2} V_I}{A} [P_2 - P_1] \approx \frac{-RTC_{B2} V_I}{A} [P_2 - P_1] \quad \text{........Eq. 6}$$
where $V_I$ is the interphase volume used as above, $V$ is the bulk-solution volume, and the identity $V_I = A \Omega$ has been applied. The approximation is valid for dilute-solute solution for which change in solvent concentration within the interphase due to adsorption is negligible. Eq. 6 is valid for any two proteins ‘$i$’ and ‘$j$’ that might have quite different MW ($MW_j \neq MW_i$) but for which we observe experimentally to exhibit similar $\delta \gamma_{lv}/\delta \ln x_2$ (i.e. the apparent Gibbs’surface excess is approximately constant for all proteins). Simultaneous solution of these two equations using ‘$i$’ and ‘$j$’ in place of the generic ‘2’ solute designation of Eq. 6 for the particular situation $C_{B,2} = C_B^{\text{max}}$ allows solution for the partition coefficients $P_i$ and $P_j$ in terms of interphase thickness and bulk concentrations:

$$\frac{[P_j - 1]}{[P_i - 1]} = \left( \frac{\Omega_j^{\text{max}}}{\Omega_i^{\text{max}}} \right) \left( \frac{C_{B,j}^{\text{max}}}{C_{B,j}^{\text{max}}} \right) = \left( \frac{\Gamma / C_{i,j}^{\text{max}}}{\Gamma / C_{i,j}^{\text{max}}} \right) \left( \frac{C_{B,j}^{\text{max}}}{C_{B,j}^{\text{max}}} \right) = \frac{P_i}{P_j} \quad \text{…………………Eq. 7}$$

where Eq. 7 makes use of the approximation $C_i = n_i/V_I \approx \Gamma/\Omega$ that is commensurate with $P >> 1$. Thus Eq. 7 leads to the conclusion that $P_j (P_j - 1) = P_i (P_i - 1)$, a condition that can be met only if $P_i = P_j$. Evidently then, similar $d\gamma_{lv}/d\ln C_B$ means that the partition coefficient $P \equiv C_i/C_B$ is approximately constant among proteins spanning nearly three decades in MW and does not significantly vary with molecular size.

**Surface Excess:** An issue that arises in the quantitative interpretation of concentration-dependent LV interfacial tensions is that activity coefficients, even of simple hydrocarbon solutes, are not typically unitary as is frequently assumed in application of Gibbs’ adsorption isotherm, and deviations from ideality have significant impact on computed values of Gibbs’
Proteins are complex polyelectrolyte solutes that are not ideal for which assumption of unit activity coefficients is no doubt quantitatively in error and apparently leads to considerable difference between real ($\Gamma_{lv} = -1/RT \frac{d\gamma_{lv}}{d\ln a}$ where $a$ is solute activity) and apparent ($\Gamma = -1/RT \frac{d\gamma_{lv}}{d\ln x_2}$, not corrected for solute activity) surface excess values. However, in this particular work, it is experimentally observed that $\Gamma$ is approximately constant for proteins with widely varying MW, strongly suggesting that any activity-related discrepancy between $\Gamma$ and $\Gamma_{lv}$ is roughly constant among these proteins. The ratio $\Gamma/\Gamma_{lv}$ measures this discrepancy and can be written in terms of an activity coefficient $\sigma \equiv a/x_2$ by noting that $\Gamma_{lv} = -a/RT (d\gamma_{lv}/da)$ and $\Gamma = -x_2/RT (d\gamma_{lv}/dx_2)$:

$$\frac{\Gamma}{\Gamma_{lv}} = \left(\frac{xd\gamma_{lv}}{dx_2}\right)\left(\frac{da}{d\gamma_{lv}}\right) = \frac{x_2}{a} \frac{d\gamma_{lv}}{dx_2} = 1/\sigma (d\sigma/dx_2) \approx c \quad \text{Eq. 8}$$

Thus, $\Gamma = c\Gamma_{lv}$ under the circumstance that the (generally unknown) activity coefficient $\sigma$ is not a strongly non-linear function of protein concentration.

**Interphase Thickness:** The maximal interphase thickness $\Omega_{\text{max}}$ that occurs when the interphase is saturated with adsorbate can be computed from Eq. 1 in combination with Eq. 2, assuming that $\Gamma = c\Gamma_{lv}$:

$$\Omega_{\text{max}} = c\Gamma_{lv} / C_{\text{max}} = \frac{\Gamma MW}{9.68 \times 10^8 \epsilon} \quad \text{Eq. 9}$$

where units of picomoles and cm have been used. Eq. 9 states that $\Omega_{\text{max}}$ increases in direct proportion to protein MW (i.e. proportional to protein volume), with a relatively thin interphase for low-MW (smaller) proteins and a thicker interphase for high-MW proteins. $\Omega_{\text{max}}$ may be
comprised of multiple layers as dictated by the partition coefficient $P$ and layer packing calculated from $N_{\text{max}} = \Omega_{\text{max}}/\delta = \Omega_{\text{max}}/2 r_v \chi$.

2.4. Results and Discussion

This portion of the paper is divided into two main parts. The first reports experimental observation of time-and-concentration-dependent liquid-vapor (LV) interfacial tension $\gamma_{\text{lv}}$ of aqueous-buffer solutions of purified human proteins with molecular weight (MW) spanning nearly three-orders-of-magnitude. The second part interprets these results according to the sphere-packing model in the general order disclosed in the preceding Theory section. Taken together, experiment and theory support the contention that water imposes uniformity in protein adsorption to the LV interface, packing molecules into an interphase region that thickens with increasing protein size (MW) at a constant partition coefficient.

2.4.1. Experimental Results

Concentration-Dependent LV Interfacial Tension: The principal experimental observations of this work were time-and-concentration-dependent liquid-vapor (LV) interfacial tension $\gamma_{\text{lv}}$ of aqueous-buffer solutions of purified human proteins with molecular weight (MW) spanning three-orders-of-magnitude. Interfacial tension measurements were made using pendant-drop tensiometry as described in the Methods and Materials section. Fig. 1 compares selected results for the anionic surfactant aerosol-OT (Fig. 1A, AOT, MW = 444 Da), human serum albumin (Fig. 1B, fatty-acid-free FAF HSA, MW = 66.3 kDa), and immunoglobulin M (Fig. 1C, IgM,
1000 kDa) in both three-dimensional (3D, \( \gamma \) as a function of time and concentration) and two-dimensional (2D, \( \gamma \) as a function concentration at specified times) representations, termed ‘\( \gamma \) curves’ herein. Note that the logarithmic-solute-concentration ordinate \( \ln C_B \) in Fig. 1 is expressed in picomolarity units (pM, \( 10^{-12} \) moles solute/L solution). It was observed that these \( \gamma \) curves were generally sigmoidal in shape, with well-defined low-concentration limits \( \gamma_o \) and a high-concentration asymptotes \( \gamma' \). Smooth curves through the data of Fig. 1 result from least-squares fitting of a four-parameter logistic equation \( \gamma = \{[(\gamma_o - \gamma')/(1+(\ln C_B / \Pi/2)^M]+ \gamma') \} \) to concentration-dependent \( \gamma \) data for each time within the observation interval, as described elsewhere.\(^1\),\(^3\),\(^5\) Empirical application of a logistic equation was a purely pragmatic approach aimed at quantifying variable parameters with a measure of statistical confidence. In this way, data fitting recovered \( \gamma_o \), \( \gamma' \), and a parameter measuring concentration-at-half-maximal-change in interfacial activity, \( \ln C_B^{\Pi/2} \) (where \( \Pi/2 = \Pi^{\max}/2 \) and \( \Pi^{\max} = \gamma_o - \gamma' \)), as well as a parameter \( M \) that measured steepness of the sigmoidal curve. Results for HSA and IgM (Fig. 1B and 1C, respectively) were similar to AOT in that sigmoidal-shaped \( \gamma \) curves connected low- and high-concentration asymptotes. Significantly more pronounced time dependence in \( \gamma \) was observed for proteins, however, especially for intermediate concentrations.

Dynamics were undoubtedly due to rate-limiting, mass-transfer and adsorption steps that slowly brought large macromolecules to the LV interface relative to the small-molecule reference compound AOT for which only limited kinetics were observed. Observation of time-dependence was important in this particular work only in so far as data demonstrate that \( \gamma \) kinetics dampen within the time frame of experimentation and achieved steady state within the 3600 sec observation window. Data collected in Table 2 refers only to steady-state measurements. The
bulk-solution concentration at which the limiting interfacial tension $\gamma'_\ell$ occurs ($C_B^{\text{max}}$) is of theoretical interest in this work and was estimated from fitted parameters compiled in Table 2 as described in the Appendix 6.1. $C_B^{\text{max}}$ is typically interpreted as the critical micelle concentration (CMC), at least for surfactants. This paper provides no evidence of micelles, for either proteins or surfactants, and so only acknowledges a bulk-phase concentration at which further increase in solute concentration did not significantly change $\gamma'_\ell$.

Fig. 2 compiles smoothed, steady-state $\gamma'_\ell$ curves for proteins selected from Table 2 spanning three decades in MW (data not shown in Fig. 2 for the sake of clarity; see Fig. 1 for examples with authentic data and Table 2 for statistics-of-fit). The Traube-rule-like progression in MW evident in the molar scaling of Fig. 2 was not at all obvious on a w/v basis (not shown) because data is compressed into a single band.\(^1\) However scaled, similarity of $\gamma'_\ell$ curves for such a diverse group of proteins is rather striking ($\Pi^{\text{max}} < 30$ dyne/cm with $\gamma'_\ell$ lying within $\pm 10$ dyne/cm) and corroborates results of previous studies of proteins covering a narrower range of MW.\(^1\)\(^9\) Bearing in mind that the $\Pi^{\text{max}}$ range for synthetic surfactants can exceed 60 dyne/cm, with $25 < \Pi^{\text{max}} < 50$ dyne/cm typical of ordinary hydrocarbon soaps and surfactants,\(^4\)\(^2\)\(^4\)\(^3\) it is apparent from data of Table 2 and Figs. 1, 2 that aqueous-protein solutions are relatively weak surfactant systems with $\Pi^{\text{max}}$ only slightly overlapping with the weakest of surfactant systems. In view of the substantial structural diversity of the human plasma proteome sampled by proteins of Table 1,\(^3\)\(^1\) it seems reasonable to conclude that variability in protein structure does not confer widely-varying LV interfacial activity; at least not in comparison to the full range available to ordinary surfactants.
Apparent Gibbs’ Surface Excess: The apparent Gibbs’ surface excess $\Gamma$ with error estimates collected in Table 2 were calculated from the slope of the linear-like region of the concentration-dependent $\gamma$ curve (i.e. the surface excess region between $\gamma^o$ and $\gamma'$ centered at $\ln C^B$; see Fig. 1) corresponding to steady-state, as described previously. The term “apparent” alerts the reader to the facts that casual application of Gibbs’ adsorption isotherm $[\Gamma = (-1/RT)d\gamma/d\ln C_B]$ treats solutes (proteins and surfactants) as isomerically-pure, non-ionized polyelectrolytes at infinite dilution with unit activity coefficients. In particular, this treatment does not explicitly take into account counter ions which, for a 1:1 ionic surfactant such as AOT dissolved in pure water, means that the actual surface excess $\Gamma_v = 1/2 \Gamma$. Thus, $\Gamma = 460 \pm 31$ picomole/cm$^2$ derived from data of Fig. 1A was found to be in good agreement with drop-volume tensiometry ($\Gamma_v = 221$ picomole/cm$^2$) and predicts an adsorbed footprint $1/\Gamma_v = 70$ Å$^2$/molecule in reasonable agreement with neutron reflectometry (NR) measurements. No counter-ion correction of apparent surface excess $\Gamma$ was necessary for polyelectrolytes dissolved in swamping concentrations of buffer salts, however, and values listed in Table 2 were taken to be proportional to the actual Gibbs’ surface excess $\Gamma_v$, as discussed in the Theory section; bearing in mind, of course, the aforementioned assumptions of purity and applicability of the infinite-dilution approximation.

Fig. 3 plots apparent $\Gamma$ values collected in Table 2 against protein MW, with error bars representing intra-experiment uncertainty calculated by propagation of fitted-parameter errors, as described previously. Inter-experiment error suggested by replicate protein preparations (sometimes different lots obtained from different vendors, see Table 1) appeared not to be significantly larger than intra-experiment uncertainty. Taken as a whole, this data supports the
contention that concentration-dependence of interfacial tension $\gamma_{lv}/\ln C_B$ was relatively constant among proteins with MW spanning nearly three-orders-of-magnitude (10–1000 kDa).

Again in view of the substantial structural diversity of the human plasma proteome sampled by proteins of Table 1, it seems reasonable to conclude that variability in protein structure does not confer widely-varying LV interfacial activity.

### 2.4.2. Theoretical Interpretation of Results

Secure interpretation of concentration-dependent interfacial tensions in terms of adsorbed concentrations and interphase thicknesses is critically dependent on availability of solvent and solute activities for protein solutions of particular interest to this work, as well as packing densities within a 3D interphase. Specific information of this kind is, for the most part, unavailable and much of the existing protein-adsorption literature is quite controversial or internally inconsistent (see ref. 3 and citations therein). Interpretive problems are exacerbated by the fact that only a narrow range of protein MW has been heretofore explored; concentrating work on relatively low-MW proteins such as albumin, lysozyme, and casein; thus comparing proteins derived from different tissues (e.g. blood, ocular, mammary) and sometimes from different species (e.g. bovine v. human). On occasion, chemically-treated proteins are used as surrogates for natural forms, such as in the delipidization of fraction (FV) albumin to fatty-acid-free (FAF) serum albumin. This work is exceptional only in that it has specifically focused on purified human globular proteins with a broad range of molecular weights. Even so, a standard of reference is required to (partially) compensate for unknown protein activity. Thus, we have chosen to ‘calibrate’ the model outlined in the Theory section to the neutron reflectometry (NR)
of FAF HSA at the LV interface reported by Lu et al.\textsuperscript{48}, recognizing that this single-point calibration to delipidated protein may bias the outcome but with the expectation that any such bias will be systematic and that general trends revealed will not be seriously compromised.

**Protein Packing within the LV Interphase:** NR resolved a single molecular layer of FAF HSA at the LV interface at saturating surface coverage, residing within a 4.8 nm thick layer.\textsuperscript{48} This finding is somewhat consistent with a protein-sphere radius $r_v = 2.7$ nm calculated from molecular-packing density calculations discussed in the Theory section or, as described in the cited NR literature, a 4X14 nm ellipsoidal molecule adsorbed with the major axis oriented parallel to the interface. The adsorbate mass/unit area deduced from NR was $2.1 \pm 0.3$ mg/m$^2$, or 3.2 picomole/cm$^2$ and construed to be equivalent to Gibbs’ surface excess $\Gamma_{lv}$.\textsuperscript{49} Interpreted in terms of Eq. 9 of the sphere-packing model, this implies that $\Omega_{lv}^{max} = 4.8 \times 10^{-7}$ cm and that $C_l^{max} = \Gamma_{lv}/\Omega_{lv}^{max} = 6.7 \times 10^6$ picomole/cm$^3$ (6.7 X10$^9$ pM or ln$C_l^{max} = 22.6$ for comparison with scaling of Figs. 1, 2, and 5). With $C_{FCC} = 1.5 \times 10^{10}$ pM for HSA (ln$C_{FCC} = 23.4$), this implies that $\varepsilon \sim 0.45$, or that the saturation interfacial concentration is approximately half of the hypothetical face-centered cubic arrangement of hard spheres with radius $r_v$, or $\Phi_p^{max} = 0.74\varepsilon \sim 1/3$. The effective-packing radius $R$ is thus 30% larger than $r_v$ with $\chi = 1.3$ (as calculated from $\delta = 2R = 2r_v\chi = 2r_v\varepsilon^{-1/3} = 7.1$ nm), which is consistent with a 3.6 nm hydrodynamic radius obtained by dynamic light scattering of albumin solutions.\textsuperscript{50} This inferred value of $\varepsilon$ might also be compared to the so-called ‘jamming limit’ of 0.55 at which adsorbing disks saturate a surface without overlap,\textsuperscript{51} and to reports of adsorbed-protein densities exceeding this limit,\textsuperscript{30,52} so long as it is borne in mind that these latter benchmarks are specific to molecular packing within a single layer (2D) and not in multiple layers (3D). It is of further interest that $C_l^{max}$ exceeds the estimated
solubility limit for HSA by a factor of ~9 ($C_{SL} = 7.5 \times 10^8$ pM, $\ln C_{SL} = 20.4$ pM or about 50 g/L), corroborating the conclusion drawn from diverse literature sources that adsorbed concentrations can be surprisingly large and that a proteinaceous interphase must indeed be a very viscous region.\footnote{3} We estimate from steady-state concentration-dependent $\gamma_l$ for FAF HSA that $\ln C_B^{\text{max}} = 14.2 \pm 0.2 (1.5 \times 10^6$ pM; see Fig. 1B and Table 2) suggesting that $P = \left( C_l^{\text{max}} / C_B^{\text{max}} \right) \approx 5 \times 10^3$, which is in reasonable agreement with early ellipsometric measurements of bovine albumin,\footnote{53} as well as other related studies reviewed in ref. 3. Furthermore, this single-point calculation of $P$ is consistent with the extrapolated value derived from the Traube-like-rule for proteins (see below), suggesting that the partition coefficient is approximately constant across the proteins of this study as deduced from the slight variation in $d\gamma_l/d\ln C_B$.

\textbf{A Traube-Like Rule for Protein Adsorption and Partition Constant:} Fig. 4 plots $C_B^{\text{max}}$ data compiled in Table 2 on logarithmic coordinates compatible with Eq. 3 of the Theory section (data corresponding to ubiquitin was estimated as described in the Appendix 6.2). Proteins fall within a monotonically-decreasing band consistent with the anticipation of a unit slope and positive intercept $[\ln C_B^{\text{max}} = (-1.4 \pm 2.8) \ln MW + (21.8 \pm 15.6); R^2 = 71.6\%]$. Interpretation of these results must take into account that the highly-simplified model of adsorption treats proteins as uniform hard spheres and does not attempt to account for structural complexities of real molecules, variations introduced by delipidization (as in the case of FAF HSA), or unfolding (denaturation) that may occur upon concentration within the interphase. Hence failure of data to quantitatively adhere to Eq. 3 is hardly surprising. Nevertheless, it is of interest to estimate $\varepsilon/P \sim 3 \times 10^{-3}$ from the nominal intercept value (bearing in mind the large error) and, by assuming $\varepsilon \sim 0.45$ from the preceding section, estimate $P \sim 1.5 \times 10^2$; which is within an order-of-magnitude of
the single-point estimate based on NR discussed above. According to Eq. 3 and Fig. 4, low-MW proteins require greater bulk-phase concentrations to saturate the interphase than higher-MW proteins. Given that $C_B^{max}$ values plotted in Fig. 4 approach 10% w/v, it is reasonable to anticipate that extrapolated $C_B^{max}$ values for yet-lower-MW proteins must equal or exceed protein-solubility limits. As a consequence, surface saturation and the related limiting interfacial tension $\gamma'_{lv}$ is not expected for low-MW proteins at fixed $P$. In this regard, it is noteworthy that $\gamma'_{lv}$ curves for low-MW proteins such as ubiquitin (10.7 kDa) fail to achieve a limiting interfacial tension at any concentration below the solubility limit\(^{15}\) (see Fig. 4 and further below).

Fig. 5 is a summary graphic showing relationships among steady-state $\gamma_{lv}$, MW, and concentration of aqueous-buffer solutions of globular proteins. Data points of Fig. 5 correspond to proteins selected from Table 1 whereas the mesh was calculated from Eq. 4a of the Theory section using the fitted value of $\varepsilon/P$ obtained as discussed above and as further detailed in Appendix 6.3. The Traube-rule effect is especially evident as viewed along the ln$C_B$ axis where increasing concentration of lower-MW proteins are required to reach the limiting interfacial tension $\gamma'_{lv}$. It is noteworthy that $\gamma'_{lv}$ is not achieved for low-MW proteins such as ubiquitin, as discussed above.

**Surface Excess and Interphase Thickness:** Again using NR of HSA as a single-point calibration by taking $\Gamma_{hv} = 3.2$ picomole/cm$^2$, the factor relating actual and apparent surface excess $c = \Gamma/\Gamma_{hv} = 179/3.2 = 56$. Eq. 9 can be used to calculate maximum interphase thickness and, from $r_v$, the number of layers required to contain different size proteins. As shown in Fig. 2.6, interphase thickness increases linearly with MW. The number of layers occupied by protein
has been calculated as increasing in steps, although it seems more likely that transitions are not so discrete; more probably partially filling an additional layer before the previous is well packed. However these layers actually populate, Eq. 9 predicts that, at fixed partition coefficient $P$, low-MW proteins such as albumin (66 kDa) fall within one layer whereas large proteins such as IgM (1000 kDa) occupy five layers. Thus, the constraint that $P$ is approximately constant for all proteins imposes the requirement that proteins with MW > 125 kDa populate more than a single layer at surface saturation.

2.5. Conclusions

The two principal experimental observations of this work were that (i) reduction in liquid-vapor (LV) interfacial tension $\gamma_{lv}$ of aqueous-buffer solutions of purified human proteins followed a regular progression in MW with the molar concentration required to reach a specified value decreasing with increasing MW and (ii) the rate-of-change in $\gamma_{lv}$ with protein concentration $d\gamma_{lv}/d\ln C_B$ was relatively constant among proteins with MW spanning nearly three-orders-of-magnitude (10–1000 kDa). The former observation was interpreted in terms of a protein-adsorption model predicated on the packing of spherically-shaped molecules with dimensions that scale with MW. The latter was rationalized as an outcome of a constant partition coefficient that entrained a fixed fraction of bulk-solution molecules within a three-dimensional interphase that thickens with increasing proteins size (MW). When calibrated to previously-reported neutron-reflectometry (NR) of albumin adsorption to the LV interface, the model permitted calculation of interphase thickness and number of molecular layers residing within this interphase. Interphase thickness was predicted to increase linearly with MW, requiring up to five
layers for large proteins (MW ~ 1000 kDa) but only a single layer for small proteins (MW < 125 kDa).

This study strongly suggests that water orchestrates a systematic pattern in protein adsorption to the LV interface that has not been evident from similar previous studies using proteins covering a narrower MW range. Realization of this pattern has followed a somewhat similar historical pathway to understanding the Traube rule, requiring routine access to purified compounds with known MW spanning a sufficiently large range that homology could be observed. In the protein case, the homology is *molecular size*, rather than chemical composition as in the Traube rule, implying that the structural variability that confers vastly different bioactivity to proteins does not greatly affect interaction energetics with water. It is these interactions that lead to the expulsion of protein from solution\textsuperscript{14} to the hydrophobic LV interface, simultaneously reducing interfacial energetics and solution concentration of a relatively hydrophobic solute\textsuperscript{11} for which water is an ambivalent solvent.\textsuperscript{54,55} *Amphilicity* is the word coined by Hartley\textsuperscript{56} in 1936 to express this ambivalent solvency from the perspective of the solute molecules (originally amphipathy). Apparently in the case of proteins, molar variability in $\gamma_{lv}$ is achieved by aggregating greater mass of similar amphiphilic character (blocks of amino acids), as opposed to accumulating greater amphilicity with MW. The extent to which this amphilicity leads to protein accumulation at the LV interface is limited by the extent to which this surface can be dehydrated, interpreted herein as protein volume fraction $\Phi^\text{max}_p \sim 1/3$. The significance of these conclusions to biomaterials science is that combinations and permutations of the 20 naturally-occurring amino acids comprising the primary sequence of mammalian proteins seems insufficient to support widely-varying LV interfacial activity, no matter how these sequences happen to fold
into higher-order structure. As a consequence, assertion of differential protein adsorption to biomaterial surfaces from heterogeneous biological mixtures (such as blood) based on molecular structure alone requires careful justification in terms of interfacial energetics,\textsuperscript{15} bearing in mind that hydrophobic hydration is a, perhaps the, key phenomenon controlling adsorption.

2.6. Appendix

2.6.1. Estimation of $C_B^{\text{max}}$

$C_B^{\text{max}}$ was calculated from fitted data by evaluating Eq. 4a of the Theory section at half-maximal change in interfacial tension $\gamma^{\frac{\Pi}{2}} = \frac{1}{2}(\gamma'_lv - \gamma''lv)$, which occurs at a bulk-phase composition $\ln C_B^{\frac{\Pi}{2}}$:

\[
\Delta \ln C_B = -RT \gamma'_{lv} = \frac{\left(\gamma'_lv - \gamma''lv\right)}{\ln C_B^{\text{max}} - \ln C_B^{\frac{\Pi}{2}}} = \frac{\left(\gamma'_lv - \gamma''lv\right)}{\ln C_B^{\text{max}} - \ln C_B^{\frac{\Pi}{2}}} \Rightarrow \ln C_B^{\text{max}} = \frac{\Pi^{\text{max}}}{2RT} + \ln C_B^{\frac{\Pi}{2}}
\]

where the identity $\Pi^{\text{max}} = \gamma'_lv - \gamma''lv$ has been used. All of the parameters in the RHS of Eq. A1 are derived from non-linear, least-squares fitting of concentration-dependent $\gamma_{lv}$ to a four-parameter logistic equation as described in the Methods and Materials section. Confidence in $C_B^{\text{max}}$ values listed in Table 2 and plotted in Fig. 4 was computed by propagation of the standard errors in best-fit parameters through Eq. A1 as given by Eq. A2. In consideration of all sources of experimental error, we conclude that $\ln C_B^{\text{max}}$ estimates are no better than about 20%.

\[
\sigma_{C_B}^2 = \sigma_{C_B}^2 + \frac{1}{4(RT \Gamma)^2} \left[ \sigma_{\gamma_{lv}}^2 - \sigma_{\gamma''lv}^2 - \left(\frac{\gamma''_{lv} - \gamma'_lv}{\Gamma^2}\right)^2 \sigma_{\Gamma}^2 \right]
\]

\text{...............Eq. A2}
where $\sigma$’s represent standard errors in $\ln C_B^{\text{max}}$ and the best-fit parameters $\ln C_B^{P2}$, $\gamma_{lv}^o$, $\gamma_{lv}'$ and $\Gamma$.

### 2.6.2. Estimation of parameters for Ubiquitin

Parameters for ubiquitin listed in Table 1 and shown in Figs. 2-5 are graphical estimates from the steady-state, concentration-dependent $\gamma_{lv}$ curve. Firm values could not be ascertained by statistical-fitting procedures described in Methods and Materials because surface saturation is not reached within solubility limits for this protein. Thus, a well-defined high concentration asymptote, $\gamma_{lv}'$ is not achieved at physically-realizable concentrations. However, the highest-concentration $\gamma_{lv} \sim 46$ dynes/cm is within the $\Pi^{\text{max}}$ of all other proteins studied. Given that $\Pi^{\text{max}}$ is conserved among the broad range of proteins, it seems reasonable to conclude that $\gamma_{lv} = 46$ dynes/cm is not too far from the hypothetical $\gamma_{lv}'$ that would be achieved if Ub was sufficiently soluble to fill the interphase. Thus, we estimate that $\Pi^{\text{max}}/2 \sim 13$ dynes/cm would occur at $\ln C_B^{P2} = 15$ pM. The low-concentration asymptote is clearly defined at $\gamma_{lv}^o = 72$ dynes/cm, consistent with the interfacial tension of saline near 25°C. An estimate of the apparent surface excess $\Gamma$ was made from the experimental data by application of the Gibbs’ adsorption isotherm. With these estimates in hand, $C_B^{\text{max}}$ was calculated as described in Appendix 6.1.

### 2.6.3. Steady-state $\gamma_{lv}$ as a function of MW and concentration

The smooth mesh through the summary graphic Fig. 5 was computed from Eq. 4a of the Theory section assuming that the nominal values $\gamma_{lv}^o = 72$, $\gamma_{lv}' = 45$ and $\Gamma = 179$ approximates all proteins of this study. Eq. 4a permits calculation on any interfacial tension within the linear-like Gibbs’ excess region (i.e. the surface excess region between $\gamma_{lv}^o$ and $\gamma_{lv}'$, centered at $\ln C_B^{P2}$; see Figs.1A, 3), above and below which calculated $\gamma_{lv}$ are truncated to $\gamma_{lv}^o$ and $\gamma_{lv}'$, respectively.
Citations


(9) Macallum, A. B. *Surface Tension and Vital Phenomena*; The University Library: Toronto, 1912.


(22) Schaff, P.; Dejardin, P. Coupling Between Interfacial Protein Adsorption and Bulk Diffusion. A Numerical Study, *Colloids and Surfaces* 1987, 24, 239.


(46) Drop-volume measurements are assumed to closely correlate with early pendant drop measurements taken near t=0 because, in a drop-volume experiment, the LV interface is continuously forming until the drop separates from the tip, rather than achieving the steady-state adsorption condition accessible to an aged pendant drop ($I = 337.3\pm28.2$ picomoles/cm² at steady-state taken near 1 hour drop age).


(49) Note that surface excess of the small molecule reference compound AOT (MW = 444 Da) reported herein is much larger than that of proteins (1000 < MW < 10 kDa), presumably because of the great disparity in molecular size.


(56) Hartley, G. S. *Actualites Scientifiques et Industrielles* 1936, 387, 4.
Table I: Purified Proteins

<table>
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<th>NAME OF PROTEIN (acronym)</th>
<th>MOLECULAR WEIGHT (kDa)</th>
<th>AS-RECEIVED FORM (mg/ml)</th>
<th>PURITY (electrophoresis) or ACTIVITY</th>
<th>VENDOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBIQUITIN (Ub)</td>
<td>10.7</td>
<td>Powder</td>
<td>98%</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>THROMBIN (FIIa)</td>
<td>35.6</td>
<td>Powder</td>
<td>1325 NIH unit/mg</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td><strong>HUMAN SERUM ALBUMIN</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fraction V (FV HSA)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Prep 1</td>
<td>66.3</td>
<td>Powder</td>
<td>96-99%</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Prep 2</td>
<td>66.3</td>
<td>Powder</td>
<td>96-99%</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Fatty Acid Free (FAF HSA)</td>
<td>66.3</td>
<td>Powder</td>
<td>96-99%</td>
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<td>PROTHROMBIN (FII)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prep 1</td>
<td>72</td>
<td>Powder</td>
<td>7.4 units/mg protein</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Prep 2</td>
<td>72</td>
<td>Powder</td>
<td>95%</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td><strong>HUMAN IgG (IgG)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Prep 1</td>
<td>160</td>
<td>Powder</td>
<td>95%</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Prep 2</td>
<td>160</td>
<td>Powder</td>
<td>95%</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Prep 3</td>
<td>160</td>
<td>Powder</td>
<td>98%</td>
<td>ICN Biomedicals</td>
</tr>
<tr>
<td>COMPLEMENT COMPONENT C1q (C1q)</td>
<td>400</td>
<td>Powder</td>
<td>Single band by immuno-electrophoresis</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>α2-MACROGLOBULIN (αmac)</td>
<td>725</td>
<td>Powder</td>
<td>90%</td>
<td>Sigma Aldrich</td>
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<td><strong>HUMAN IgM (IgM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prep 1</td>
<td>1000</td>
<td>Solution (0.8)</td>
<td>98%</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Prep 2</td>
<td>1000</td>
<td>Solution (0.8)</td>
<td>98%</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Prep 3</td>
<td>1000</td>
<td>Solution (2.1)</td>
<td>96%</td>
<td>ICN Biomedicals</td>
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## TABLE II: STEADY-STATE PROTEIN PARAMETERS

<table>
<thead>
<tr>
<th>NAME OF PROTEIN (acronym)</th>
<th>$\gamma_0^o$ (dynes/cm)</th>
<th>$\gamma'_o$ (dynes/cm)</th>
<th>$\ln C_B$ (pM)</th>
<th>$M$ (dimensionless)</th>
<th>$I_{\textrm{max}}^o$ (dynes/cm)</th>
<th>Apparent $I^\dagger$ (picomoles/cm$^2$)</th>
<th>$\ln C_B^{\max}$ (pM)</th>
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<tbody>
<tr>
<td>UBIQUITIN (Ub)*</td>
<td>72</td>
<td>46</td>
<td>15</td>
<td>-</td>
<td>26</td>
<td>146</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>THROMBIN (FIIa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17.41±0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72.23±0.31</td>
<td>47.6±1.5</td>
<td>14.44±0.26</td>
<td>-11.00±1.3</td>
<td>24.1±1.5</td>
<td>167 ±18</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>17.41±0.18</td>
</tr>
<tr>
<td>HUMAN SERUM ALBUMIN</td>
<td>Fraction V (FV HSA)</td>
<td>72.3±1.2</td>
<td>50.3±1.2</td>
<td>12.14±0.28</td>
<td>-9.9±2.5</td>
<td>21.4±1.2</td>
<td>160±21</td>
</tr>
<tr>
<td></td>
<td>Prep 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14.92±0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70.8±1.1</td>
<td>46.2±2.5</td>
<td>12.44±0.53</td>
<td>-7.3±2.2</td>
<td>25.5±2.5</td>
<td>129±25</td>
</tr>
<tr>
<td></td>
<td>Prep 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16.26±0.39</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>HUMAN IgG (IgG)</td>
<td>Prep 1</td>
<td>70.50±0.73</td>
<td>43.1±2.4</td>
<td>12.72±0.35</td>
<td>-10.8±2.9</td>
<td>28.6±2.4</td>
<td>207±33</td>
</tr>
<tr>
<td></td>
<td>Prep 2</td>
<td>70.51±0.73</td>
<td>43.0±2.4</td>
<td>12.72±0.34</td>
<td>-10.8±2.8</td>
<td>28.6±2.4</td>
<td>207±32</td>
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<td></td>
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<td></td>
<td></td>
<td>15.40±0.26</td>
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<tr>
<td>HUMAN IgM (IgM)</td>
<td>Prep 1</td>
<td>70.48±0.57</td>
<td>48.7±3.2</td>
<td>13.04±0.69</td>
<td>-8.6±2.1</td>
<td>23.1±3.2</td>
<td>130±34</td>
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<tr>
<td></td>
<td>Prep 2</td>
<td>71.13±0.57</td>
<td>51.6±1.9</td>
<td>13.54±0.41</td>
<td>-10.4±2.7</td>
<td>20.1±1.9</td>
<td>134±24</td>
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<td></td>
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<td></td>
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<td></td>
<td>16.49±0.29</td>
</tr>
<tr>
<td></td>
<td>Prep 3</td>
<td>71.09±0.42</td>
<td>56.48±0.92</td>
<td>14.65±0.19</td>
<td>-20.1±5.1</td>
<td>15.21±0.92</td>
<td>177±21</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>16.26±0.17</td>
</tr>
<tr>
<td>COMPLEMENT COMPONENT C1q (C1q)</td>
<td>71.59±0.54</td>
<td>54.2±1.6</td>
<td>11.27±0.38</td>
<td>-14.2±4.1</td>
<td>17.5±1.6</td>
<td>194±33</td>
<td>13.08±0.34</td>
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<tr>
<td>$\alpha_2$-MACROGLOBULIN (α mac)</td>
<td>71.96±0.36</td>
<td>57.21±0.57</td>
<td>9.54±0.15</td>
<td>-19.4±5.7</td>
<td>14.47±0.57</td>
<td>266±21</td>
<td>10.65±0.15</td>
</tr>
<tr>
<td>HUMAN IgM (IgM)</td>
<td>Prep 1</td>
<td>70.98±0.39</td>
<td>51.4±1.2</td>
<td>9.92±0.19</td>
<td>-13.2±3.5</td>
<td>20.3±1.2</td>
<td>230±26</td>
</tr>
<tr>
<td></td>
<td>Prep 2</td>
<td>71.65±0.55</td>
<td>50.2±3.1</td>
<td>11.64±0.35</td>
<td>-14.2±4.2</td>
<td>21.5±3.1</td>
<td>232±59</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
<td>13.50±0.22</td>
</tr>
<tr>
<td></td>
<td>Prep 3</td>
<td>70.51±0.59</td>
<td>55.4±1.3</td>
<td>10.58±0.30</td>
<td>-11.7±3.3</td>
<td>16.3±1.3</td>
<td>149±25</td>
</tr>
</tbody>
</table>

Notes: * Ubiquitin(10.7 kDa) does not reach surface saturation within solubility limits; reported values are graphical estimates (see Appendix 6.2).
† Apparent $I^\dagger$ is proportional to Gibbs’ surface excess $I_n^o$ (see Theory section).
**Figure 1:** Interfacial tension profiles in 3D ($\gamma_{lv}$ as a function of analysis time (drop age) and logarithmic (natural) solution concentration $C_B$) and 2D ($\gamma_{lv}$ as a function of logarithmic solution concentration $C_B$ at selected times) formats comparing Aerosol-OT (AOT, panel A), human serum albumin (FAF HSA, panel B), and human immunoglobulin-M (IgM, panel C, preparation 3, Table 1). In each case, solute concentration $C_B$ is expressed in picomoles/L (pM). Symbols in 2D panels represent time slices through 3D representations (filled circle = 0.25 sec, open circle = 900 sec, filled triangles = 1800 sec, and open triangles = 3594 sec; annotations in panel A indicate maximum and half-maximum spreading pressure). Notice that adsorption kinetics dominate IgM adsorption, requiring 1 hour to reach steady state, whereas kinetics dampen within about 2000 sec for HSA. Dynamic effects dampen within 900 sec for AOT, the small-molecule reference surfactant.
**Figure 2:** Comparison of steady-state, concentration-dependent $\eta$ for proteins selected from Table 1 spanning three decades in MW (only statistically-fit curves shown for clarity; see Fig. 1 for similar plots including authentic data and Table 2 for statistics of fit). Molar scaling reveals a Traube-rule-like ordering in which it is observed that high-MW proteins reduce $\eta$ to any arbitrary value at lower molarity than low-MW proteins (arrow).
**Figure 3:** Apparent Gibbs’ surface excess $\Gamma$ as a function of protein MW calculated from concentration-dependent $\gamma_{lv}$ for multiple preparations of proteins as listed in Table 1 (Open circle = preparation 1, filled circle = preparation 2, Filled triangle = preparation 3) and inset expands low-MW region. Data point corresponding to ubiquitin (Ub, filled diamond) was estimated as described in the Appendix 6.2. Error bars represent uncertainty computed by propagation of experimental errors into compiled $\Gamma$ values (see Results section). Dashed line represents arithmetic mean of $\Gamma$ values listed in Table 2.
Apparent $\Gamma$ (picomoles/cm$^2$)

MW (kDa)

Ub  
FIIa  
FAF HSA  
FV HSA  
FII  
IgG  
αmac  
C1q  
IgM

$179 \pm 27$
Figure 4: Relationship between the surface-saturating bulk solution concentration $C_B^{\text{max}}$ and protein MW (Open circle = preparation 1, filled circle = preparation 2, filled triangle = preparation 3). Linear regression through the data yielded a slope of $-1.4 \pm 2.8$ consistent with the expectation of unit slope and an intercept $21.8 \pm 15.6$ ($R^2 = 71.6\%$). Notice that low-MW proteins require greater bulk-phase concentrations to saturate the interphase than higher-MW proteins.
Figure 5: Traube-rule-like dependence of concentration-dependent $\gamma_n$ for proteins selected from Table 1 spanning three decades in MW (reading from left to right on MW axis: Ub, FIIa, FAF HSA, FV HSA preparation 1, FII preparation 1, IgG preparation 2, $\alpha$ mac, C1q, IgM preparation 4). Data points are superimposed on a mesh calculated from Eq. 4a of the theory section (see Appendix 6.3 for details). The Traube-rule effect is especially evident as viewed along the $\ln C_B$ axis where monotonically-increasing concentrations of lower-MW proteins are required to reach a universal limiting interfacial tension $\gamma_n^\prime$. 
**Figure 6:** Monotonic increase in interphase thickness with protein molecular weight (left-hand axis). A fixed partition coefficient requires that higher-MW proteins (MW > 125 kDa) occupy multiple layers (right-hand axis), predicting up to five layers for proteins such as IgM (1000 kDa). Transition between layers is shown as occurring in discrete steps although it is more likely that occupation of a subsequent layer occurs before the preceding is completely packed.
### Glossary of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Protein activity</td>
</tr>
<tr>
<td>A</td>
<td>Area of interphase (cm²)</td>
</tr>
<tr>
<td>c</td>
<td>Proportionality constant, ( c \equiv \Gamma/\Gamma_{lv} )</td>
</tr>
<tr>
<td>( C_B )</td>
<td>Bulk solution concentration (moles/volume)</td>
</tr>
<tr>
<td>( C_B^{max} )</td>
<td>Bulk solution concentration at limiting interfacial tension (moles/volume)</td>
</tr>
<tr>
<td>( C_B^{DF2} )</td>
<td>Bulk solution concentration at half-maximal-change in interfacial activity (moles/volume)</td>
</tr>
<tr>
<td>( C_B^{*} )</td>
<td>Bulk solution concentration at arbitrary interfacial tension, ( \gamma^*_v ) (moles/volume)</td>
</tr>
<tr>
<td>( C_{FCC} )</td>
<td>Face-Centered-Cubic close packing concentration (moles/volume)</td>
</tr>
<tr>
<td>( C_I )</td>
<td>Interphase concentration (moles/volume)</td>
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<tr>
<td>( C_I^{max} )</td>
<td>Maximal interphase concentration (moles/volume)</td>
</tr>
<tr>
<td>( C_{SL} )</td>
<td>Solubility limit concentration (moles/volume)</td>
</tr>
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<td>( \chi )</td>
<td>Proportionality constant, ( \chi \equiv R/r_v )</td>
</tr>
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<td>( \delta )</td>
<td>Interphase thickness element (cm)</td>
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<td>( \varepsilon )</td>
<td>Packing efficiency</td>
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<td>( \Phi_p^{max} )</td>
<td>Volume fraction of protein in the interphase</td>
</tr>
<tr>
<td>( \Phi_p^{max} )</td>
<td>Maximum volume fraction of protein in the interphase</td>
</tr>
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<td>( \gamma_{lv} )</td>
<td>Liquid-vapor (LV) interfacial tension (dynes/cm)</td>
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<td>( \gamma'_{lv} )</td>
<td>Low-concentration asymptote of a concentration-dependent ( \gamma_v ) curve (dynes/cm)</td>
</tr>
<tr>
<td>( \gamma''_{lv} )</td>
<td>High-concentration asymptote of a concentration-dependent ( \gamma_v ) curve (dynes/cm)</td>
</tr>
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<td>( \gamma^*_v )</td>
<td>Arbitrary interfacial tension (dynes/cm)</td>
</tr>
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<td>( \Gamma_{lv} )</td>
<td>Actual Gibbs’ surface excess (moles/area)</td>
</tr>
<tr>
<td>( \Gamma )</td>
<td>Apparent Gibbs’ surface excess (moles/area), ( \Gamma = c\Gamma_{lv} )</td>
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<td>( \Gamma_{lv}^{o} )</td>
<td>Moles of water per-unit-area of interface when ( \Phi_w = 1 ) (moles/area)</td>
</tr>
<tr>
<td>M</td>
<td>Parameter fitted to concentration-dependent ( \gamma_v ) curve</td>
</tr>
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<td>( n_I )</td>
<td>Total protein moles within the interphase (moles), ( n_I = n_a + n_B )</td>
</tr>
<tr>
<td>( n_a )</td>
<td>Moles of adsorbate within the interphase (moles)</td>
</tr>
<tr>
<td>( n_B )</td>
<td>Moles of protein contributed by bulk phase (moles)</td>
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<td>Moles of component 1 in a 2-component solution</td>
</tr>
<tr>
<td>( n_2 )</td>
<td>Moles of component 2 in a 2-component solution</td>
</tr>
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<td>N</td>
<td>Number of slabs of interphase</td>
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<td>Avogadro number</td>
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<td>( P )</td>
<td>Partition coefficient, ( P \equiv C_I/C_B )</td>
</tr>
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<td>( \Pi )</td>
<td>Spreading pressure (dynes/cm), ( \Pi \equiv \gamma''<em>{lv} - \gamma'</em>{lv} )</td>
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<tr>
<td>( \Pi^{max} )</td>
<td>Maximum spreading pressure (dynes/cm)</td>
</tr>
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<td>( r_v )</td>
<td>Protein radius (cm)</td>
</tr>
<tr>
<td>( R )</td>
<td>Effective radius (cm), ( R \equiv \chi r_v )</td>
</tr>
<tr>
<td>R</td>
<td>Universal Gas Constant (ergs/°K mol)</td>
</tr>
<tr>
<td>( \sigma )</td>
<td>Activity coefficient</td>
</tr>
<tr>
<td>T</td>
<td>Temperature (°K)</td>
</tr>
<tr>
<td>( V_I )</td>
<td>Interphase volume (cm³)</td>
</tr>
<tr>
<td>( V_p )</td>
<td>Protein molar volume (cm³/mole)</td>
</tr>
<tr>
<td>( \Omega )</td>
<td>Total molar volume (cm³/mole)</td>
</tr>
<tr>
<td>x</td>
<td>Mole fraction</td>
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Chapter 3

Mixology of Protein Solutions and the Vroman Effect

Abstract

Mixing rules stipulating both concentration and distribution of proteins adsorbed to the liquid-vapor (LV) interphase from multi-component aqueous solutions are derived from a relatively straightforward protein-adsorption model. Accordingly, proteins compete for space within an interphase separating bulk-vapor and bulk-solution phases on a weight, not molar, concentration basis. This results in an equilibrium weight-fraction distribution within the interphase that is identical to bulk solution. However, the absolute interphase concentration of any particular protein adsorbing from an \( m \)-component solution is \( \frac{1}{m} \)th that adsorbed from a pure, single-component solution of that protein due to competition with \( m-1 \) constituents. Applied to adsorption from complex biological fluids such as blood plasma and serum, mixing rules suggest that there is no energetic reason to expect selective adsorption of any particular protein from the mixture. Thus, dilute members of the plasma proteome are overwhelmed at the hydrophobic LV surface by the thirty classical plasma proteins occupying the first-five decades of physiological concentration. Mixing rules rationalize the experimental observations that (i) concentration-dependent liquid-vapor interfacial tension \( \gamma_{lv} \) of blood plasma and serum (comprised of about 490 different proteins) cannot be confidently resolved, even though serum is substantially depleted of coagulable proteins (e.g. fibrinogen); and (ii) \( \gamma_{lv} \) of plasma is startlingly similar to that of purified protein constituents. Adsorption-kinetics studies of human albumin (66.3 kDa) and IgM (1000 kDa) binary mixtures revealed that relatively sluggish IgM molecules displace faster-moving albumin molecules adsorbing to the LV surface. This Vroman-effect-like process leads to an equilibrium \( \gamma_{lv} \) reflecting the linear combination of w/v concentrations at the surface predicted by theory. Thus, the Vroman effect is interpreted as a natural outcome of protein reorganization to achieve an equilibrium interphase composition dictated by a firm set of mixing rules.
3.1. Introduction

Protein adsorption is widely accepted within the biomaterials community to be among the first steps in the biological response to materials that ultimately determines biocompatibility in end use (see ref. 1 and citations therein). As a consequence, a great deal of effort has been expended toward understanding the biochemical activity of proteins in the adsorbed state\(^2\) and how these surface-bound proteins trigger a rich panoply of macroscopic biological outcomes when a biomaterial is brought into contact with different biological milieu or used in different physiological compartments (the \textit{in vitro} or \textit{in vivo} biological response). Biomedical surface science\(^3\) of protein adsorption has also received considerable attention because interfacial energetics (related to biomaterial surface hydration)\(^4,5\) control the amount of protein adsorbed under different physical conditions (protein-solution concentration, biomaterial surface energy, temperature, \textit{etc.}), which of course influences biocompatibility as well.

A popular research strategy in both of these pursuits has been the study of purified proteins that are thought to be representative members of the mammalian proteome (see, for examples refs. 6-9). An implicit assumption underlying this strategy is that behavior of an intact biological system (\textit{e.g.} whole blood plasma/serum or tissue/cell extracts) consisting of a plurality of proteins can be inferred from that of the individual constituents, even though there is no readily apparent, \textit{a priori} reason to expect that this ‘piece-at-a-time’ postulate is valid.\(^10\) That is to say, there is no strong precedent suggesting that a simple sum-of-the-parts-equals-the-whole equation is applicable to the protein-adsorption problem. Indeed, the so-called ‘Vroman effect’ (see refs. 6,7,11-29 and citations therein) strongly suggests otherwise. Leo Vroman first observed that adsorption from plasma or serum occurred through a complex series of adsorption-displacement
steps in which low-molecular-weight (MW) proteins arriving first at a surface are displaced by relatively higher MW proteins arriving later. Certain proteins, such as albumin, are observed to be relatively resistant to displacement at hydrophobic surfaces whereas others, such as high molecular weight kininogen, readily displaces fibrinogen.\textsuperscript{30} Exact molecular mechanisms underlying this process have not yet been resolved and the Vroman effect remains one of the quintessential mysteries of biomaterials surface science.\textsuperscript{29} All of this is to say that there are no specific ‘mixing rules’ stipulating how interfacial behavior of complex protein mixtures can be deduced from the behavior of single-protein solutions.

This chapter discusses mixing rules derived from a relatively straightforward theory of protein adsorption that reveals how individual proteins comprising a mixture compete for space at a surface in the adsorption process. Reported results are specific to the liquid-vapor (LV) interface, a molecularly-smooth hydrophobic surface where interfacial energetics can be directly and sensitively measured by tensiometric (surface thermodynamic) techniques.\textsuperscript{5} Insights into protein adsorption may thus be relevant to purely hydrophobic solid surfaces where dispersion forces predominate, although this is not proven by this work, but probably do not directly extend to hydrophilic surfaces where more chemically-specific interactions between proteins and the surface may occur.\textsuperscript{4} Using pendant-drop tensiometry, we find that concentration-dependent interfacial tension $\gamma_{lv}$ of a broad array of purified human proteins spanning three decades in molecular weight (MW) are quite similar to one another and surprisingly similar to that of plasma and serum, when protein concentration is scaled on a weight/volume (w/v) basis. These experimental outcomes are rationalized in terms of the mixing model, as are adsorption-kinetic studies of binary mixtures that illuminate the cause of the Vroman effect.
3.2. Materials and Methods

**Purified Proteins and Protein Mixtures:** Human albumin (FV), IgG, and IgM were used as received from Sigma-Aldrich and were the highest purity available (>96%) as assessed by SDS PAGE. Mass, concentration, and molecular weights supplied with purified proteins were accepted without further confirmation. Human platelet-poor plasma (citrated) was prepared from outdated (within 2 days of expiration) lots obtained from the Hershey Medical Center Blood Bank. Human serum was prepared in 15 mL batches by recalcification with 0.1 M CaCl$_2$ at 5:1 v/v plasma:calcium ratio in clean-glass scintillation vials for about 15 min. Ref. 31 discloses all details related to protein-solution preparation including serial dilutions of protein stock solutions (usually 10 mg/mL) that were performed in 96-well microtiter plates by (typically) 50:50 dilution in phosphate buffered saline solution (PBS). PBS was prepared from powder (Sigma Aldrich) in distilled-deionized (18 MΩ) water. Interfacial tension of PBS and water was checked periodically by Wilhelmy-balance tensiometry.

**Liquid-Vapor Interfacial Tension Measurements:** LV interfacial tensions $\gamma_{lv}$ reported in this work were measured by pendant-drop tensiometry (PDT) using a commercial automated tensiometer (First Ten Angstroms Inc., Portsmouth VA) applying techniques discussed in detail elsewhere. Briefly, the tensiometer employed a Tecan liquid-handling robot to aspirate between 10-12 µL of solutions contained in a 96-well microtiter plate prepared by the serial-dilution protocol mentioned above. The robot was used to reproducibly transfer the tip with fluid contents into a humidified (99+ % RH) analysis chamber and dispense between 6-11 µL pendant drops (smaller drop volume required for lower interfacial tensions) within the focal plane of a
magnifying camera. These and all other aspects of pendant-drop analysis were performed under computer control. Precision of $\gamma_{lv}$ was about 0.5 mN/m based on repeated measurement of the same pendant drop. The instrument was calibrated against pure water interfacial tension and further confirmed on occasion against Wilhelmy-balance tensiometry. The analysis chamber was thermostated to a lower-limit of 25±1 °C by means of a computer-controlled resistive heater. Upper-temperature limit was, however, not controlled but rather floated with laboratory temperature, which occasionally drifted as high as 29 °C during summer months. Thus, reported $\gamma_{lv}$ values are probably not more accurate than about 1 mN/m on an inter-sample basis considering the small but measurable variation of water interfacial tension with temperature. This range of accuracy is deemed adequate to the conclusions of this report which do not strongly depend on more highly-accurate $\gamma_{lv}$ that is quite difficult to achieve on a routine basis with the multiplicity of protein solutions investigated herein.

**Computation and Data Representation:** Computational, statistical, and theoretical methods used in this work have been discussed in detail elsewhere.\textsuperscript{5,32,33} Briefly, time-dependent $\gamma_{lv}$ data corresponding to solutions at different w/v concentration $C_B$ were recovered from PDT files and correlated with concentrations, leading to a matrix of results with row values representing concentration and time (in sec) as column values. It was generally observed that $\gamma_{lv}$ data takes on a sigmoidal shape when plotted on logarithmic-concentration axes,\textsuperscript{5,32} with a well-defined low-concentration asymptote $\gamma^{\omega}_{lv}$ and a high-concentration asymptote $\gamma^{\prime}_{lv}$. Successive non-linear least-squares fitting of a four-parameter logistic equation

$$
(\gamma_{lv} = \{((\gamma^{\omega}_{lv} - \gamma^{\prime}_{lv})/(1 + (\ln C_B^{z/2} / \ln C_B^M)\gamma_{lv}) + \gamma^{\prime}_{lv})\})
$$

to concentration-dependent $\gamma_{lv}$ data for each
time within the observation interval quantified $\gamma''_v$ and $\gamma'_v$ parameters with a measure of statistical uncertainty. Fitting also recovered a parameter measuring concentration-at-half-maximal-change in interfacial activity, $\ln C_\Pi^{1/2}$ (where $\Pi / 2 = 1/2 \Pi^{\text{max}}$ and $\Pi^{\text{max}} \equiv \gamma''_v - \gamma'_v$), as well as a parameter $M$ that measured steepness of the sigmoidal curve. This empirical, multi-parameter fitting to concentration-dependent $\gamma_v$ data was a purely pragmatic strategy that permitted quantification of best-fit protein and surfactant characteristics but is not a theory-based analysis.\textsuperscript{5,32,33} Three-dimensional (3D) representations of time-and-concentration-dependent $\gamma_v$ data were created in Sigma Plot (v8) from the data matrix discussed above and overlain onto fitted-mesh data computed from least-squares fitting. Two-dimensional (2D) representations were created from the same data matrices at selected observation times.

3.3. Theory

Protein Adsorption Model: Previous work developed a model of protein adsorption predicated on the interfacial packing of hydrated spherical molecules with dimensions scaling as a function of MW.\textsuperscript{1,31} This strategy was similar to that adopted by Ostuni \textit{et al.}\textsuperscript{34} in which sphere packing was taken to be the simplest, physically-relevant model that might yield semiquantitative description of general trends in protein adsorption. This model was not designed to account for the myriad complexity and variations among proteins that no doubt invalidate such a simple conceptual construction at a detailed level of investigation. However, general predictions were found to explain adsorption energetics of a broad spectrum of plasma proteins spanning nearly three decades in molecular weight (MW). Briefly outlining the core ideas behind the model for the purposes of this paper, the LV surface was modeled as a 3D interphase region with volume $V_I$ (in cm$^3$) that separates bulk-solution and bulk-vapor phases. Protein molecules with radius
\[ r_v = 6.72 \times 10^8 MW^{1/3} \] (packing-volume radius in cm for MW expressed in kDa) adsorb from the bulk phase into this interphase region, occupying one or more layers depending on protein size (MW) and solution concentration. Stated somewhat more precisely, protein partitions from the bulk phase to the interphase region, achieving a fixed concentration ratio governed by a partition coefficient \( P \equiv C_I / C_B \); where \( C_I \) is the interphase concentration (\( C_I \equiv n_I / V_I \) if \( n_I \) is the total number of moles of protein within the interphase) and \( C_B \) is the corresponding bulk concentration (both in moles/cm\(^3\)). Interestingly, it was found that \( P \) was essentially invariant among diverse group of proteins studied (\( P \approx 150 \)), meaning that proteins adsorb to the LV interface at concentrations exceeding 150-fold bulk-solution concentration. Protein size and repulsion between molecules place an upper bound on maximal interphase concentration denoted \( C_I^{\text{max}} \). \( C_I^{\text{max}} \) can also be expressed in terms of fractional volume occupied by protein \( \Phi_p^{\text{max}} = C_I^{\text{max}} V_p \) (dimensionless); where protein molar volume \( V_p = 4/3 \pi r_p^3 N_A \) (in cm\(^3\)/mole) if \( N_A \) is the Avogadro number. Elaboration of this model and calibration to experimental neutron-reflectivity data on albumin adsorption revealed that \( \Phi_p^{\text{max}} \approx 1/3 \) and was, like \( P \), essentially invariant among diverse group of proteins studied. In other words, the LV-interphase capacity was found to be limited by the extent to which this interphase can be dehydrated through displacement of water by adsorbed protein molecules. Thus, the controlling role of water in protein adsorption was emphasized, in general agreement with independently-developed theory.\(^{35}\)

\( \Phi_p^{\text{max}} \) can be further quantified in the more familiar units of weight/volume (w/v) concentration \( W_I^{\text{max}} \) by expanding the definition above explicitly in terms of \( r_v \):
\[
\Phi_p^{\text{max}} = C_i^\text{max} V_p = C_i^\text{max} \left[ \frac{4}{3} \pi MW_i \left(6.72 \times 10^{-8}\right)^3 \right] = 7.65 \times 10^2 W_i^{\text{max}} \frac{1}{3} \Rightarrow W_i^{\text{max}} = 4.36 \times 10^{-4} \text{ kg/cm}^3 = 436 \text{ mg/mL}
\]  

where Eq. (1) recognizes that molar concentration is converted to w/v concentration by MW.

Importantly, Eq. (1) reveals that a proteinaceous interphase saturates at a fixed w/v (not molar) concentration and corroborates the conclusion drawn from diverse literature sources that adsorbed protein concentrations can be surprisingly large.\(^{36}\) Eq. (1) also anticipates the experimental observation that \(\gamma_h\) curves for diverse proteins spanning three decades of MW appear more similar than dissimilar when scaled on a w/v basis\(^ {31}\) because the maximum interphase concentration is very similar for all proteins in w/v units.

**Protein Mixtures – Binary Solutions:** A fixed interphase capacity for protein coupled with a nearly constant partition coefficient imposes significant restrictions on the fractional contribution of individual proteins adsorbing to the LV surface from mixed solution. Consider first a binary solution comprised of proteins “i” and “j” with different MW, each at a bulk w/v concentration equal or exceeding \(W_B^{\text{max}} / P\) so that interphase saturation is assured. Accordingly, Eq. (1) must be rewritten to accommodate contributions from \(i\) and \(j\), each occupying space dictated by the product \(C_i V_p\):

\[
\Phi_p^{\text{max}} = C_{i,j} V_{p,i} + C_{i,j} V_{p,j} = \frac{4}{3} \pi N_A \left(6.72 \times 10^{-8}\right)^3 \left[ C_{i,i} MW_i + C_{i,j} MW_j \right] = 7.65 \times 10^2 \left[ W_{i,i} + W_{i,j} \right] \approx \frac{1}{3} \Rightarrow \left[ W_{i,i} + W_{i,j} \right] = 436 \text{ mg/mL}
\]  

Eq. (2) states that constant \(\Phi_p^{\text{max}}\) forces the interphase to be populated by both \(i\) and \(j\) proteins such that the summed weight concentrations equals the fixed interphase capacity of \(\sim 436\)
mg/mL. Given that $P$ is approximately constant for all proteins and both $i, j$ are at equal bulk concentration $W_\text{max}^B$ sufficient to individually fill the interphase (by model construction), it seems reasonable to assume that each protein must compete equally for space within the interphase. Hence, at equilibrium, proteins $i, j$ must be represented at the surface by equal w/v concentrations. It was thus concluded that $W_{i, i} = W_{i, j} = \frac{1}{2} W_{\text{max}, i} = \frac{1}{2} (PW_\text{max}^B)$. Notably, the interphase concentration of either protein adsorbed from the binary solution is half that adsorbed from a single-protein solution.

If, however, the $i^{th}$ protein comprising the binary solution is below $W_\text{max}^B$ and thus not capable of individually saturating the interphase, it cannot compete for space as effectively as in the preceding proposition, contributing at most $\frac{1}{2} PW_{B,i}$. Again in consideration of the fact that $P$ is approximately constant for all proteins, it seems reasonable that proteins $i, j$ compete for space within the interphase on a purely concentration basis so that

$$W_{i, i}^\text{max} = (W_{i, i} + W_{i, j}) = \frac{1}{2} P (W_{B,i,j} + W_{B,j, i}^\text{max}).$$

Thus, the $j^{th}$ protein dominates the saturated interphase (on a w/v basis) by an amount proportionate to the weight excess of protein $j$ over $i$ in the bulk solution. Now, if neither protein $i$ nor $j$ individually exceed $W_B^\text{max}$ but $(W_{B,i} + W_{B,j}) \geq W_B^\text{max}$, then the logic of the mixing model insists that each protein competes for space according to

$$W_{i, i}^\text{max} = \frac{1}{2} P (W_{B,i} + W_{B,j}).$$

A survey of many different purified blood-plasma proteins spanning three decades in MW shows that concentration-dependence of $\gamma_{lv}$ was very similar among this disparate group of molecules.\textsuperscript{1}
However, no two proteins were found to be identical in this regard. Instead, it was found that each protein retained a kind of ‘interfacial signature’ written in the tension at interphase saturation $\gamma'_i$ (occurring at $W_B^{\text{max}}$) that falls within the 20 mN/m band characteristic of all proteins studied.\textsuperscript{1,31} If $i$ and $j$ are two such proteins in a binary mixture, then it can be expected that this interfacial-tension signature will be expressed in a manner dependent on relative proportions of $i$ and $j$. That is to say, if $\Pi_i^{\text{max}} \equiv \left(\gamma_i^o - \gamma_i'\right)$ and $\Pi_j^{\text{max}} \equiv \left(\gamma_j^o - \gamma_j'\right)$ are spreading pressures of pure $i$ and $j$ at $W_B^{\text{max}}$ for each (where $\gamma_i^o = 71.97$ mN/m at 25 $^\circ$C is the interfacial tension of phosphate-buffer-saline diluent), then the observed spreading pressure

$$\Pi_{\text{obs}}^{\text{max}} \equiv \left(\gamma_i^o - \gamma_j^o\right)_{\text{obs}}$$

of an $i, j$ mixture should vary with the relative proportion of each component within the interphase. Expressing this proportion as a weight fraction $f_{i,j}^{\text{max}} \equiv \left(\frac{W_{i,j}}{W_{i,i} + W_{i,j}}\right)$ and recalling that $P_{i,j} \equiv \left(\frac{W_{i,j}}{W_{i,i}}\right)$, then it follows that $f_{i,j}^{\text{max}} = \left(\frac{W_{B,j}}{W_{B,i} + W_{B,j}}\right) \equiv f_{i,j}^{\text{max}}$ (where the superscript ‘max’ is retained to emphasize the restriction that $(W_{i,i} + W_{i,j}) \geq W_{i,j}^{\text{max}}$). It can be expected that $\Pi^{\text{max}}_{\text{obs}}$ should vary in some way with the bulk composition $f_{i,j}^{\text{max}}$. We are unaware of any theoretical precedent for such a combining formula for interfacial tensions and so tender the linear-combination of Eq. (3):

$$\Pi_{\text{obs}}^{\text{max}} = \Pi_i^{\text{max}} (1 - f_{i,j}^{\text{max}}) + \Pi_j^{\text{max}} f_{i,j}^{\text{max}} = \Pi_i^{\text{max}} - f_{i,j}^{\text{max}} (\Delta \Pi^{\text{max}})$$

(3)

where $\Delta \Pi^{\text{max}} \equiv \Pi_i^{\text{max}} - \Pi_j^{\text{max}}$. Eq. (3) stipulates that $\Pi_{\text{obs}}^{\text{max}}$ varies between the two boundaries $\Pi_i^{\text{max}}$ and $\Pi_j^{\text{max}}$ as a function of weight-fraction composition, assuming of course that the mixing rule of Eq. (2) and subordinate relationships discussed above are valid.
**Protein Mixtures – Multi-component Solutions:** The logic applied above to derive the binary mixing rule can be extended to a more complex solution of $m$ proteins that collectively saturate the interphase, leading to a generalized mixing rule applicable to multi-component protein solutions:

$$\frac{1}{m} \sum_{i} W_{i} = \frac{P}{m} \sum_{i} W_{i} \Rightarrow \sum_{i} W_{i} = \frac{m}{P} W_{i} \max \sim \frac{4.36 \times 10^{-4}}{1.5 \times 10^{-2} \cdot m}$$

Eq. (4) states that the w/v distribution of proteins within the LV interphase of a solution of $m$ proteins (at equilibrium) is identical to that of the bulk phase. But the w/v concentration of any particular protein is diluted by a factor of $m$ relative to the maximal interphase concentration that would have otherwise been achieved from a pure, single-component solution of any particular solution constituent. That is to say, the effective partition coefficient $P_{\text{eff}} = \frac{P}{m}$ for a particular protein in an $m$ component solution is lower than the constant partition coefficient $P$ for a purified, single-component solution. This is a direct outcome of a competition among proteins on an equal w/v concentration basis for a fixed fraction of space within the interphase volume.

### 3.4. Results

**Plasma, Serum, and Purified Proteins:** Fig. 1 collects time-and-concentration-dependent ‘$\gamma_{h}$ curves’ for human immunoglobulin G (hIgG, panel A), human-blood plasma (HP, panel B), and serum derived from this plasma (HS, panel C). Results are given in both three-dimensional (3D, $\gamma_{h}$ as a function of time and concentration) and two-dimensional (2D, $\gamma_{h}$ as a function concentration at specified times) representations. Note that the logarithmic-solute-dilution
ordinate \ln C_B in Fig. 1 is expressed in either w/v or v/v units of parts-per-trillion (PPT, grams solute/10^{12} grams solution for purified proteins and mL/10^{12} mL solution for plasma/serum). Both purified-protein solutions and protein mixtures exhibited the biosurfactant property of adsorbing to the LV interphase, causing a reduction in \gamma_{lv} as a function of bulk solution concentration \( C_B \). Adsorption results in sigmoidally-shaped, concentration-dependent \( \gamma_{lv} \) curves on a \ln C_B axis, with a well-defined low-concentration asymptote \( \gamma_{lv}^o \) and a high-concentration asymptote \( \gamma_{lv}^{'} \).5

We observed that most proteins within the \( 10 \leq MW \leq 1000 \) kDa achieve a limiting \( \gamma_{lv}^{'} \) within the 1 hr observation period employed in this work.1 Results shown in Fig. 1A for hIgG is somewhat exceptional in this regard and arguably did not reach equilibrium. Either the bulk solution concentration was insufficient to fully saturate the surface (in spite of being near a solubility limit) or longer drop age was required to achieve equilibrium. Analysis disclosed in ref. 31 strongly suggests that the latter is more likely, with slight but detectable change in \( \gamma_{lv}^{'} \) possibly due to slow change in adsorbate configuration at the LV surface. In any event, \( \gamma_{lv}^{'} \) parameters collected in Table 1 for three separate preparations of hIgG should be interpreted as projected equilibrium values based on statistical fitting of data, as described in the Methods and Materials section. Characteristic parameters listed in Table 1 for albumin, plasma, and serum are more certain in this regard because a secure limiting \( \gamma_{lv}^{'} \) was achieved (see Fig. 1). Fig. 2 graphically compares equilibrium, concentration-dependent \( \gamma_{lv}^{'} \) data for hIgG, plasma, and serum on a single set of axis where the surrounding band represents 99% confidence intervals around
the best-fit human-plasma data listed in Table 1. It is apparent from this data that concentration-dependent $\gamma_{lv}$ of hIgG, plasma, and serum cannot be confidently distinguished.

**Binary Protein Mixtures:** Fig. 3 compares time-dependent $\gamma_{lv}$ of hIgM and FV HSA solutions mixed in various proportions at fixed total protein concentration (see ref. 1 for more details of HSA and IgM interfacial properties). Fig. 3A,B corresponds to experiments performed at a total protein concentration of 2.1 mg/mL whereas Fig. 3C,D corresponds to 27 µg/mL total protein. These results were representative of a broader experimental agenda summarized in Table 2. At each composition ranging from 100% HSA to 100% hIgM (column 1 of Table 2), $\gamma_{lv}$ was observed to asymptotically approach a characteristic steady-state value $\gamma_{lv}'$. Comparison of Fig. 3A and 3.3C shows that whereas steady state was arguably reached at 2.1 mg/mL total protein, $\gamma_{lv}$ drifted continuously lower with time at 27 µg/mL total protein. For the 2.1 mg/mL total protein case, steady-state $\gamma_{lv}'$ was estimated by simply averaging the final 25 $\gamma_{lv}$ observations. Results were expressed in terms of steady-state spreading pressure $\Pi_{obs} = \left( \gamma_{lv}^o - \gamma_{lv}' \right)$ listed in columns 3-4 of Table 2; where $\gamma_{lv}^o \equiv 71.9$ mN/m consistent with the interfacial tension of water at 25 °C. Fig. 3B plots $\Pi_{obs}$ against % HSA composition, where the line drawn through the data corresponds to Eq. (3) of the theory section (error bars correspond to the standard deviation of the mean $\gamma_{lv}$). It is emphasized that this line is not a statistical fit but rather an analytic function since Eq. (3) contains no parameters that are not experimentally determined.
Equilibrium $\Pi_{obs}$ could not be reliably estimated in the 27 $\mu$g/mL total protein case because $\gamma_{hv}$ did not achieve steady-state. However, it was noted that data corresponding to $t > 900$ sec was quite linear on $t^{1/2}$ coordinate (Fig. 3D, see annotations) following a lag phase that was especially noticeable in the 100% IgM case (columns 4-5 of Table 2 compile linear-least-squares parameters of corresponding to $t^{1/2} > 30$ data). Interestingly, this lag phase substantially disappeared upon mixing 25% albumin with 75% IgM and the time decay in $\gamma_{hv}$ was very similar to that observed for 100% albumin solutions. In fact, slopes of the $t^{1/2}$ curves for each protein composition were statistically identical.

3.5. Discussion

Plasma and Serum: An extensive survey of concentration-dependent $\gamma_{hv}$ of human-blood proteins spanning nearly 3 decades of MW ($10 \leq MW \leq 1000$) has revealed only modest differences within this diverse group.\(^1\) Herein we report that concentration-dependent $\gamma_{hv}$ for plasma and serum are effectively the same (see Figs. 1 and 2), even though serum is compositionally distinct from plasma by virtue of being depleted of fibrinogen - one of the twelve most abundant proteins (2.5-4.5 mg/mL).\(^{37}\) Taken together, experimental results show that $\gamma_{hv}$ of blood plasma and serum are very similar to that of purified constituents. These findings supplement the long-known, but heretofore unexplained, observation that $\gamma_{hv}$ of plasma derived from different (normal) mammalian species (bovine, ovine, canine, human…) fall within a narrow 5 mN/m window,\(^{38-45}\) in spite of significant differences in plasma proteome among species.\(^{46}\)
The similarity between purified protein solutions and complex mixtures stands in strong contrast to the general expectation that compositional/structural differences among proteins should also result in quite different adsorption energetics and commensurately different concentration-dependent $\gamma_{lv}$. Indeed, preferential/selective adsorption of proteins has long been linked to different biological responses evoked by different materials (see ref. 2 and citations therein). However, detailed analysis of $\gamma_{lv}$ data strongly suggests that the interaction energetics of water with (globular blood) proteins do not, in fact, vary substantially across a broad span of MW.$^{1,31}$ This conserved amphilicity among different proteins manifests itself in a substantially invariant partition coefficient $P = C_I / C_B \sim 150$; where $C_I$ and $C_B$ are interphase and bulk solution concentrations, respectively. Hence, all proteins adsorb to the LV interface at the same ratio-to-bulk concentration and there is no energetic reason to expect selective adsorption of any particular protein from the mixture. Furthermore, as long as the bulk solution contains sufficient total protein concentration to saturate the interphase, then saturating interfacial tension $\gamma_{lv}$ will not vary significantly with bulk phase composition.

At 60-85 mg/mL total protein (including fibrinogen), plasma and serum is well over the $\sim 3$ mg/mL required to saturate the LV surface. According to the discussion above, depletion or concentration of one particular protein over another will not measurably affect $\gamma_{lv}$. So it happens that concentration-dependent $\gamma_{lv}$ of blood plasma is nearly identical to that of serum, even though serum is substantially depleted of fibrinogen. This further explains how it happens that $\gamma_{lv}$ does not significantly vary among mammalian species with different blood-protein composition. Simply stated, any combination of blood-protein constituents behave similarly in
water because individual constituents behave similarly. This extension of the venerable *similia similibus solventur* (like dissolves like) rule of miscibility occurs because the energetics of hydrophobic hydration\(^48\) of blood proteins is approximately constant across a broad range of MW.\(^1,31\)

Mixing rules articulated in the Theory section suggest that protein adsorption to the LV surface from multi-component solutions can be accurately viewed as a competition for space within the interphase region. Competition is on a on a w/v basis, not molar, so that each competing protein is represented within the interphase at the same w/v fraction as in the bulk solution phase at equilibrium. The plasma/serum proteome is comprised of at least 490 proteins, with a natural abundance that varies over more than 10 orders of magnitude.\(^49\) Only the highest-concentration members of the proteome can be expected to measurably affect concentration-dependent \(\gamma_{lv}\).

For example, we have reported that blood factor XII is only weakly surface-active at physiological concentration of 4 mg/100 mL.\(^31\) Bearing this in mind, Eq. (4) interprets total plasma/serum protein as \(60 < \sum_{i} W_{bi} < 85\) mg/mL, or \(20 < m < 28\), which is roughly consistent with the 30 classical plasma proteins\(^37\) that occupy the first five decades of physiological concentration.\(^49\) In other words, the effective partition coefficient \(P_{\text{eff}} \equiv \frac{P}{m} \sim \frac{150}{30} = 5\) for important coagulation proteins such as FXII at a hydrophobic surface. We thus conclude that statistical representation of yet rarer plasma proteins within this hydrophobic interphase must be vanishingly small. Depletion of coagulation proteins in the conversion of plasma to serum certainly changes \(m\) but does not alter the total w/v composition within the interphase because \(\sum W_{bi} \gg W_{b}^{\text{max}}\). As a consequence concentration-dependent \(\gamma_{lv}\) curves of plasma and serum are
nearly identical because other protein constituents compete for the interfacial vacancies effectively created by removal of proteins consumed in the coagulation process (e.g. conversion of fibrinogen to insoluble fibrin).

**Binary Protein Mixtures and the Vroman Effect:** Mixing two proteins with slightly different characteristic γv in different proportions at fixed total protein concentration provides a means of testing mixing rules articulated in the Theory section by using interfacial tension as a kind of tracer of interphase composition. Fig. 3 summarizes results of such an approach at two total protein concentrations. Fig. 3A shows that limiting γv is achieved at 2.1 mg/mL for 100% hIgM or HSA. Fig. 3B shows that Eq. (3) is in nearly quantitative adherence to the data listed in Table 2, demonstrating that the weight-fraction combining formula for the interfacial tension of protein mixtures closely simulates reality; at least for the 2.1 mg/mL HSA/hIgM solutions that come to equilibrium within the time frame of γv observation (1 hr). It is thus concluded that the binary mixing rule of Eq. (2) and subordinate assumptions accurately specify the interphase protein composition at equilibrium. However, steady-state is not achieved for 27 µg/mL total-protein solutions regardless of HSA/hIgM relative composition.

It is noteworthy that the lag phase in γv dynamics observed in the 100% hIgM case (see annotations in Fig. 3D) was completely eliminated upon replacement with only 25% albumin (while maintaining constant total protein composition). Little doubt this occurred because diffusion/mass transfer of HSA molecules (66.3 kDa) to the interphase region was much faster than that of hIgM (1000 kDa), so that initial kinetics was dominated by HSA adsorption at t < 900 sec. However, from the discussion above, it is apparent that steady-state interphase
composition is a mixture controlled by w/v proportions of the bulk solution, not the dynamics that lead to the final composition. Presumably then, transient accumulation of HSA within the interphase is eventually accommodated by dilution with hIgM. This adsorption-displacement mechanism is consistent with the Vroman effect mentioned in the Introduction section. However, Vroman effect at the LV interface is herein interpreted as a process of adjusting interphase composition to achieve the composition dictated by mixing rules rather than attributing the cause to specifics of molecular composition or molecular characteristics (see as examples refs. 30,50,51 and citations therein).

It is of further interest that post-lag-phase kinetic data was quite linear on $t^{1/2}$ coordinates for all protein compositions listed in Table 2 (see (Fig. 3D), suggesting that approach to steady state was dominated by diffusion and followed $\sqrt{\frac{Dt}{\pi}}$ kinetics. However, slopes through this data region were statistically identical for all total-protein compositions ranging from 100% hIgM to 100% FV HSA (Table 2). This finding is inconsistent with the substantial size disparity between these two proteins. Thus, we suspect that linearity on $t^{1/2}$ coordinates is happenstance and that kinetics are rather controlled by an adsorption process, not simple diffusion, possibly related to molecular exchange reactions occurring within the various layers comprising the mixed interphase. However interpreted, it is clear that much more work is required to evaluate mechanistic alternatives.
3.6. Conclusions

A relatively simple model of protein adsorption to the liquid-vapor (LV) interface leads to mixing rules stipulating the equilibrium protein composition of the interphase region formed by adsorption from a solution of \( m \) constituents. Proteins compete for space at the LV interphase on a weight, not molar, concentration basis. As a consequence, the equilibrium weight-fraction composition of the interphase is identical to that of the bulk phase. However, interphase concentration of any particular protein is diluted by a factor of \( m \) relative to that which would have otherwise been achieved from a pure, single-component solution. That is to say, each protein of an \( m \) protein mixture achieves a interphase concentration \( C_I \) that is dictated by an effective partition coefficient \( P_{ef} = \frac{P}{m} \); where \( P = \frac{C_I}{C_B} \) and \( C_B \) is the bulk-protein concentration. For complex biological fluids such as blood plasma and serum, mixing rules thus imply that dilute members of the proteome are overwhelmed at the LV interphase by the thirty classical plasma proteins occupying the first-five decades of physiological concentration. Liquid-vapor interfacial tension \( \gamma_{lv} \) of single-protein solutions, binary-protein mixtures, blood plasma and serum measured by pendant drop tensiometry is completely consistent with mixing theory in that steady-state concentration-dependent \( \gamma_{lv} \) among these specimens cannot be clearly distinguished when scaled by a w/v concentration. Furthermore, equilibrium (steady-state) \( \gamma_{lv} \) of albumin and IgM binary mixtures precisely follows a simple combining rule predicated on mixing theory. Adsorption-kinetics studies of these binary solutions strongly suggest that slow-moving IgM molecules displace faster-moving albumin molecules in a Vroman-effect-like process leading to steady state. The Vroman effect is thus interpreted as a natural outcome of surface reorganization to achieve the equilibrium interphase composition dictated by a firm set of mixing rules.
Citations


(19) Elwing, H.; Askendal, A.; Lundstrom, I. *Competition Between Adsorbed Fibrinogen and High-molecular-weight Kininogen on Solid Surfaces Incubated in Human*


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<td>-8.6±2.1</td>
<td>23.1±3.2</td>
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</tr>
<tr>
<td>Prep 1</td>
<td>71.13±0.57</td>
<td>51.6±1.9</td>
<td>18.60±0.39</td>
<td>-10.4±2.7</td>
<td>20.1±1.9</td>
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<tr>
<td>Prep 3</td>
<td>71.09±0.42</td>
<td>56.48±0.92</td>
<td>19.72±0.19</td>
<td>-20.1±5.1</td>
<td>15.21±0.92</td>
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<tr>
<td>HUMAN IgM (hIgM)</td>
<td>70.98±0.39</td>
<td>51.4±1.2</td>
<td>16.82±0.19</td>
<td>-13.2±3.5</td>
<td>20.3±1.2</td>
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<tr>
<td>Prep 1</td>
<td>71.65±0.55</td>
<td>50.2±3.1</td>
<td>18.52±0.35</td>
<td>-14.2±4.2</td>
<td>21.5±3.1</td>
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<td></td>
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<tr>
<td>Prep 3</td>
<td>70.51±0.59</td>
<td>55.4±1.3</td>
<td>17.59±0.12</td>
<td>-11.7±3.3</td>
<td>16.3±1.3</td>
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<tr>
<td>HUMAN PLASMA (HP)</td>
<td>71.70±0.62</td>
<td>48.55±0.71</td>
<td>19.56±0.19</td>
<td>-23.3±3.6</td>
<td>23.15±0.71</td>
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<tr>
<td>Prep 1</td>
<td>71.38±0.74</td>
<td>45.5±1.1</td>
<td>19.19±0.26</td>
<td>-10.9±1.6</td>
<td>26.5±1.1</td>
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<tr>
<td>HUMAN SERUM (HS)</td>
<td>70.54±0.46</td>
<td>47.61±0.62</td>
<td>19.91±0.17</td>
<td>-23.4±3.7</td>
<td>24.08±0.62</td>
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<tr>
<td>Prep 1</td>
<td>72.64±0.72</td>
<td>45.64±0.69</td>
<td>18.47±0.18</td>
<td>-13.4±1.6</td>
<td>26.33±0.69</td>
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Table II: Time-Dependent $\gamma_v$ of FV-HSA in hIgM Solutions

<table>
<thead>
<tr>
<th>% FV HSA in hIgM</th>
<th>2.1 mg/mL Total Protein Steady-State Parameters</th>
<th>27 $\mu$g/mL Total Protein Kinetic Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\gamma_v$ (mN/m)</td>
<td>$\Pi_{obs}$ (mN/m)</td>
</tr>
<tr>
<td>0</td>
<td>56.21±0.22</td>
<td>15.76±0.22</td>
</tr>
<tr>
<td>25</td>
<td>54.59±0.17</td>
<td>17.39±0.17</td>
</tr>
<tr>
<td>50</td>
<td>53.92±0.11</td>
<td>18.17±0.11</td>
</tr>
<tr>
<td>75</td>
<td>52.15±0.12</td>
<td>19.82±0.12</td>
</tr>
<tr>
<td>100</td>
<td>51.42±0.17</td>
<td>20.55±0.17</td>
</tr>
</tbody>
</table>
**Figure 1**: Interfacial tension profiles in 3D ($\gamma_v$ as a function of analysis time (drop age) and logarithmic (natural) solution concentration $C_p$) and 2D ($\gamma_v$ as a function of logarithmic solution concentration $C_p$ at selected times) formats comparing (human) immunoglobulin-G (hIgG, panel A, preparation 1, Table 1), plasma (HP, panel B, preparation 1, Table 1), and serum (HS, panel C, preparation 1, Table 1). In each case, solute concentration $C_p$ is expressed in either w/v or v/v units of parts-per-trillion (PPT, grams solute/10^{12} grams solution for hIgG and mL/10^{12} mL solution for plasma/serum). Symbols in 2D panels represent time slices through 3D representations (filled circle = 0.25 sec, open circle = 900 sec, filled triangles = 1800 sec, and open triangles = 3594 sec; annotations in panel A indicate maximum and half-maximum spreading pressure). Similar interfacial activity is observed for both protein mixtures as well as pure protein solutions.
**Figure 2:** Comparison of steady-state, concentration-dependent $\gamma_k$ data for (human) plasma (HP, preparation 2, Table 1), serum (HS, preparation 2, Table 1), and IgG (hIgG, preparation 3, Table 1) on a single concentration axis, showing that protein mixtures cannot be confidently distinguished from a purified protein. Band represents 99% confidence intervals around best-fit-human plasma data. (open circle = HP, filled square = HS, filled triangle = hIgG).
**Figure 3:** Interfacial tension $\gamma_{lv}$ of (human) FV HSA and hIgM mixtures in different proportions (A, B at 2.1 mg/mL; C, D at 27 µg/mL total protein concentration). Symbols represent different proportions of FV HSA in hIgM (filled circle = 100%, open square = 25%, filled triangle = 0% FV HSA in hIgM). Equilibrium (steady state) is reached within the 3600 sec drop age of the PDT mixture experiment at 2 mg/mL (Panel A), while $\gamma_{lv}$ drifted continuously lower with time at 27 µg/mL (Panel C). Panel B plots the observed spreading pressure ($\Pi_{obs}$) as a function of proportion of FV HSA in hIgM, with error bars corresponding to the standard deviation of the mean $\gamma_{lv}$. Line representing Eq.3 (see theory) demonstrates close correlation with experimental results. Panel D plots $\gamma_{lv}$ as a function of $t^{1/2}$, revealing a linear trend (following a lag phase of $t < 900$ sec) that is statistically identical for each of the proportions of FV HSA in hIgM. See Table 2 for parameters from linear-fit to data for $t > 900$ seconds.
<table>
<thead>
<tr>
<th><strong>Glossary of Symbols</strong></th>
<th></th>
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</thead>
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<tr>
<td>$C_B$</td>
<td>Bulk solution concentration (moles/volume)</td>
</tr>
<tr>
<td>$C_B^{\text{max}}$</td>
<td>Bulk solution concentration at limiting interfacial tension (moles/volume)</td>
</tr>
<tr>
<td>$C_B^{\pi/2}$</td>
<td>Bulk solution concentration at half-maximal-change in interfacial activity (moles/volume)</td>
</tr>
<tr>
<td>$C_I$</td>
<td>Interphase concentration (moles/volume)</td>
</tr>
<tr>
<td>$C_I^{\text{max}}$</td>
<td>Maximal interphase concentration (moles/volume)</td>
</tr>
<tr>
<td>$D$</td>
<td>Diffusion Coefficient</td>
</tr>
<tr>
<td>$f_{i,j}^{\text{max}}$</td>
<td>Weight fraction of protein $i$ within the interphase</td>
</tr>
<tr>
<td>$f_{j,i}^{\text{max}}$</td>
<td>Weight fraction of protein $j$ within the interphase</td>
</tr>
<tr>
<td>$f_{B,i}^{\text{max}}$</td>
<td>Weight fraction of protein $i$ within the bulk</td>
</tr>
<tr>
<td>$f_{B,j}^{\text{max}}$</td>
<td>Weight fraction of protein $j$ within the bulk</td>
</tr>
<tr>
<td>$\Phi_p$</td>
<td>Volume fraction of protein in the interphase</td>
</tr>
<tr>
<td>$\Phi_p^{\text{max}}$</td>
<td>Maximal volume fraction of protein in the interphase</td>
</tr>
<tr>
<td>$\gamma_{\text{lv}}$</td>
<td>Liquid-vapor (LV) interfacial tension (mN/m)</td>
</tr>
<tr>
<td>$\gamma_{\text{lv}}'$</td>
<td>Low-concentration asymptote of a concentration-dependent $\gamma_\nu$ curve (mN/m)</td>
</tr>
<tr>
<td>$\gamma_{\text{lv}}''$</td>
<td>High-concentration asymptote of a concentration-dependent $\gamma_\nu$ curve (mN/m)</td>
</tr>
<tr>
<td>$m$</td>
<td>Number of components in a multi-component solution</td>
</tr>
<tr>
<td>$M$</td>
<td>Parameter fitted to concentration-dependent $\gamma_\nu$ curve</td>
</tr>
<tr>
<td>$n_i$</td>
<td>Moles of protein within the interphase</td>
</tr>
<tr>
<td>$N_A$</td>
<td>Avogadro number</td>
</tr>
<tr>
<td>$P$</td>
<td>Partition coefficient, $P \equiv C_I / C_B$</td>
</tr>
<tr>
<td>$P_{\text{eff}}$</td>
<td>Effective Partition coefficient of a protein in a multi-component solution, $P_{\text{eff}} \equiv \frac{P}{m}$</td>
</tr>
<tr>
<td>$\Pi$</td>
<td>Spreading pressure (mN/m), $\Pi = \gamma_{\text{lv}}' - \gamma_{\text{lv}}''$</td>
</tr>
<tr>
<td>$\Pi^{\text{max}}$</td>
<td>Maximum Spreading pressure (mN/m)</td>
</tr>
<tr>
<td>$\Pi_i^{\text{max}}$</td>
<td>Maximum Spreading pressure of protein $i$ in a binary mixture of proteins $i$ and $j$ (mN/m)</td>
</tr>
<tr>
<td>$\Pi_j^{\text{max}}$</td>
<td>Maximum Spreading pressure of protein $j$ in a binary mixture of proteins $i$ and $j$ (mN/m)</td>
</tr>
<tr>
<td>$\Delta \Pi^{\text{max}}$</td>
<td>Difference in spreading pressure between 2 proteins in a binary mixture of proteins $i$ and $j$</td>
</tr>
<tr>
<td>$\Delta \Pi_i^{\text{max}}$</td>
<td>$\equiv \Pi_i^{\text{max}} - \Pi_j^{\text{max}}$ (mN/m)</td>
</tr>
<tr>
<td>$\Pi_{\text{obs}}^{\text{max}}$</td>
<td>Maximum Observed Spreading pressure (mN/m)</td>
</tr>
<tr>
<td>$r_v$</td>
<td>Protein radius (cm)</td>
</tr>
<tr>
<td>$t$</td>
<td>Time (seconds)</td>
</tr>
<tr>
<td>$t^{1/2}$</td>
<td>Sqrt(time) (s$^{1/2}$)</td>
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<tr>
<td>$V_I$</td>
<td>Interphase volume (cm$^3$)</td>
</tr>
<tr>
<td>$V_p$</td>
<td>Protein molar volume (cm$^3$/mole)</td>
</tr>
<tr>
<td>$W_B^{\text{max}}$</td>
<td>Bulk weight/volume concentration</td>
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<tr>
<td>$W_I^{\text{max}}$</td>
<td>Interphase weight/volume concentration</td>
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Chapter 4

Liquid-Vapor Interfacial Tension of Blood Plasma, Serum and Purified Protein Constituents Thereof

Abstract

A systematic study of water-air (liquid-vapor, LV) interfacial tension \( \gamma_{lv} \) of blood plasma and serum derived from four different mammalian species (human, bovine, ovine and equine) reveals nearly identical concentration-dependence (\( \frac{d\gamma_{lv}}{d \ln C_B} \); where \( C_B \) is plasma/serum dilution expressed in v/v concentration units). Comparison of results to a previously-published survey of purified human-blood proteins reveals that \( \frac{d\gamma_{lv}}{d \ln C_B} \) of plasma and serum is surprisingly similar to that of purified protein constituents. It is thus concluded that any combination of blood-protein constituents will be substantially similar because \( \frac{d\gamma_{lv}}{d \ln C_B} \) of individual proteins are very similar. Experimental results are further interpreted in terms of a recently-developed theory emphasizing the controlling role of water in protein adsorption. Accordingly, the LV interphase saturates with protein adsorbed from bulk solution at a fixed weight-volume concentration (~436 mg/mL) independent of protein identity or mixture. As a direct consequence, \( \frac{d\gamma_{lv}}{d \ln C_B} \) of purified proteins closely resembles that of mixed solutions and does not depend on the relative proportions of individual proteins comprising a mixture. Thus variations in the plasma proteome between species are not reflected in \( \frac{d\gamma_{lv}}{d \ln C_B} \) nor is serum different from plasma in this regard, despite being depleted of coagulation proteins (e.g. fibrinogen). A comparison of pendant-drop and Wilhelmy-balance tensiometry as tools for assessing protein \( \gamma_{lv} \) shows that measurement conditions employed in the typical Wilhelmy plate approach fails to achieve the steady-state adsorption state that is accessible to pendant-drop tensiometry.
4.1. Introduction

Clinical interest in the water-air (liquid-vapor, LV) interfacial tension $\gamma_{lv}$ of blood plasma and serum extends back at least eight decades to the pioneering work of Morgan and Woodward who, in 1913, used the venerable drop-weight method to demonstrate that $\gamma_{lv}$ of blood serum was much lower than that of water. Related work further showed that pathological conditions such as syphilis, typhoid, tuberculosis, and cancer led to statistically-significant reduction in serum $\gamma_{lv}$. Otherwise, however, it was found that plasma/serum $\gamma_{lv}$ was remarkably consistent within and among species (human, canine, porcine, equine and ovine); with a lower $\gamma_{lv}$ limit near 45 mN/m and an upper bound no greater than 48 mN/m for normal donors. Studies carried out between 1916-1929 by thought leaders of the day such as William Harkins and Pierre DuNuoy debated the best method for measuring equilibrium $\gamma_{lv}$ of biological fluids (see also the 1929 work of Jolin). DuNuoy and Harkins reported that $\gamma_{lv}$ of plasma was about 10 mN/m higher than that reported by Morgan and Woodward. Still later in 1935, Zozaya undertook a comprehensive study of human blood serum, reporting an average value of 54.2 mN/m. Recent work by Kratochvil et al. reports values within the 60-68 mN/m range; with yet higher values for plasma derived from patients with disease conditions such as rheumatoid arthritis, proliferative glomerulonephritis, some tumors of the central nervous system, and disorders of the urogenital tract.

Our main interest in blood plasma and serum interfacial tension lies not in the dissimilarities observed between normal and pathological groups but rather in the impressive similarity in $\gamma_{lv}$ of plasma and serum prepared from normal blood of diverse species. We find this consistency
remarkable in view of the substantial differences in plasma proteome across species. And of course serum is substantially depleted of fibrinogen relative to plasma, removing one of the five most abundant proteins (2.5-4.5 mg/mL). Specifically, we are curious to know in detail how it happens that $\gamma_w$ values for these proteinaceous mixtures are so similar in light of the general expectation that compositional/structural differences among proteins should result in quite different adsorption energetics and commensurately different concentration-dependent $\gamma_w$.

Stated somewhat more precisely, we wish to know the ‘mixing rules’ that relate protein type or MW and bulk-phase composition to observed $\gamma_w$ and how these rules explain adsorption from complex proteinaceous milieu such as blood plasma and serum.

This paper reports a systematic survey of concentration-dependent water-air (liquid-vapor, LV) interfacial tension $\gamma_w$ of blood plasma and serum derived from human and animal species, measured by both pendant drop and Wilhelmy balance tensiometry (PDT and WBT, respectively). PDT results generally corroborate that of Morgan and Woodward mentioned above in that we find considerable consistency in interfacial tension across species with an average $\gamma_w \sim 45$ mN/m. Comparison of PDT and WBT results suggest that historical lack of agreement between investigators studying plasma/serum $\gamma_w$ is related to the slow achievement of steady-state protein adsorption that effectively invalidates tensiometric methods in which the LV interface is continuously moving or forming (as in typical applications of WBT or drop-weight tensiometry). We further report that concentration-dependent $\gamma_w$ of plasma and serum is surprisingly similar to that of purified constituents by comparing results to survey of human-
blood proteins\textsuperscript{14}. A newly-developed theory of protein adsorption\textsuperscript{13,15} is employed to reconcile experimental findings.

4.2. Materials and Methods

Purified Proteins and Protein Mixtures: Human platelet-poor plasma (citrated) was prepared from outdated (within 2 days of expiration) lots obtained from the Hershey Medical Center Blood Bank. Human serum was prepared in 15 mL batches by recalcification with 0.1 M \( \text{CaCl}_2 \) at 5:1 v/v plasma:calcium ratio in clean glass scintillation vials for about 15 min. Bovine, ovine and equine plasma and serum were used as received from Hemaresource and Supply Inc. (Aurora, OR) and were not subject to any additional fractionation/purification steps. Purified bovine albumin, both Fraction V (FV) and Fatty Acid Free (FAF), were used as received from Sigma-Aldrich and were the highest purity available (>96\% as assessed by SDS PAGE). Mass, concentration, and molecular weights supplied with purified proteins were accepted without further confirmation. Ref.\textsuperscript{14} discloses details of protein purity and protein-solution preparation including serial dilutions of protein stock solutions (usually 10 mg/mL) that were performed in 96-well microtiter plates by (typically) 50:50 dilution in phosphate buffered saline solution (PBS) prepared from powder (Sigma Aldrich) in distilled-deionized (18 M\( \Omega \)) water (interfacial tension of PBS and water was checked periodically by Wilhelmy-balance tensiometry).

Liquid-Vapor Interfacial Tension Measurements: LV interfacial tensions \( \gamma_{lv} \) reported in this work were measured by either Wilhelmy-balance tensiometry (WBT) or pendant-drop tensiometry (PDT). Unless specifically indicated otherwise, results refer to PDT performed on a commercial automated tensiometer (First Ten Angstroms Inc., Portsmouth VA). Briefly, the
tensiometer employed a Tecan liquid-handling robot to aspirate between 10-12 µL of solutions contained in a 96-well microtiter plate prepared by the serial-dilution protocol mentioned above. The robot was used to reproducibly transfer the tip with fluid contents into a humidified (99+ % RH) analysis chamber and dispense between 6-11 µL pendant drops (smaller drop volume required for lower interfacial tensions) within the focal plane of a magnifying camera. These and all other aspects of pendant-drop analysis were performed under computer control. Precision of $\gamma_n$ was about 0.5 mN/m based on repeated measurement of the same pendant drop. The instrument was calibrated against pure water interfacial tension and further confirmed on occasion against WBT. The analysis chamber was thermostated to a lower-limit of 25±1 °C by means of a computer-controlled resistive heater. Upper-temperature limit was, however, not controlled but rather floated with laboratory temperature, which occasionally drifted as high as 29 °C during summer months. Thus, reported $\gamma_n$ values are probably not more accurate than about 1 mN/m on an inter-sample basis considering the small but measurable variation of water interfacial tension with temperature. This range of accuracy is deemed adequate to the conclusions of this report which do not strongly depend on more accurate $\gamma_n$ that is difficult to achieve on a routine basis with the multiplicity of protein solutions investigated herein.

WBT was performed using a commercial computer-controlled instrument (Camtel CDCA 100, Royston UK) applying techniques discussed in detail elsewhere.° Solvent-and-plasma-discharge-cleaned glass coverslips were used as the plate (Fisher brand 22X30 mm #1) and solutions (approximately 10 mL) were contained in disposable polystyrene beakers (Fisher) previously determined not to measurably affect interfacial tension of water contained therein. No attempt was made to thermostat the balance and all reported measurements were obtained at
ambient laboratory temperature. Also, no attempt was made to correct for the (presumably small but not measured) variation in the perimeter of glass coverslips used as plates in the Wilhelmy method.

**Computation and Data Representation:** Computational, statistical, and theoretical methods used in this work have been discussed in detail elsewhere \(^\text{16}\). Briefly, time-dependent \(\gamma_v\) data corresponding to solutions at different w/v or v/v concentration \(C_B\) (for purified proteins or protein mixtures respectively) were recovered from PDT files and correlated with concentrations, leading to a matrix of results with row values representing concentration and time (seconds) as column values. It was generally observed that \(\gamma_v\) data take on a sigmoidal shape when plotted on logarithmic-concentration axes \(^\text{16}\), with a well-defined low-concentration asymptote \(\gamma_v^o\) and a high-concentration asymptote \(\gamma_v^\prime\). Successive non-linear least-squares fitting of a four-parameter logistic equation \(\gamma_v = \{(\gamma_v^o - \gamma_v^\prime)/(1 + (\ln C_B^{\Pi/2} / \ln C_B^\Pi)^M)\} + \gamma_v\) to concentration-dependent \(\gamma_v\) data for each time within the observation interval quantified \(\gamma_v^o\) and \(\gamma_v^\prime\) parameters with a measure of statistical uncertainty. Fitting also recovered a parameter measuring concentration-at-half-maximal-change in interfacial activity, \(\ln C_B^{\Pi/2}\) (where \(\Pi/2 = 1/2\Pi^{\max}\) and \(\Pi^{\max} \equiv \gamma_v^o - \gamma_v^\prime\)), as well as a parameter \(M\) that measured steepness of the sigmoidal curve. This empirical, multi-parameter fitting to concentration-dependent \(\gamma_v\) data was a purely pragmatic strategy that permitted quantification of best-fit protein and surfactant characteristics but is not a theory-based analysis \(^\text{16}\). Three-dimensional (3D) representations of time-and-concentration-dependent \(\gamma_v\) data were created in Sigma Plot (v8) from the data matrix discussed above and
overlaid onto fitted-mesh data computed from least-squares fitting. Two-dimensional (2D) representations were created from the same data matrices at selected observation times.

4.3. Results

The principal experimental observations of this work were time- and concentration-dependent water-air (liquid-vapor, LV) interfacial tension $\gamma_{lv}$ of human and animal (bovine, ovine, equine) blood plasma and serum. $\gamma_{lv}$ of serially-diluted, whole plasma or serum was measured using either or both pendant-drop (PDT) and Wilhelmy-balance tensiometry (WBT), as detailed in the Materials and Methods section, resulting in graphical constructions herein referred to as ‘$\gamma_{lv}$ curves’. Figures 1-3 are $\gamma_{lv}$ curves summarizing experimental results. Table I compiles quantitative results from statistical fitting of steady-state (equilibrium) $\gamma_{lv}$ data for each species studied. Purified proteins listed in Table I (FV and FAF BSA) supplement a previously-published survey of purified blood proteins spanning three decades in molecular weight (MW) 14.

Fig. 1 compiles representative PDT results for human (HS, panel A), ovine (OS, panel B), and equine blood serum (ES, panel C). Concentration-dependent $\gamma_{lv}$ was observed to follow a sigmoidal-like function on logarithmic concentration axis; yielding parameters $\gamma_{lv}^o$, $\gamma_{lv}^\prime$, $\ln C_B^{11/2}$, and $M$ when statistically-fit to a four-parameter logistic equation as described in Materials and Methods. In each case represented in Fig. 1, $\gamma_{lv}$ was observed to change with time to a final, steady state $\gamma_{lv}^\prime$ value. Dynamics were undoubtedly due to protein mass-transfer and adsorption-kinetic effects. Characteristic $\gamma_{lv}$-curve parameters (see Methods and Materials) listed in
columns 2-6 of Table I compare PDT and WBT results. Parameters for PDT represent the mean of the final 25 $\gamma_{lv}$ curves recorded within the 1-hour observation time of the PDT experiment, with standard deviation of the mean $\gamma_{lv}$ used as a measure of statistical uncertainty. Parameters for WBT result from best fit to a single, concentration-dependent $\gamma_{lv}$ curve because no time dependence was recorded in WBT experiments. Here, uncertainty was taken to be the standard-error-of-the-fit to the four-parameter logistic equation (Methods and Materials). Note that WBT was applied only to plasma and serum solutions because the high volume requirement (~ 10 mL) made WBT impractical for purified proteins available in only small quantities. Replicate preparations for HP, HS, and albumin (bovine FV and FAF) provide a sense for the variation in results associated with the whole analytical process leading from concentrate preparation, serial dilution, and finally $\gamma_{lv}$ measurement. Close inspection of columns 2 and 3 of Table I reveals that $\gamma_{lv}^0$ parameters (pure PBS) obtained by PDT and WBT were not significantly different. However, WBT $\gamma_{lv}'$ values (column 3) were frequently (9/12 cases) higher than PDT by about 10 mN/m. This discrepancy between techniques propagates directly into differences in $\Pi_{max}$ values listed in column 6. Likewise, $\ln C_B^{1/2}$ (column 4) and $M$ (column 5) parameter estimates from PDT and WBT $\gamma_{lv}$ curves were in substantial disagreement. Taken together, this data shows that PDT and WBT give different measures of concentration-dependent $\gamma_{lv}$ for protein mixtures. In fact, we have found that PDT $\gamma_{lv}$ curves most strongly resemble that of WBT at early observation times within the 1 hr PDT measurement window (not shown), suggesting that lack of agreement between PDT and WBT was related to dynamic effects (mass transport and adsorption kinetics).
Fig. 2 graphically compares representative PDT steady-state $\gamma_\nu$ curves for human, bovine, ovine and equine plasma serum on a single set of axis where the surrounding band encloses data within 99% confidence intervals around the best-fit human-plasma data listed in Table I. Fig. 2 demonstrates that plasma and serum of diverse species could not be confidently distinguished based on this data. Fig. 3 is similar to Fig. 2 comparing new-born, calf, and adult bovine plasma/serum; where the band encloses 99% confidence intervals around the best-fit, adult-bovine-plasma data listed in Table I. Fig. 3 demonstrates that plasma and serum derived from different age groups of a single species could not be confidently distinguished based on this data. However, the high-concentration asymptote $\gamma_\nu^{'}$ among bovine species appears not as well-defined as observed for other species studied (Fig. 1), for as-yet unresolved reasons. However, Figs. 1-3 collectively show that there is a striking similarity in $\gamma_\nu$ curves for plasma and serum across both donor species and age. Serum was, of course, compositionally distinct from plasma by virtue of being depleted of fibrinogen in the process of coagulating plasma.

**4.4. Discussion**

**Quantitative Comparison of PDT and WBT:** It is common knowledge in modern surface science that proteins exhibit slow (relative to low MW surfactants) mass transport and adsorption kinetics that depend strongly on molecular size (MW; see, for example, refs. \textsuperscript{17,18} and citations therein). These dynamics are responsible for the time dependence observed in interfacial tensions \textsuperscript{19}. In the early 1900’s, however, these phenomena were being observed for the first time (through the pioneering work of DuNouy, Morgan, and Harkins briefly reviewed in the Introduction section), as were consequences of changing LV surface area during $\gamma_\nu$ measurement. Meniscus stretching in WBT measurements or expansion of droplets in drop-
weight tensiometry conspires with adsorption kinetics by inducing movement of protein to
freshly-formed surface (see refs. 19,20 for more discussion). As a consequence, adsorption may
not reach equilibrium and \( \gamma \) not achieve the lowest possible value. Thus, historical debate in
the literature regarding the equilibrium value of plasma/serum \( \gamma \) is not surprising in retrospect,
given the widespread reliance on either drop-weight or DuNouy ring tensiometry in that era.
Discrepancies among more recent investigators 9,10 may also be related to dynamic effects in
plasma/serum \( \gamma \) measurements.

We attribute the significant differences between WBT and PDT measurement of concentration-
dependent \( \gamma \) of plasma/serum quantified in Table I to a failure to achieve steady-state
adsorption in WBT measurements 14. By contrast, the stagnant drop used in PDT yields a
verifiable steady-state (equilibrium; see Fig. 1) and a commensurately lower \( \gamma \) than observed in
WBT. Furthermore, we contend that WBT, as applied in this work, does not measure adsorption
kinetics in the same way as PDT due to quite different mass-transfer and adsorption processes
occurring at the moving plate-meniscus region compared to the stagnant-drop interface. These
differences are negligible at very low protein concentrations (WBT and PDT each yielding \( \gamma \sim
72 \text{ mN/m consistent with pure PBS}) but become increasingly important at higher concentration;
leading to significant discrepancies in \( \gamma \), \( \ln C_B^{1/2} \), and \( M \) between tensiometric methods 14.
Interestingly, the average PDT \( \gamma \sim 45 \text{ mN/m was in close (but undoubtedly fortuitous)}
agreement with the work of Morgan and Woodward 1. WBT \( \gamma \sim 52 \text{ mN/m closely matches that
of Harkins}^3, presumably because WBT and drop-weight tensiometry (as applied by
investigators) undershoots steady state in approximately the same way. Hence, we contend that
neither drop-weight nor WBT (with a continuously moving solid-liquid interface) are appropriate methods for measuring protein $\gamma_{lv}^{14,16,21}$.

**Concentration-Dependent $\gamma_{lv}$ of Plasma, Serum, and Protein Constituents Thereof:**

Similarity in concentration-dependent $\gamma_{lv}$ of blood plasma and serum obtained from normal donors of diverse species and age groups is very intriguing in light of the substantial differences in protein composition. Observations collected in Figs. 1-3 corroborating general trends of experimental work dating back to the early 1900’s are all the more compelling in light of our recent findings that $\frac{d\gamma_{lv}}{d\ln C_p}$ of a broad range of purified blood-protein constituents are likewise similar $^{14}$. Furthermore, we find that the saturating minimum interfacial tension $\gamma'_v$ falls within a relatively narrow 20 mN/m band characteristic of all proteins and mixtures studied. Experiment thus suggests that $\frac{d\gamma_{lv}}{d\ln C_p}$ of any combination of blood-proteins is very similar because $\frac{d\gamma_{lv}}{d\ln C_p}$ of individual protein constituents are very similar $^{14,15}$. All taken together, we find this evidence suggestive of a heretofore unresolved mechanistic commonality underlying protein adsorption from complex mixtures to the LV surface.

Our recently-developed theory of protein adsorption to the LV surface $^{13,15}$ asserts that the mechanistic commonality mentioned above is the behavior of water at interfaces $^{22-24}$. Theory suggests that interfacial capacity for protein is controlled by the extent to which interfacial water can be displaced by adsorbing protein molecules. This maximal interfacial capacity can be expressed either as a maximal volume fraction or, equivalently, a maximum weight/volume (not molar) concentration denoted $W_i^{\max}$ at which $\gamma'_v$ occurs. Calibration of theory to experimental
neutron-reflectivity or light scattering data has revealed that $W_i^{\text{max}} \sim 436 \text{ mg/mL}$, corroborating the conclusion drawn from diverse literature sources that adsorbed protein concentrations can be surprisingly large \(^{22}\). Importantly, $W_i^{\text{max}}$ is found not to be dependent on protein identity or MW but rather is a generic, water-limited adsorption capacity for protein. Furthermore, $W_i^{\text{max}}$ can be attained by any mixture of proteins, which is of relevance in the interpretation of concentration-dependent $\gamma_i$ of multi-component protein mixtures such as blood plasma or serum, as further discussed below.

First, fixed $W_i^{\text{max}}$ immediately explains the experimental observation that $\gamma_i$ curves for diverse purified proteins spanning three decades of MW appear more similar than dissimilar when scaled on a w/v basis \(^{15}\). Serial dilution of a protein solution at surface-saturating concentration $W_i^{\text{max}}$ exhibiting the characteristic interfacial tension $\gamma_i'$ will trace similar concentration dependence because $\frac{d\gamma_i}{d \ln C_b}$ of individual proteins are very similar. Second, fixed $W_i^{\text{max}}$ rationalizes the experimental observation that $\gamma_i$ curves for plasma and serum are substantially similar, regardless of species or age of normal donor. At 60-85 mg/mL total protein (including fibrinogen) \(^{25}\), plasma and serum is well over the $\sim 3$ mg/mL required to saturate the LV surface at $W_i^{\text{max}}$, achieving minimum interfacial tension $\gamma_i'$ (see Fig. 1A). Depletion of coagulation proteins in the conversion of plasma to serum certainly changes the number and concentration of individual protein constituents in bulk solution, but does not alter $W_i^{\text{max}}$ because the bulk-solution concentration is well above that required to saturate the LV surface. As a consequence, concentration-dependent $\gamma_i$ curves of plasma and serum are nearly identical
because other protein constituents compete for the interfacial vacancies effectively created by removal of proteins consumed in the coagulation process (e.g. conversion of fibrinogen to insoluble fibrin). Third, although variations in plasma proteome among species and age groups lead to a different weight-fraction distribution of proteins in the bulk phase and surface, these differences do not affect surface saturation ($W_i^{\text{max}} \sim 436 \text{ mg/mL}$). And, because $\frac{d\gamma_i}{d \ln C_i}$ of individual protein constituents are very similar, the net observed $\gamma_i$ curves for plasma derived from different species are not significantly different.

However, fixed $W_i^{\text{max}}$ does not explain why $\gamma_i$ of plasma/serum from abnormal (pathological) donors is (reportedly) different from that of normal donors (see Introduction). Perhaps disease states ‘contaminate’ plasma with unknown compounds that affect $\gamma_i$ or accurate measurement of plasma $\gamma_i$. Given the rather substantial impact methods of $\gamma_i$ measurement have on results, we are inclined to suspect that some or all of the 5-10 mN/m discrepancies between normal and pathological plasma $\gamma_i$ can be traced directly to metrological issues. Values as high as 60-68 mN/m$^9$,$^{10}$ cannot be reconciled with our theoretical interpretation of plasma $\gamma_i$. In a more general sense, it is difficult to rationalize how plasma $\gamma_i$ can rise to such high levels without invoking an improbable, wholesale dilution of blood. Another experimental observation not reconciled by theory is failure of bovine plasma (at various ages) to achieve a sharp plateau (Fig. 3). Bovine plasma is unlike that of other species studied in this regard (compare Figs 1-2) for as yet unresolved reasons.
4.5. Conclusions

The principal conclusion to be drawn from this and related work\textsuperscript{14,15} is that differences in concentration-dependent water-air (liquid-vapor, LV) interfacial tension $\frac{d\gamma_{lv}}{d\ln C_b}$ among blood plasma, serum, and purified constituents thereof are surprisingly small. Likewise, differences in $\frac{d\gamma_{lv}}{d\ln C_b}$ among plasma and serum derived from disparate species (bovine, ovine, human) or within a species at different stages of development (new-born, calf and adult bovine) are not resolvable within 99% statistical confidence. Although these experimental observations extend and corroborate results of work published in the early part of the 20\textsuperscript{th} century\textsuperscript{1-4,6}, collective implications for protein-adsorption mechanisms have apparently been considered only recently\textsuperscript{13}. Our interpretation is that the insensitivity of $\frac{d\gamma_{lv}}{d\ln C_b}$ to substantial differences in plasma proteome among donor species, and the obvious difference between plasma and serum chemistry within these groups, arises from an invariance in protein amphilicity (interaction energetics with water) with protein type (molecular weight or size)\textsuperscript{14}.

Adsorption of proteins from heterogeneous aqueous solution can be accurately viewed as a competition for space in the LV surface region\textsuperscript{13}. LV surfaces of concentrated protein solutions such as plasma or serum are always saturated with protein. Thus, enrichment or depletion of any particular protein in bulk solution does not have a significant effect on $\frac{d\gamma_{lv}}{d\ln C_b}$ because substitution of one protein for another within the surface region does not affect surface saturation.
Acknowledgments

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Citations


(4) Nouy, P. L. D. *Surface Equilibria of Biological and Organic Colloids*; The Chemical Catalog Co.: New York, 1926; Vol. 27.


(20) Valkovska, D. S.; Shearman, G. C.; Bain, C. D.; Darton, R. C.; Eastoe, J.


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## Table 1
Comparison of Pendant-Drop Tensiometry (PDT) and Wilhelmy Balance Tensiometry (WBT) of Purified Proteins and Protein Mixtures

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Figure 1: Interfacial tension profiles in 3D ($\gamma_{iv}$ as a function of analysis time (drop age) and logarithmic (natural) solution concentration $C_{iv}$ ) and 2D ($\gamma_{iv}$ as a function of logarithmic solution concentration $C_{iv}$ at selected times) formats comparing human (HS, panel A, preparation 2, Table I), ovine (OS, panel B), and equine (ES, panel C) serum. In each case, solute concentration $C_{iv}$ is expressed in v/v units of parts-per-trillion (PPT, mL/10^{12} mL solution). Symbols in 2D panels represent time slices through 3D representations (filled circle = 0.25 sec, open circle = 900 sec, filled triangles = 1800 sec, and open triangles = 3594 sec; annotations in panel A indicate maximum ($\Pi_{max}$) and half-maximum spreading pressure ($1/2 \Pi_{max}$)). Concentration-dependent interfacial tensions of blood serum are nearly identical across diverse species.
**Figure 2:** Comparison of steady-state, concentration-dependent $\gamma_h$ data for human

(HP, filled circle, preparation 2, Table I), ovine (OP, open circle), equine (EP, open square) and bovine (BP, open triangle) plasma on a single concentration axis. Band represents 99% confidence intervals around best-fit-human plasma data, suggesting that species cannot be confidently distinguished from this data. In each case, solute concentration $C_B$ is expressed in v/v units of parts-per-trillion (PPT, mL/10^{12} mL solution).
Figure 3: Comparison of steady-state (t = 3600 sec), concentration-dependent $\gamma_\nu$ data for bovine plasma collected at different ages. Filled circles = adult bovine plasma (BP), open squares = calf plasma (CP) and open triangle = new-born calf plasma (NBCP). Band represents 99% confidence intervals around adult plasma, suggesting that age groups cannot be confidently distinguished from this data. In each case, solute concentration $C_\nu$ is expressed in v/v units of parts-per-trillion (PPT, mL/10^{12} mL solution).
Chapter 5

Scaled Interfacial Activity of Proteins at a Hydrophobic Solid/Aqueous-Buffer Interface

Abstract

Contact-angle goniometry confirms that interfacial energetics of protein adsorption to the hydrophobic solid-water (solid-liquid, SL) surface is not fundamentally different than adsorption to the water-air (liquid-vapor, LV) interface measured by pendant-drop tensiometry. Adsorption isotherms of 9 globular blood proteins with molecular weight (MW) spanning 10-1000 kDa on methyl-terminated self-assembled monolayer surfaces demonstrate that (i) proteins are weak surfactants, reducing contact angles by no more than about 15° at maximum solution concentrations (~10 mg/mL); (ii) the corresponding dynamic range of spreading pressure $\Pi_a < 20$ mN/m; and (iii) the maximum spreading pressure $\Pi_a^{\text{max}}$ for these diverse proteins falls within a relatively narrow 5 mN/m band. As with adsorption to the LV interface, we find that concentration scaling substantially alters perception of protein interfacial activity measured by $\Pi_a$. Proteins appear more similar than dissimilar on a weight/volume basis whereas molarity scaling reveals a systematic ordering by MW, suggesting that adsorption is substantially driven by solution concentration rather than diversity in protein amphility. Scaling as a ratio-to-physiological-concentration demonstrates that certain proteins exhibit $\Pi_a^{\text{max}}$ at-and-well-below physiological concentration whereas others require substantially higher solution concentration to attain $\Pi_a^{\text{max}}$. Important among this latter category of proteins is blood factor XII, assumed by the classical biochemical mechanism of plasma coagulation to be highly surface active, even in the presence of overwhelming concentrations of other blood constituents such as albumin and immunoglobulin that are shown by this work to be among the class of highly-surface-active proteins at physiologic concentration. The overarching interpretation of this work is that water plays a dominant, controlling role in the adsorption of globular-blood proteins to hydrophobic surfaces and that energetics of hydration control the amount of protein adsorbed to poorly-water-wettable biomaterials.
5.1. Introduction

The protein adsorption problem – how (glyco)proteins selectively collect at biomaterial surfaces from complex solutions such as blood; the structure-property relationships connecting surface chemistry/energy to the extent and specificity of protein adsorption; and the manner by which surface-bound protein directs ‘biological responses’ such as blood coagulation, cell/tissue adherence, or fouling of medical devices – remains one of the quintessential unsolved mysteries of biomaterials surface science. And yet a full understanding of protein adsorption is essential to prospective, fundamentals-driven design of biomaterials. This is because protein adsorption is among the first molecular-level interactions with a biomaterial that ultimately lead to biocompatibility - or lack thereof (see, for example, refs. 1-5 and citations therein).

Our work probing each of the above aspects of the protein adsorption problem strongly implicates water as a moderating or mediating agent.\textsuperscript{6-10} We have pursued the long-standing hypothesis that surface interactions with water and reorganization of ions near the water-contacting surface precede protein adsorption,\textsuperscript{11} forming a hydration layer extending some (generally unknown) distance from the water-contacting surface with which protein molecules interact.\textsuperscript{10} This layer is variously referred to as bound water\textsuperscript{12-14} or vicinal water\textsuperscript{6,15} or as an interphase;\textsuperscript{16,17} depending on the context and application. However termed, we find that vicinal water controls protein adsorption to surfaces by resisting displacement with adsorbing protein molecules. In turn, resistance to displacement depends on the affinity of water for the surface,\textsuperscript{10,17} and so protein adsorption is found to scale with surface energy (water wettability).\textsuperscript{18-20} According to this view, water-wettable surfaces (a.k.a hydrophilic)\textsuperscript{21} with strongly-bound vicinal water adsorb little-or-no protein directly onto the surface; although this does not
necessarily rule out collection of protein near the surface in a manner that does not require dehydration of the surface.\textsuperscript{6,10,22-24} By contrast, proteins can adsorb directly onto poorly-water-wettable (hydrophobic) surfaces by displacing relatively weakly-bound vicinal water.\textsuperscript{18-20}

Detailed examination of these basic principles operating at the water-air (liquid-vapor, LV) interface, a molecularly-smooth hydrophobic surface, strongly suggests that water does indeed orchestrate a clearly-discernable, systematic pattern in protein adsorption.\textsuperscript{25} Specifically, we find that: (i) variation in globular blood protein “amphilicity” (interaction energetics with water) with composition spanning 3 decades of molecular weight (MW) is surprisingly modest; (ii) the equilibrium partition coefficient $P$ (ratio of interphase-to-bulk concentration) is relatively constant across this diverse group of proteins ($10^2 < P < 10^3$; with $P \sim 150$ taken as nominal value); (iii) interphase capacity for protein is limited by the extent to which the LV surface can be dehydrated by displacement of interfacial water by adsorbing protein; and that (iv) molar concentrations required to fill the LV interphase follow a “homology in protein size” consistent with packing hydrated spheroidal molecules within this space.\textsuperscript{16} This latter effect is particularly interesting because it predicts that molar-interphase concentrations scale inversely with protein MW and, as a consequence, multiple layers of higher-MW proteins (MW > 125 kDa) are required to fully fill the LV surface. For example, one molecular layer of albumin (MW = 66.3 kDa) is predicted to fully saturate the LV surface whereas two layers of IgG (MW = 160 kDa) are required (both of which have been confirmed experimentally),\textsuperscript{26,27} and five layers of IgM (MW = 1000 kDa) form at the saturated LV surface\textsuperscript{16} (which apparently awaits experimental verification).
It turns out that fixed LV surface capacity for protein at approximately constant $P$ imposes significant constraints on the protein adsorption problem. That is to say, because protein adsorption is more about solvent than adsorbate, a tractable quasi-thermodynamic theory describes a phenomenon that would otherwise be overwhelmingly complex for more than just a few proteins in solution. In fact, a straightforward set of “mixing rules” stipulates both concentration and weight-fraction distribution of proteins adsorbed to the LV interphase from multi-component aqueous solutions such as blood plasma or serum at equilibrium.\textsuperscript{28} These mixing rules rationalize the long-known but otherwise unexplained observations that (i) LV interfacial tension $\gamma_{lv}$ of blood plasma and serum is nearly identical, in spite of the fact that serum is substantially depleted of coagulation proteins such as fibrinogen; and (ii) $\gamma_{lv}$ of plasma and serum derived from human, bovine, ovine, and equine blood is practically identical, even though there are substantial differences in the plasma proteome among these species.\textsuperscript{29} Thus, in summary, we find that protein adsorption to the LV surface, including certain aspects of the Vroman effect,\textsuperscript{28} can be quite well understood on the basis of the behavior of water at surfaces.

This paper discloses results of an investigation of protein adsorption to a well-defined, hydrophobic solid-water (solid-liquid, SL) interface. Methyl-terminated self-assembled thiol monolayers (SAMs) on gold-coated semiconductor-grade silicon wafers exhibiting water contact angles $\theta_a \sim 110^\circ$ are used as test substrata. Time-and-concentration-dependent contact angles are used to measure adsorption energetics of (globular) proteins spanning 3 decades in MW in a manner that parallels the above-cited studies of protein adsorption to the LV surface. We find that the basic pattern observed at the LV surface is repeated at the hydrophobic SL surface,
supporting our contention that water is the significant controller of protein adsorption to biomaterial surfaces.

5.2. Materials and Methods

**Purified Proteins and Synthetic Surfactants:** Table 1 compiles pertinent details on proteins and surfactants used in this work. Protein purity was certified by the vendor to be no less than the respective values specified in Column 4 of Table 1, as ascertained by electrophoresis (SDS-PAGE or IEP). Mass, concentration, and molecular weights supplied with purified proteins were accepted without further confirmation. Issues associated with protein purity, especially contamination with surfactants, and the potential effect on measured interfacial tensions were discussed in detail in ref. 25. The single value given in Table 1 for physiological concentration of human proteins (Column 5) applied in this work was middle of the range listed by Putnam.\(^{30}\) Serial dilutions of protein stock solutions (usually 10 mg/mL) were performed in 96-well microtiter plates by (typically) 50:50 dilution in phosphate buffered saline solution (0.01 M PBS, 0.14 M NaCl, 0.003M KCl) prepared from powder (Sigma Aldrich) in distilled-deionized (18 M\(\Omega\)) water using procedures detailed in ref. 25 (interfacial tension of PBS and water was checked periodically by Wilhelmy-balance tensiometry). Between 24-30 dilutions were prepared in this manner, covering a dynamic range between \(10^{-10}\) to 1 % (w/v), taking care to mix each dilution by repeated pipette aspiration and avoiding foaming of concentrated solutions.

**Surfaces:** Methyl-terminated self assembled monolayer surfaces (SAMs) were prepared according to standard methods of surface engineering. Details involved have been reported elsewhere.\(^{31-35}\) Alkanethiol (Aldrich Chemical Co., Milwaukee, WI) and ethanol (commercial
reagent-grade) were used as-received, without further purification. Samples were stored in the thiol solution until use, and were rinsed with ethanol just prior to an experiment.

**Tensiometry & Goniometry:** Liquid-vapor interfacial tensions required by this work were measured by Pendant Drop Tensiometry (PDT) as described in refs. 16,25,28,29. Contact angle and wettability methods applied in this work have been disclosed in detail elsewhere, including verification that measured advancing angles ($\theta_a$) were in statistical agreement with that obtained by Wilhelmy balance tensiometry.$^{36,37}$ Receding angles ($\theta_r$) were shown to be not as reliable as $\theta_a$. Consequently, only $\theta_a$ was analyzed in this work. Briefly, for the purposes of this paper, $\theta_a$ measurements were made using a commercial automated tilting-plate goniometer (TPG, First Ten Angstroms Inc., Portsmouth VA). The goniometer employed a Tecan liquid-handling robot to aspirate 12 µL of solutions contained in a 96-well microtiter plate prepared by the serial-dilution protocol mentioned above. We found that dip-coating of disposable polypropylene tips in a commercial perfluorocarbon hydrophobizing agent (NYEBAR, NYE Lubricants Inc.; Bedford, MA) followed by water wash and air drying greatly aided dispensing of drops, especially at the highest protein concentrations (lowest $\gamma_v$). This coating procedure was shown not to measurably affect interfacial tensions or contact angles. The robot was used to reproducibly transfer the tip with fluid contents into a humidified (99+ % RH) analysis chamber and dispense 10 µL drops of protein solution onto the surface of test substrata (see below) held within the focal plane of a magnifying camera. These and all other aspects of tilting-plate goniometry were performed under computer control. Proprietary algorithms supplied by the vendor were used to deduce contact angles from drop images captured at a programmed rate by a frame grabber. Typically, 600 images were captured at a rate of 1 image every 6 sec following
0.25 sec delay to permit vibrations of the expelled drop to dampen. Drop evaporation rates within the humidified chamber deduced from computed-drop volumes (based on image analysis) were observed to vary with solute concentration, generally ranging from approximately 25 nL/min for pure water to 10 nL/min for solute solutions > 0.1% w/v. The impact of this evaporation rate over the 60 min time frame of the experiment was apparently negligible, as gauged from the behavior of purified surfactants discussed in the results section and in ref. 37. Precision of $\theta_a$ was about 0.5° based on repeated measurement of the same drop. The analysis chamber was thermostated to a lower-limit of 25±1 °C by means of a computer-controlled resistive heater. Upper-temperature limit was not controlled but rather floated with laboratory temperature, which occasionally drifted as high as 29 °C during summer months. Thus, reported $\theta_a$ values were probably not more accurate than about 1° on an inter-sample basis considering the small, but measurable, variation of water interfacial tension with temperature. This range of accuracy was deemed adequate to the conclusions of this report which do not strongly depend on more highly accurate $\theta_a$ that is difficult to achieve on a routine basis. Instead, veracity of arguments raised herein depend more on a breadth of reliable measurements made across the general family of human proteins.

Test substrata were held on a rotating, tilting-plate platform driven by stepper motors under computer control. Substrata were allowed to come to equilibrium within the sample-chamber environment for no less than 30 min before contact angle measurements were initiated. The platform was programmed to tilt at 1°/sec from horizontal to 25° after the drop was deposited on the surface by the robot. The optimal (incipient rolling) tilt angle was found to be 25° and 15° for solutions of proteins and surfactants, respectively. The first 120 sec (20 images) monitored
evolution of the advancing angle. At the end of the 1 hr $\theta_a$ measurement period, the platform was programmed to return to horizontal and rotate 15° to the next analysis position along the periphery of the semiconductor wafer. This process was repeated for all dilutions of the protein under study so that results reported for each protein were obtained on a single test surface, eliminating the possibility of substratum-to-substratum variation within reported results. We observed that the contact angle of a pure PBS droplet slowly decreased with time from the initial value of $108^\circ < \theta_a^o < 106^\circ$ at $t = 0$ to $104^\circ < \theta_a^o < 102^\circ$ at $t = 1$ hr; where $\theta_a^o$ is the pure buffer contact angle and the range of reported results corresponds to all of the 17 methyl-terminated SAM surfaces analyzed during the course of this work. We attribute this systematically-varying wettability to slow “hydration” of SAM surfaces, which may include dissolution of thiol from the surface into solution, but have no specific proof this was the cause. This same phenomenon apparently affects measurement of protein-solution contact angles because we observed that the whole contact angle isotherm ($\theta_a$ vs. concentration) slowly shifted lower with time (see Fig. 1B). We do not believe this slight but apparently unavoidable attribute of thiol-SAMs on silicon wafers negatively affects the veracity of conclusions drawn herein which were based on final, steady-state measurements made at ~ 1 hr analysis time.

**Computation and Data Representation:** Computational, statistical, and theoretical methods used in this work have been discussed in detail elsewhere.\textsuperscript{17-19} In brief, time-dependent $\theta_a$ data corresponding to protein dilutions (see above) were recovered from TPG files and correlated with concentrations, leading to a matrix of results with row values representing concentration and time (in sec) as column values. It was generally observed that $\theta_a$ isotherms were sigmoidal in shape when plotted on logarithmic-concentration axes,\textsuperscript{17,18} with well-defined low-
concentration asymptotes \( \theta_a^o \) and high-concentration asymptotes \( \theta_a' \) (see Fig. 1 for examples).

Successive non-linear least-squares fitting of a four-parameter logistic equation

\[
\theta_a = \frac{\theta_a^o - \theta_a'}{1 + (\ln C_B^{\Theta/2} / \ln C_B)^M + \theta_a'}
\]

to contact angle isotherms data for each time within the observation interval quantified \( \theta_a^o \) and \( \theta_a' \) parameters with a measure of statistical uncertainty.

Fitting also recovered a parameter measuring concentration-at-half-maximal-change in \( \theta_a \), \( \ln C_B^{\Theta/2} \) (where \( \Theta / 2 = 1/2 \Theta_{\text{max}} \) and \( \Theta_{\text{max}} = \theta_a^o - \theta_a' \)), as well as a parameter \( M \) that measured steepness of the sigmoidal curve. This multi-parameter fitting to concentration-dependent \( \theta_a \) data was a purely pragmatic strategy that permitted quantification of best-fit protein and surfactant characteristics but is not a theory-based analysis.\(^{17-19}\) Three-dimensional (3D) representations of time-and-concentration \( \theta_a \) data were created in Sigma Plot (v8) from the data matrix discussed above and overlain onto fitted-mesh data computed from least-squares fitting.

Two-dimensional (2D) representations were created from the same data matrices at selected observation times. Measured \( \theta_a \) were converted to advancing adhesion tension \( \tau_a = \gamma_v \cos \theta_a \) for general interpretation,\(^{17}\) where \( \gamma_v \) is the interfacial tension of the contact-angle fluid. Adhesion tensions \( \tau_a^o = \gamma_v^o \cos \theta_a^o \) (pure saline) and \( \tau_a' = \gamma_v' \cos \theta_a' \) (at the minimum contact angle observed \( \theta_a' \)) were computed with fitted parameters \( \gamma_v^o \) and \( \gamma_v' \) reported in ref. 25 for the proteins under investigation. Smoothed adhesion-tension isotherms (\( \tau_a \) vs. \( \ln C_B \)) were computed from smoothed \( \theta_a \) obtained from \( \theta_a \) isotherms above, using smoothed \( \gamma_v \) values computed from best-fit parameters reported in ref. 25. Likewise, smoothed spreading pressure isotherms (\( \Pi_a \) vs. \( \ln C_B \)) were computed from smoothed \( \tau_a \) curves where \( \Pi_a \equiv (\tau_a - \tau_a^o) \).
5.3. Results and Discussion

Results and discussion are combined into one section because it is efficient to introduce and discuss the different facets of the work in the sequence that follows and then combine separate observations in the Conclusions section. The presentation follows the basic outline used in a companion paper published in this journal describing protein adsorption to the LV surface.25

First, general characteristics of the quantitative data are described, comparing proteins to a surfactant reference compound and placing data into an overall context for interpretation. Results are then interpreted graphically by scaling concentration-dependent SL interfacial tensions in three different ways that give different insights into protein interfacial activity and provoke interpretation in terms of the behavior of water at surfaces.

**General Aspects of the Data:** Table 2 compiles quantitative results of this work. Contact angle parameters $\theta_a^\circ$, $\theta_a'$, $\ln C_B^{\theta/2}$ and M listed in cols 2-5 of Table 2 are the mean fitted values corresponding to final 25 $\theta_a$ curves recorded within the 60-minute time frame of the TPG experiment. Listed error is standard deviation of this mean. Corresponding adhesion tensions $\tau_a^\circ$ and $\tau_a'$ (Columns 6, 7) were computed from $\theta_a^\circ$ and $\theta_a'$ values, respectively, with uncertainty estimates computed by propagation of error in $\theta_a$ and $\gamma_H$ measurements (Methods and Materials). Maximum “spreading pressure” $\Pi_a^{\text{max}} \equiv (\tau_a' - \tau_a)$ (Column 8) was computed directly from aforementioned $\tau_a$ values and associated uncertainty again estimated by propagation of error. Replicate protein preparations were studied for Ub, FV HSA, FXII, IgM and $\alpha_2$-macroglobulin. Different vendors were used as a means of controlling for discrepancies that might arise from sourcing (Table 1). In consideration of all sources of error ranging from
solution preparation to data reduction (including purity of commercial protein preparations discussed in ref. 25), we conclude that variation associated with manipulation and dilution of protein concentrates (~10 mg/mL; includes transfer losses by adsorption to pipettes, vials, and wells; slight but inevitable foaming of proteins brought into solution from powder; etc.) overwhelms $\theta_a$ measurement error discussed in the Materials and Methods and that differences between preparations or vendors were insufficient to affect basic conclusions of this work.

**Quantitative Comparison of Proteins and Surfactants:** Concentration-dependent contact angles of protein and surfactant solutions ($\theta_a$ isotherms) on methyl-terminated SAMs fell into one of two categories, identified herein as Type 1, 2. Type 1 $\theta_a$ curves were clearly sigmoidal with distinct low- and high-concentration plateau (see, as examples, Figs. 1 A and B) measured by $\theta_a^\circ$ and $\theta_a$ parameters. Steady-state was apparently achieved in Type 1 adsorption at-or-before 1 hr drop age whereupon kinetic effects in $\theta_a$ were no longer resolvable at any concentration (less than about 1°). No semblance of a $\theta_a^\circ$ plateau was reached at the highest concentrations studied for Type 2 proteins (ubiquitin and $\alpha_2$-macroglobulin; see Fig. 1C for an example). Only graphical estimates are provided in Table 2 for Type 2 protein parameters since firm values could not be ascertained by statistical fitting procedures described in the Methods and Materials section. No attempt was made to estimate the exponential parameter M for Type 2 proteins. It was not evident from this work whether these proteins would exhibit Type 1 behavior if yet-higher concentrations were available for study. We note, however, that kinetic effects had fully dampened for Type 2 adsorption within the interval 3500 < t <3600 sec of the TPG experiment. This strongly suggests that steady-state had in fact been achieved, but that
higher solution concentrations were required to saturate the surface and achieve a verifiable $\theta_a$ plateau. Moreover, we note that large proteins such as IgM achieved steady state over the 60 min observation interval, confirming that mass transport and adsorption kinetics could indeed be completed within the time frame of the TPG experiment, even for much larger proteins. We thus conclude that Type 2 adsorption was concentration- rather than time-limited. Indeed, theoretical interpretation of protein adsorption to the LV interface suggests that surface-saturating concentrations for small proteins such as ubiquitin (10.7 kDa) exceed solubility limits, explaining why a verifiable $\theta_a$ plateau was not achieved for this protein. This same argument cannot be applied to results obtained for a molecule as large as $\alpha_2$-macroglobulin (725 kDa), however, and the outcome for this protein stands exceptional, not only to others listed in Table 1 but also to studies of adsorption of this protein to the LV surface.

$\theta_a^o$ (column 2, Table 2) is a measure of variability in surface-to-surface preparation procedures measured with a droplet of pure PBS in contact with the surface for 1 hr. Scanning down the rows of column 2, it is apparent that $\theta_a^o$ data fell between 104° and 100°. Much of this variability can probably be attributed to differences in substratum “hydration” that gives rise to a small but clearly-observable decrease in $\theta_a^o$ with time (see arrow annotation, Fig. 1B and Methods and Materials). Adsorption of either protein or surfactant to the solid surface from aqueous solution leads to measurable, concentration-dependent decrease in $\theta_a$ to lower limit values $\theta_a$ collected in column 3. In the case of proteins, this decrease in contact angle was quite modest, generally less than 15°, and showed little discernable trend among the proteins listed in Table 1. By contrast, the surfactant reference standard SDS gave rise to about 44° decrease in $\theta_a$. 
Adsorption energetics are best gauged in terms of adhesion tension $\tau_a = \gamma_{lv} \cos \theta_a$ because $\tau$ is linearly related to free energy of adsorption $\Delta G_{ads}$ that simultaneously accounts for adsorption to the LV and SL surface.$^{6,17-19}$ Columns 6 and 7 of Table 2 collect $\tau^*_a$ and $\tau^*_a$ parameters (corresponding to measured $\theta^*_a$ and $\theta^*_a$) and converts these to “spreading pressure” $\Pi_{a}^{\text{max}} \equiv \left( \tau^*_a - \tau^*_a \right)$ in column 8. Examination of $\Pi_{a}^{\text{max}}$ values shows that albumin and $\alpha_2$-macroglobulin exhibited the strongest biosurfactancy whereas C1q was weakest on this scale. However, from a broader perspective, it is apparent that proteins studied were more alike than dissimilar, as has been noted in adsorption to the LV surface.$^{16,25,28,29}$ Proteins clearly exhibited modest surfactancy compared to SDS for which $\Pi_{a}^{\text{max}} = 31 \text{ mN/m}.$ The free energy of protein adsorption to the hydrophobic surface $\Delta G_{ads}^o = -RT \ln P$ must be commensurately modest and, based on a partition coefficient $P \sim 150$ (see Introduction), $\Delta G_{ads}^o = -RT \ln P \sim -5RT$; consistent with estimates for lysozyme, myoglobin, and $\alpha$-amylase determined by hydrophobic interaction chromatography.$^{38}$

With the above in mind, it is of interest to estimate the extent that adsorption depletes a fluid phase in contact with a hydrophobic surface because it provides a tangible sense of protein surfactancy. Adopting 2 mg/m$^2$ as a nominal protein concentration adsorbed to the surface of a hypothetical 10 $\mu$L spherical drop (2.2X10$^{-5}$ m$^2$ surface area) from a surface-saturating 3 mg/mL bulk-phase composition (e.g. albumin adsorbed to the LV surface as determined by neutron reflectometry),$^{16,26}$ it is evident that only 47 ng or about 0.15 % of bulk-phase protein is removed by adsorption. Thus, it can be concluded that protein adsorbed from a contact-angle droplet to a hydrophobic surface does not significantly deplete the bulk fluid phase. That is to
say, proteins are weak surfactants. Indeed, it may be reasonably concluded from this simple analysis that proteins do not adsorb avidly to hydrophobic surfaces, as is commonly acknowledged, if solution depletion is used as the measure of adsorption. At the same time, however, it must be borne in mind that a partition coefficient $P \sim 150$ means that interphase concentrations expressed in mass or moles per-unit-volume (not mass or moles per-unit-surface area) are 150X bulk-phase compositions (i.e. $\sim 450 \text{ mg/mL}$ at surface saturation). Certainly, from this point of view, proteins do adsorb avidly to a hydrophobic surface. These seemingly divergent perspectives of protein adsorption can be reconciled by recognizing that the (LV or SL) interphase is very thin, between 1 and 5 molecular layers for the proteins studied herein, with a commensurately low total volume that effectively magnifies surface concentrations expressed in per-unit-volume dimensions. Thus, even though protein saturates a hydrophobic interphase at volume fractions as high as 1/3, the total mass of protein within this interphase is sparingly small in comparison to that contained in a macroscopic volume of bulk solution.

It is of special interest to the development of hemocompatible materials that FXII did not exhibit extraordinary adsorption behavior ($\Pi^\text{max}_a \sim 15 \text{ mN/m}$) because FXII adsorption from whole blood or plasma to procoagulant materials is thought to potentiate the intrinsic pathway of coagulation. As will be discussed subsequently, FXII surface activity is substantially mitigated by a low-physiologic concentration that does not sustain a high level of interfacial activity.

**Graphical Interpretation of Results:** Time-and-concentration-dependent $\theta_a$ for the anionic surfactant SDS, purified protein HSA (Fraction V, FV HSA), and $\alpha_2$-macroglobulin are compared in Fig. 1 in both three-dimensional (3D, $\theta_a$ as a function of time and concentration)
and two-dimensional (2D, $\theta_a$ as a function concentration at specified times) representations. Examining first 3D and 2D representations of SDS interfacial activity (Fig. 1A) which serves as a reference compound, it was observed that the $\theta_a$ curve was sigmoidal in shape, with a well-defined low-concentration asymptote $\theta_ao$ and a high-concentration asymptote $\theta_a'$ characteristic of Type 1 adsorption. In this latter regard, SDS and HSA exhibited similar concentration-limiting behavior (compare Fig. 1 A and B) that is typically interpreted as formation of a critical micelle concentration (CMC), at least for surfactants. This paper provides no evidence of micelles, for either proteins or surfactants, and so only acknowledges a limiting behavior at which further increase in solute concentration did not measurably change $\theta_a$. Unlike SDS, however, adsorption/mass transfer kinetics significantly affected early-time $\theta_a$ measurements of HSA solutions, although steady state was achieved well before the final observation time at 3600 sec. Kinetic effects were even more pronounced for large proteins such as $\alpha_2$-macroglobulin (Fig. 1C) for which Type 2 adsorption behavior was observed. Thus Fig. 1 captures the significant adsorption trends quantified in Table 2.

**Scaled Interfacial Activity:** Fig. 2 traces the sequential interpretation of steady-state, concentration-dependent $\theta_a$ data (Fig. 2A, observed at 1 hr drop age) in terms of $\tau_a$ (Fig. 2B) and spreading pressure $\Pi_a$ (Fig. 2C) isotherms for the proteolytic enzyme thrombin (blood factor FIIa). Smooth curves through the data (see Methods and Materials) provide guides to the eye. Figs. 3 A, B and C compare $\Pi_a$ curves for proteins selected from Table 1 to cover the full range of molecular weight (MW) studied in this work. Concentration is scaled in three different ways in Fig. 3 for direct comparison; by weight (Fig. 3A), molarity (Fig. 3B), and ratio-to-
physiological concentration, $C_p$ (Fig. 3C). Only smoothed curves corresponding to steady-state (equilibrium) are shown for the sake of clarity, but representative $\theta$, $\tau$, and $\Pi$ curves with authentic data are amply illustrated in Figs. 1 and 2. The following discusses results of each scaling method in order of appearance on Fig. 3. These different scaling methods sharpen general impressions about the energetics of protein adsorption that are particularly pertinent to the controlling role of water in protein adsorption discussed in the Introduction.

Fig. 3A compares $\Pi$ curves with $C_p$ expressed in parts-per-trillion (i.e. PPT, grams solute/10^{12} g solvent, so that the ln $C_p$ scale has positive values at all concentrations). Weight scaling is applicable to purified proteins, protein mixtures, and surfactants alike, permitting comparison of interfacial activity on a single concentration axis.\textsuperscript{17-19} Two features are readily apparent from Fig. 3A. First, the dynamic range of $\Pi \sim 20$ mN/m is consistent with weak surfactancy, as discussed in the previous section, and is similar to that observed for these proteins at the LV surface ($15 < \Pi < 30$ mN/m). Second, $\Pi_{max}$ lies within a relatively narrow 5 mN/m band for this diverse set of proteins (with MW spanning nearly three orders of magnitude; see Table 1). Thus, it is evident from Fig. 3A that, on a weight basis, proteins exhibit quite similar SL interfacial activity, mirroring the general experience with these proteins at the LV surface.\textsuperscript{25}

Bearing in mind the great range in MW spanned by proteins in Fig. 3, it is reasonable to conclude that commensurate variability in protein structure does not confer widely varying SL interfacial activity; at least not in comparison to the full range available to ordinary surfactants.

However convenient weight scaling may be, it is nevertheless true that free-energy and stoichiometry scale on a molar basis. Thus, for the purpose of better understanding interfacial
energetics, it is useful to express bulk concentration $C_B$ in molar units. We have chosen to
express solute dilution $C_B$ in picomoles/L (i.e. picomolarity, pM, $10^{-12}$ moles solute/L solution so
that the $\ln C_B$ scale has positive values for all dilutions) for solutes with a known (nominal) MW.
This approach is applicable to purified proteins and surfactants but not to chemically-undefined
protein mixtures such as plasma and serum. Variability in interfacial activity among the diverse
purified proteins reported in Fig. 3 is much more evident on a molar than weight basis (compare
Fig. 3A to Fig. 3B). Of course, molar scaling does not alter observations regarding the range of
$\Pi_a$ mentioned above, but it does effectively expand the concentration axis by moving higher-
MW proteins (such as IgM) to the left and lower-MW proteins (such as albumin and ubiquitin) to
the right. It is evident from Fig. 3B that high-MW proteins reduce $\Pi_a$ at lower molarity than
low-MW proteins, again repeating a general observation made at the LV surface for these
proteins. The inference taken from Fig. 3B is that protein concentration required to reduce $\Pi_a$
to a specified value decreases with MW in a manner loosely consistent with the addition of a
generic amino-acid-building-block having an “average amphility” that increases MW but does
not radically change protein interfacial activity. Otherwise, if MW increased by addition of
amino-acid-building-blocks with highly-variable amphility, then $\Pi_a$ would be expected to be a
much stronger function of protein MW than is observed in Fig. 3B. Thus, it appears that molar
variability in $\Pi_a$ is achieved by aggregating greater mass of similar amphiphilic character, as
opposed to accumulating greater amphility with increasing MW.

Weight and molar scaling are very useful experimental and conceptual constructs that may turn
out to have little direct relevance to the in vivo biological response to materials because these
scales do not account for the widely-varying natural abundance of the many different proteins comprising the mammalian proteome. Thus, for the purpose of better understanding protein interfacial activity within a physiologic context, we have found it useful to ratio $C_B$ to nominal (mean) physiological concentration $C_P$ and express $C_B / C_P$ on a logarithmic scale ($\log (C_B / C_P)$). In this way, the physiologic condition can be readily identified at $\log (C_B / C_P) = 0$ with more-dilute-protein solutions lying to the left (negative $\log (C_B / C_P)$ values) and more-concentrated solutions lying to the right (positive $\log (C_B / C_P)$ values; see dashed vertical line on Fig. 3C). This approach is applicable to purified proteins for which humoral or cellular concentrations are known. Thus, Fig. 3C reveals a different kind of diversity in protein interfacial activity than discussed above in reference to weight or molar scales. Certain proteins, notably albumin, IgG and IgM, produced the full $\Pi^\text{max}$ range well below physiologic concentration ($\Pi_a$ data lies substantially to the left of $\log (C_B / C_P) = 0$). By contrast, C1q and prothrombin (FII) required full physiological concentration to express $\Pi^\text{max}_a$ whereas ubiquitin was not fully surface active at-and-well-above physiologic concentration. Again, these results closely correspond to the behavior noted at the LV surface. And just as observed at the LV surface, only a limited portion of the available $\Pi^\text{max}_a$ range was exhibited by blood factor XII ($\Pi_a$ data lie to the right of $\log (C_B / C_P) = 0$), as will be discussed separately below in reference to Fig. 4 because this has special significance in blood coagulation. Thus, it appears that $\log (C_B / C_P)$ is a pragmatic scaling of interfacial activity with relevance to biomedical materials that reveals concentration-driven diversity in interfacial activity observed among humoral and cellular proteins.
Fig. 4 summarizes results obtained for blood factor FXII. Activation of FXII by contact/adsorption to procoagulant surfaces is thought to potentiate the intrinsic pathway of blood coagulation (see refs. 43,44 and citations therein). Thus, understanding interfacial activity of FXII is important toward a full appreciation of the contact activation mechanism\textsuperscript{45-47} and anticoagulation as well.\textsuperscript{48} Fig. 4 is prepared in basically the same format as Fig. 3C except that $\Pi_a$ curves here correspond to different observation times up to 1 hr drop age and demonstrates that FXII $\Pi_{a_{\text{max}}}$ was not achieved at physiological concentrations. Moreover, $\Pi_{a_{\text{max}}}$ was achieved only after long equilibration times (> 1800 sec). These adsorption kinetics must be interpreted in terms of the blood-plasma-coagulation process that can be complete within 300 sec or so when plasma is saturated with high-surface-area procoagulants.\textsuperscript{46} Results obtained with purified FXII are thus seemingly inconsistent with the traditional biochemical mechanism of contact activation of blood plasma coagulation that asserts rapid FXII adsorption onto hydrophilic procoagulant surfaces. FXII adsorption, and FXIIa desorption for that matter, must occur in the presence of overwhelming concentrations of other blood proteins, notably albumin and IgG that this work demonstrates to be much more surface active on both molar and physiological-concentration scales (Fig. 3B, C). Furthermore, we note that FXII adsorption to the SL interface is not rapid relative to other proteins such as albumin, as might be expected for a protein with putatively enhanced interfacial activity. Although mechanisms of adsorption to hydrophilic (efficient procoagulant) surfaces are not necessarily the same as those leading to adsorption to hydrophobic (inefficient procoagulant) surfaces, it is the general experience that much more protein is adsorbed to hydrophobic surfaces.\textsuperscript{39,47} On this basis, we expect that FXII would exhibit even less adsorption to hydrophilic procoagulants than we observe at the hydrophobic SL
surface within a time-frame relevant to coagulation. All taken together, these studies of FXII adsorption to the hydrophobic SL and LV interfaces support our contention that FXII does not adsorb directly onto hydrophilic procoagulant surfaces in a manner that displaces water (surface dehydration) and that contact activation of FXII → FXIIa occurs by some process other than suggested by the traditional mechanism.46-48

5.4. Conclusions

Concentration-dependent contact angles $\theta_a$ of buffered-protein solutions on methyl-terminated self-assembled monolayer (SAM) surfaces have been measured for selected globular-blood proteins spanning 3 decades in molecular weight (MW). Observed protein $\theta_a$ was reduced by no more than 15° at maximum protein concentrations (~10 mg/mL). Adsorption energetics were interpreted in terms of adhesion tension $\tau_a = \gamma_{lv} \cos \theta_a$ and spreading pressure $\Pi_a = (\tau_a - \tau_a^\circ)$ adsorption isotherms; where $\gamma_{lv}$ is concentration-dependent liquid-vapor (LV) interfacial tension of the fluid phase, $\tau_a^\circ$ is the adhesion tension corresponding to minimum contact angle $\theta_a^\circ$, and $\tau_a^\circ$ is the adhesion tension of pure buffer. Results closely mirrored that reported in a companion paper published in this journal describing protein adsorption to the hydrophobic water-air (liquid-vapor, LV) interface.25 On this basis, it is concluded that the physical chemistry of protein adsorption to the solid-liquid (SL) interface is not remarkably different than that controlling protein adsorption to the LV interface. In particular, it was found that maximum spreading pressure fell within a relatively narrow $10 < \Pi_a^{max} < 20 \text{ mN/m}$ band for all proteins studied. Results confirm that proteins are, in general, weak surfactants in comparison to synthetic detergents (represented herein by the surfactant reference compound sodium dodecyl
sulfate, SDS). The free energy of adsorption to hydrophobic surfaces is thus found to be quite modest ($\Delta G_{\text{ads}}^o \sim -5RT$) and the total protein adsorbed from a contact-angle droplet a small percentage of the total available in solution. However, adsorbed concentrations in mass or moles per-unit-volume are surprisingly large ($\sim$450 mg/mL)$^{6,16,28}$ because adsorbate collects within an thin (SL or LV) interphase, arguably no more than a few protein molecular layers thick.

Three different methods of scaling $\Pi_a$ isotherms were explored: weight, molarity, and ratio-to-physiologic concentration. On a weight basis, proteins among the group studied appeared more similar than dissimilar, especially when viewed from the perspective of the full range available to synthetic surfactants. We interpret this observation to mean that there is insufficient amphiphilic diversity (variation in the interaction energetics with water) among the proteins studied to support wide-ranging interfacial activity at the hydrophobic SL interface, even though protein composition varied significantly among proteins studied. Molar scaling revealed that SL interfacial activity followed a progression in MW, with the concentration required to reach a specified $\Pi_a$ value decreasing with increasing MW. This progression in MW is interpreted to mean that molar variability in $\Pi_a$ is achieved by aggregating greater mass of similar amphiphilic character (blocks of amino acids), as opposed to accumulating greater amphilicity with MW. The significance of this observation is that it suggests that the structural variability that confers profoundly different bioactivity does not greatly affect interaction energetics in water that drive adsorption to the LV interface. Scaling interfacial activity to physiological concentration revealed that certain proteins, such as albumin and IgG, achieved $\Pi_a^{\text{max}}$ at-and-well below physiologic concentration whereas others, notably blood factor XII (Hageman factor), required
concentration by almost two-fold above the nominal-physiologic concentration to express the full range of $\Pi_{a}^{\text{max}}$ characteristic of this protein.

In summary, results reported herein support the overall conclusion that combinations and permutations of the 20 naturally-occurring amino acids comprising the primary sequence of mammalian proteins is insufficient to support widely-varying interfacial activity at hydrophobic surfaces (LV or SL), no matter how these sequences happen to fold into higher-order structure.\(^{25}\) Furthermore, we contend that protein concentration, not diversity in molecular structure, is the significant energetic driver of adsorption to hydrophobic surfaces. The overarching interpretation is that water plays a dominate, controlling role in the adsorption of globular blood proteins to hydrophobic surfaces and that the mechanism of protein adsorption can be quite comprehensively understood from this perspective.

Outcomes of this work are in general agreement with other investigations of the interfacial energetics of protein adsorption. In particular, the pioneering work of Tripp, Magda, and Andrade\(^{49}\) revealing that “…mesoequilibrium surface tension [of 8 globular proteins]…did not vary greatly between different proteins…” is relevant to this investigation. The term ‘mesoequilibrium’ was wisely applied by Tripp et al. because, in general, it is technically challenging to unambiguously prove fully-reversible adsorption (or thermodynamic reversibility of any process for that matter). And in the particular case of proteins, there is the expectation from a burgeoning literature base that proteins ‘denature’ over time (see ref. 50 and citations therein). Denaturation can include changes in molar free volume/interfacial area, loss of higher-order structure with concomitant change in specific bioactivity, and irreversible adsorption. Of
course, tensiometric methods applied by Tripp *et al.* and us are effectively blind to these molecular processes, except insofar as denaturation may lead to time-varying interfacial tensions and contact angles. Our measurements achieved, or asymptotically approached, a well-defined steady-state within the hour observation window applied, suggesting that putative ‘denaturation processes’ either had an insignificant impact on results or occurred significantly faster/slower than the time frame of experimentation. Given the similarity in adsorption energetics to hydrophobic LV and SL surfaces among the broad array of proteins studied (including those of Trip *et al.*), and the general expectation that denaturation is a slow process, we are inclined to conclude that either denaturation did not significantly affect results (perhaps accounting for small-but-measurable differences among proteins) or the denaturation effect was astonishingly similar among very different proteins. With regard to irreversible adsorption, we note that experiments examining competitive adsorption between albumin and IgM at the LV surface demonstrated protein displacement (Vroman effect) that followed a simple mass-balance exchange, strongly suggesting that neither albumin nor IgM was irreversibly adsorbed to this surface.

Even in view of the similarity among studies of the interfacial energetics of protein adsorption to hydrophobic surfaces, it is difficult to fully reconcile our findings with all other previous investigations of protein adsorption. But then it is also challenging to find substantive commonality within and among this prior art, let alone extend its margins to embrace new findings. Thus, there should be little surprise that results reported herein may seem exceptional relative to certain particular studies selected from this expansive literature base. In this connection, it seems useful to step back from the details momentarily and point out that relating
mass to energy inventories of protein adsorption will require more than casual comparison of experimental results. For this purpose, a theory of protein adsorption is required. And until such a theory is available, comparison of mass measurements (by solution depletion, gravimetry, or spectroscopy for examples) to interfacial energetics (by calorimetry, chromatography, or tensiometry for examples) should be made with due caution.
Citations


(43) Mitropoulos, K. A. The Levels of FXIIa Generated in Hyman Plasma on an Electronegative Surface are Insensitive to Wide Variation in the Concentration of FXII, Prekallikrein, High Molecular Weight Kininogen or FXI, Thromb. Haemost. 1999, 82, 1033.

(44) Mitropoulos, K. A. High Affinity Binding of Factor FXIIa to an Electronegative Surface Controls the Rates of Factor XII and Prekallirien Activation in vitro, Thrombosis Research 1999, 94, 117.


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Figure 1: Contact angle isotherms in 3D ($\theta_a$ as a function of analysis time (drop age) and logarithmic (natural) solution concentration $C_a$) and 2D ($\theta_a$ as a function of logarithmic solution concentration $C_a$ at selected times) formats comparing sodium dodecyl sulfate (SDS, panel A), human serum albumin (FV HSA, panel B, preparation 1, Table 2), and $\alpha_2$-macroglobulin (panel C, preparation 1, Table 2). In each case, solute concentration $C_b$ is expressed in picomoles/L (pM) on a natural logarithmic scale. Symbols in 2D panels represent time slices through 3D representations (filled circle = 0.25 sec, open circle = 900 sec, filled triangles = 1800 sec, and open triangles = 3594 sec; annotations in panel A indicate maximum and half-maximum contact angle reduction). Notice that adsorption kinetics dominated $\alpha_2$-macroglobulin adsorption whereas steady-state was achieved within about 1000 sec for HSA, and nearly no adsorption kinetics is detected for SDS. Note also decrease in $\theta_a$ with time, attributed to slow hydration of the SAM surface (arrow annotation, Panel B; see Results section).
Figure 2: Sequential interpretation of a steady-state (3600 sec drop age) contact angle adsorption isotherm for thrombin (FIIa) - Panel A, advancing contact angles, $\theta_a$; panel B, advancing adhesion tension, $\tau_a$; panel C, advancing spreading pressure $\Pi_a$. Smoothed curves through the data are guides to the eye. Annotations identify low- and high- concentration asymptotes for contact angles ($\theta_a^-, \theta_a^+$), adhesion tensions ($\tau_a^-, \tau_a^+$) respectively, and maximum spreading pressure $\Pi_a^{\text{max}}$ that are used to characterize isotherms (Table 2).
**Figure 3:** Spreading pressure $\Pi_a$ isotherms scaled three different ways for selected proteins spanning three decades in molecular weight – by natural logarithmic weight-volume (panel A), picomolarity (panel B), and logarithmic (base 10) ratio-to-physiological concentration (panel C). Smooth curves are guides to the eye. Protein interfacial activity appears more similar than dissimilar when viewed on a weight basis but diversity among proteins becomes more apparent when scaled on a molar or ratio-to-physiological concentration. Physiologic scaling (panel C) shows that FV HSA, IgG, and IgM (preparations 1, Table 1) were fully surface active at-and-below physiological concentrations whereas C1q, FII, and Ub required nearly 2X concentration to achieve maximum spreading pressure.
Figure 4: Spreading pressure $\Pi_a$ isotherms of blood factor FXII (preparation 1, Table 1), scaled as a ratio-to-physiological concentration. Smooth curves at 0.25, 900, 1800, and 3594 sec drop age illustrate FXII adsorption kinetics. FXII is weakly surface active at physiologic concentrations (see Fig. 3 for comparison to other proteins such as FVHSA, IgG). Note that maximum spreading pressure was achieved only at two decades of concentration higher than physiologic concentration.
\[ \Pi_a (\text{mN/m}) \]

\[ \log [C_B/C_P] \]
Chapter 6

Interfacial Energetics of Globular-Blood Protein Adsorption
to a Hydrophobic Solid/Aqueous-Buffer Interface

Abstract

Adsorption isotherms of nine (9) globular proteins with molecular weight (MW) spanning 10-1000 kDa confirm that interfacial energetics of protein adsorption to a hydrophobic solid/aqueous-buffer (solid-liquid, SL) interface are not fundamentally different than adsorption to the water-air (liquid-vapor, LV) interface. Adsorption dynamics dampen to a steady-state (equilibrium) within a 1 hr observation time and protein adsorption appears to be reversible, following expectations of Gibbs’ adsorption isotherm. Adsorption isotherms constructed from concentration-dependent advancing contact angles $\theta_a$ of buffered-protein solutions on methyl-terminated, self-assembled monolayer surfaces show that maximum advancing spreading pressure, $\Pi_{a}^{\text{max}}$, falls within a relatively narrow $10 < \Pi_{a}^{\text{max}} < 20$ mN/m band characteristic of all proteins studied, mirroring results obtained at the LV surface. Furthermore, $\Pi_{a}$ isotherms exhibited a ‘Traube-rule-like’ progression in MW similar to the ordering observed at the LV surface wherein molar concentrations required to reach a specified spreading pressure $\Pi_{a}$ decreased with increasing MW. Finally, neither Gibbs’ surface excess quantities $[\Gamma_{sl} - \Gamma_{sv}]$ nor $\Gamma_{lv}$ varied significantly with protein MW. The ratio

\[
\frac{[\Gamma_{sl} - \Gamma_{sv}]}{\Gamma_{lv}} \sim 1,
\]

implying both that $\Gamma_{sv} \sim 0$ and chemical activity of protein at SL and LV surfaces was identical. These results are collectively interpreted to mean that water controls protein adsorption to hydrophobic surfaces and that the mechanism of protein adsorption can be understood from this perspective for a diverse set of proteins with very different composition.
6.1. Introduction

Protein adsorption is one of the most fundamental, unsolved problems in biomaterials surface science. Practical importance of the problem is related to the fact that protein adsorption is among the first steps in the acute biological response to materials that dictates biocompatibility, and hence utility in medical-device applications. As a consequence of these scientific and pragmatic factors, the protein-adsorption problem has attracted considerable research attention from diverse fields of inquiry ranging from biomaterials to physics. An undercurrent flowing through much of this research seems to be that the amount of protein adsorbed to a surface is primarily controlled by short-range, pair-wise interactions between protein molecules and adsorbent surface (consider, for example, the RSA model of protein adsorption). Adsorbed protein is frequently assumed to be irreversibly surface bound in a monolayer arrangement; see for example and citations therein. However, presumption of irreversible adsorption remains controversial in the literature and multilayering of protein has been experimentally demonstrated in a number of cases.

Furthermore, the apparent specificity/selectivity of adsorption from multi-component solutions is frequently attributed to variations in protein molecular structure that give rise to differential interactions with a particular adsorbent.

It is our contention that this view of protein adsorption to surfaces does not properly account for the role of water in the process and, in so doing, fails to discern unifying trends in protein adsorption. For example, literature illustrations depict protein and adsorbent surfaces without juxtaposing hydration layers, one layer for protein and one for surface, and do not contemplate how these layers are displaced or coalesced as protein and surface come into close contact. Many modern computational models probing surface-protein interactions regard water as a complicating feature
that can be ignored for the sake of reasonable computational time; see refs. 15-17 and citations therein. When water is included in such models, it is usually only those molecules directly adjacent to the protein that comprise the ‘bound-water layer’, classically measured by $\delta$ in grams-water-per-gram-protein $^{18-20}$; where $\delta \sim 0.35 \text{ g/g}$ is found to be a representative average value $^{20}$. This protein-bound water layer falls well short of the volume which must be displaced when a protein molecule approaches a hydrated adsorbent surface. That is to say, since two objects cannot occupy the same space at the same time, a volume of interfacial water at least equal to the partial specific volume $\nu^o$ $(0.70 \leq \nu^o \leq 0.75 \text{ cm}^3/\text{g protein})$ of the adsorbing protein must move $^{21}$. If protein adsorbs in multilayers, then clearly much more water must be displaced. Some or all of this interfacial water is bound to the adsorbent surface to an extent that varies with surface energy $^{2,22}$. Consequently, protein adsorption is found to scale with water wettability $^{23-25}$, underscoring need to incorporate surface hydration explicitly into protein-adsorption models. Indeed, accounting for water in protein adsorption has become a significant preoccupation of quartz crystal microbalance (QCM) practitioners because QCM not only measures adsorbed protein mass but also “trapped”$^{26}$ or “intra-layer”$^{27}$ or “hydrodynamically coupled”$^{28}$ water.

We have made use of a simplified ‘core-shell’ model of globular proteins in which spheroidal molecules are represented as a packed core surrounded by a hydration shell. The core has a radius $r_v$ that scales with molecular weight ($\text{MW}^{1/3}$) and the hydration shell has a thickness such that the ensemble radius $R = \chi r_v$ equals the hydrodynamic radius $^{10}$; where $\chi$ is taken to be a generic factor for all proteins. Calibration to human serum albumin (FV HSA) dimensions reveals that $R = 1.3 r_v$ (30% larger than $r_v$) and contains about 0.9 g water/g protein. Hence, the hydration layer accommodated by this model is $\sim 3X$ greater than $\delta$. Calibrated to neutron-reflectivity (NR) of
albumin adsorption to surfaces, this model suggests that protein saturates the hydrophobic surface region by packing to nearly face-centered-cubic (FCC) concentrations wherein hydration shells touch but do not overlap. We propose that osmotic repulsion among hydrated protein molecules limits interphase capacity. Stated another way, protein adsorption is limited by the extent to which the hydrophobic interface can be dehydrated through displacement of interfacial water by adsorbing protein. Accordingly, protein adsorption is viewed as being more about solvent than protein itself; a perspective in sharp contrast to the prevailing paradigm mentioned above.

This water-oriented perspective on protein adsorption presents a considerable simplification of the protein-adsorption process and, as a result, a tractable quasi-thermodynamic theory can be sketched out for a phenomenon that would otherwise be overwhelmingly complex for more than just a few proteins in solution. We find that this theory naturally explains the experimentally-observed ‘Traube-rule progression’ in which molar concentrations required to fill the liquid-vapor (LV) surface follow a homology in protein size, consistent with packing hydrated spheroidal molecules within this space. A relatively straightforward set of “mixing rules” follow directly, stipulating both concentration and weight-fraction distribution of proteins adsorbed to the LV surface from multi-component aqueous solutions such as blood plasma or serum. These mixing rules rationalize the long-known-but-otherwise-unexplained observations that (i) LV interfacial tension $\gamma_{lv}$ of blood plasma and serum is nearly identical, in spite of the fact that serum is substantially depleted of coagulation proteins such as fibrinogen; and (ii) $\gamma_{lv}$ of plasma and serum derived from human, bovine, ovine, and equine blood is practically identical, even though there are substantial differences in plasma proteome among these species.
This paper discloses results of an investigation of globular-blood protein adsorption to a well-defined, hydrophobic solid/aqueous-buffer (solid-liquid, SL) interface. Methyl-terminated, self-assembled thiol monolayers (SAMs) on gold-coated semiconductor-grade silicon wafers exhibiting water contact angles $\theta_a \sim 110^\circ$ are used as test substrata. Time-and-concentration-dependent contact angles measure adsorption energetics of (globular) proteins spanning 3 decades in MW in a manner parallel to the above-cited studies of protein adsorption to the LV surface. We find that the basic pattern observed at the LV surface is repeated at the hydrophobic SL surface, supporting our contention that water is the significant controller of protein adsorption to surfaces.

6.2. Materials and Methods

Purified Proteins and Synthetic Surfactants: Table 1 compiles pertinent details on proteins and surfactants used in this work. Protein purity was certified by the vendor to be no less than the respective values listed in Column 4 of Table 1, as ascertained by electrophoresis (SDS-PAGE or IEP). Mass, concentration, and molecular weights supplied with purified proteins were accepted without further confirmation. Issues associated with protein purity, especially contamination with surfactants, and the potential effect on measured interfacial tensions have been discussed elsewhere. The single value given in Table 1 (Column 5) for physiological concentration of human proteins applied in this work was middle of the range listed by Putnam. Serial dilutions of protein stock solutions (usually 10 mg/mL) were performed in 96-well microtiter plates by (typically) 50:50 dilution in phosphate buffered saline solution (PBS; 0.14 M NaCl, 0.003M KCl) prepared from powder (Sigma Aldrich) in distilled-deionized (18.2 M\(\Omega\)-cm) water using procedures detailed in ref. Between 24-30 dilutions were prepared in this manner, covering a dynamic range between $10^{-10}$.
to 1 % (w/v), taking care to mix each dilution by repeated pipette aspiration and avoiding foaming of concentrated solutions.

**Surfaces:** Methyl-terminated, self-assembled monolayer surfaces (SAMs) were prepared according to standard methods of surface engineering. Briefly, silicon wafers were pre-cleaned in hot 1:4 H₂O₂ (30%)/H₂SO₄ followed by rinsing with distilled-deionized H₂O and absolute ethanol. Gold-coated wafers were prepared by vapor deposition of chromium and gold (99.99% purity) from resistively-heated tungsten boats onto clean 3-in. diameter silicon wafers at about 1 x 10⁻⁸ torr base pressure in a cryogenically pumped deposition chamber. The sample was not allowed to rise above ~40°C during the evaporation. Film thicknesses, monitored with a quartz crystal oscillator, were typically 15 nm and 200 nm for chromium and gold, respectively. Chromium was deposited prior to gold to enhance adhesion to the substrate. After deposition, the chamber was backfilled with research-grade nitrogen. Gold-coated samples were removed and immersed in 1mM solutions of 1-hexadecanethiol (CH₃(CH₂)₁₅SH) in ethanol, contained in glass jars at ambient temperature, for at least 3 days.

The alkanethiol (Aldrich Chemical Co., Milwaukee, WI) and ethanol (commercial reagent-grade) were used as-received, without further purification. Samples were stored in the thiol solution until use and were rinsed with ethanol just prior to an experiment.

**Tensiometry & Goniometry:** Liquid-vapor interfacial tensions required by this work were measured by Pendant Drop Tensiometry (PDT) as described in refs. ¹⁰,¹¹. Tilting-Plate Goniometry (TPG) was performed using a commercial-automated goniometer (First Ten Angstroms Inc., Portsmouth, VA). Advancing contact angles (θₐ) applied in this work have been verified to be in
statistical agreement with those obtained by Wilhelmy Balance Tensiometry (WBT) and Captive-Drop Goniometry (CDG), as detailed in ref. 35. Receding angles ($\theta_r$) were shown to be not as reliable as $\theta_a$ and, as a consequence, only $\theta_a$ was analyzed in this work. The tilting-plate goniometer (TPG) employed a Tecan liquid-handling robot to aspirate 12 µL of solutions contained in a 96-well microtiter plate prepared by the serial-dilution protocol mentioned above. The robot was used to reproducibly transfer the tip with fluid contents into a humidified (99+ % RH) analysis chamber and dispense 10 µL drops of protein solution onto the surface of test substrata (see below) held within the focal plane of a magnifying camera. These and all other aspects of TPG were performed under computer control. Proprietary algorithms supplied by the vendor were used to deduce contact angles from drop images captured at a programmed rate by a frame grabber. Typically, 600 images were captured at a rate of 1 image every 6 sec following 20 sec delay to permit vibrations of the expelled drop to dampen. Drop evaporation rates within the humidified chamber deduced from computed-drop volumes (based on image analysis) were observed to vary with solute concentration, generally ranging from approximately 25 nL/min for pure water to 10 nL/min for solute solutions > 0.1% w/v. The impact of this evaporation rate over the 60 min time frame of the experiment was apparently negligible, as gauged from the behavior of purified surfactants discussed in the Results section. Precision of $\theta_a$ was about 0.5° based on repeated measurement of the same drop. The analysis chamber was thermostated to a lower-limit of 25±1 °C by means of a computer-controlled resistive heater. Upper-temperature limit was not controlled but rather floated with laboratory temperature, which occasionally drifted as high as 29 °C during summer months. Thus, reported $\theta_a$ values were probably not more accurate than about 1° on an inter-sample basis considering the small, but measurable, variation of water interfacial tension with temperature. This range of accuracy was deemed adequate to the conclusions of this report which do not strongly depend on more highly
accurate $\theta_a$ that is difficult to achieve on a routine basis. Instead, veracity of arguments raised herein depend more on a breadth of reliable measurements made across the general family of human proteins.

Test substrata were held on a rotating, tilting-plate platform driven by stepper motors under computer control. Substrata were allowed to come to equilibrium within the sample-chamber environment for no less than 30 min before contact angle measurements were initiated. The platform was programmed to tilt at $1^\circ$/sec from horizontal to $25^\circ$ after the drop was deposited on the surface by the robot. The optimal (incipient rolling) tilt angle was found to be $25^\circ$ and $15^\circ$ for solutions of proteins and surfactants respectively. The first 20 images monitored evolution of the advancing angle. At the end of the 1 hr $\theta_a$ measurement period, the platform was programmed to return to horizontal and rotate $15^\circ$ to the next analysis position along the periphery of the semiconductor wafer. This process was repeated for all dilutions of the protein under study so that results reported for each protein were obtained on a single test surface, eliminating the possibility of substratum-to-substratum variation within reported results.

$\theta_a$ measurements by TPG employed in this work were verified against Wilhelmy-balance tensiometry (WBT) and found to agree within a percentage difference of 2.5±1.9% for $50^\circ < \theta_a < 120^\circ$. It is worthwhile mentioning in this context that WBT itself is inappropriate for studies of protein adsorption at the SL interface (at least as applied herein) because (i) the technique requires thin plates that are difficult to two-side coat with gold for thiol-SAM preparation, (ii) WBT generally requires high solution volumes (~10 mL) that greatly exceed availability of purified proteins, and (iii) the moving three-phase line deposits solute (protein or surfactant) at the SV
interface making interpretation of the Gibbs’ surface excess parameter $[\Gamma_{av} - \Gamma_{sw}]$ highly ambiguous.

Overall, we have found the tilting-plate method applicable to measuring adsorption, at least for hydrophobic surfaces, and suitable for 1 hr equilibration times if a humidified chamber is used to control evaporation. However, it was observed that SAM surfaces were slightly unstable and subject to “hydration” that led to a systematic decrease in water/PBS contact angles with time. These hydration dynamics were observed to be more pronounced on SAM surfaces that had been incubated for long periods (> 3 d) in the 100% RH atmosphere of the PDT analysis chamber (not shown). However, we do not believe this slight but apparently unavoidable attribute of SAMs on silicon wafers negatively affects the veracity of conclusions based on final, steady-state $\Pi_a$ measurements made at ~ 1 hr analysis time.

**Computation and Data Representation:** Computational, statistical, and theoretical methods used in this work have been discussed in detail elsewhere. Briefly, time-dependent $\theta_a$ data corresponding to protein dilutions (see above) were recovered from TPG files and correlated with concentrations, leading to a matrix of results with row values representing concentration and time (in sec) as column values. It was generally observed that $\theta_a$ isotherms were sigmoidal in shape when plotted on logarithmic-concentration axes, with well-defined low-concentration asymptote $\theta_a^{\theta_a}$ and high-concentration asymptote $\theta_a^{\prime}$ (see Fig. 1). Successive non-linear least-squares fitting of a four-parameter logistic equation

$$\theta_a = \frac{\theta_a^{\prime} - \theta_a^{\theta_a}}{1 + (\ln C_B^{\theta_a}/2 \ln C_B^{\prime})^{\theta_a}} + \theta_a'$$

to contact angle isotherms data for each time within the observation interval quantified parameters $\theta_a^{\prime}$ and $\theta_a^{\theta_a}$ with a measure of statistical uncertainty. Fitting also recovered a parameter measuring concentration-at-half-maximal-
change in $\theta_a$, $\ln C_B^{0/2}$ (where $\Theta / 2 = 1/2 \Theta_{\text{max}}$ and $\Theta_{\text{max}} \equiv \theta_a^\prime - \theta_a^{\prime\prime}$), as well as a parameter $M$ that measured steepness of the sigmoidal curve. This multi-parameter fitting to concentration-dependent $\theta_a$ data was a purely pragmatic strategy that permitted quantification of best-fit protein and surfactant characteristics but is not a theory-based analysis $^{1,10,11,13,23,24}$. Three-dimensional (3D) representations of time-and-concentration-dependent $\theta_a$ data were created in Sigma Plot (v8) from the data matrix discussed above and overlain onto fitted-mesh computed from least-squares fitting. Two-dimensional (2D) representations were created from the same data matrices at selected observation times. Measured $\theta_a$ were converted to advancing adhesion tension $\tau_a = \gamma_{lv} \cos \theta_a$ for general interpretation $^1$; where $\gamma_{lv}$ was the interfacial tension of the contact-angle fluid. Adhesion tensions, $\tau_a^o = \gamma_{lv}^o \cos \theta_a^o$ (pure saline) and $\tau_a^' = \gamma_{lv}^' \cos \theta_a^'$ (at the minimum contact angle observed $\theta_a^'$) were computed with fitted parameters $\gamma_{lv}^o$ and $\gamma_{lv}^'$ reported in refs. $^{10,11}$ for the proteins under investigation. Smoothed adhesion-tension isotherms ($\tau_a$ vs. $\ln C_B$) were computed from smoothed $\theta_a$ using smoothed $\gamma_{lv}$ values computed from best-fit parameters reported in refs. $^{10,11}$. Likewise, smoothed spreading pressure isotherms ($\Pi_a$ vs. $\ln C_B$) were computed from smoothed $\tau_a$ curves, where $\Pi_a \equiv (\tau_a - \tau_a^*)$.

6.3. Theory

Adsorption Isotherms: Adsorption of surface-active solutes (surfactants, where the term includes both synthetic detergents and proteins) can affect liquid-vapor (LV), solid-vapor (SV), or solid-liquid (SL) interfacial tensions, thus producing a change in measured contact angles $\theta$ as given by the Young equation $\tau \equiv \gamma_{lv} \cos \theta = \gamma_{sv} - \gamma_{sl}$; where $\tau$ is adhesion tension and $\gamma$ is the interfacial
tension at the interface denoted by subscripts. Thus, contact angles can be used to monitor adsorption to solid surfaces; see refs. \(^1,2,3,4\) and citations therein. Contact-angle isotherms are graphical constructions that monitor effects of adsorption by plotting advancing contact angles \(\theta_a\) against \(\ln C_B\) (see Fig. 1 for examples); where surfactant bulk-phase concentrations \(C_B\) range from \(10^{-10}\) to \(1\%\) (w/v, see Materials and Methods). Contact-angle isotherms were sequentially interpreted in terms of adhesion tension (\(\tau_a\) vs. \(\ln C_B\)) and spreading pressure (\(\Pi_a\) vs. \(\ln C_B\)) isotherms; where \(\tau_a = \gamma_o \cos \theta_a\), \(\Pi_a = (\tau_a - \tau_a^0)\), \(\gamma_o\) is the LV interfacial tension of the fluid at \(C_B\), and \(\tau_a^0\) is the adhesion tension of pure buffer (\(\gamma_o = 71.97\text{mN/m at 20°C}\)). We monitored time dependence of all three isotherm forms but herein interpret only final measurements that achieve or approach steady-state (equilibrium). Issues associated with adsorption reversibility are discussed in the Results section. Secure interpretation of measured \(\theta_a\) in terms of \(\tau_a\) depends on accurate knowledge of \(\gamma_o\) at the bulk-phase surfactant concentration in equilibrium with SL and LV interfaces. Thus, solute depletion of the bulk phase by adsorption may require correction of as-prepared bulk-phase concentration \(C_B\). However, agreement between (uncorrected) tensiometry and instrumental methods of measuring adsorption for surfactants (see Table 3) suggests solute-depletion was not a serious issue for surfactant standards. Likewise, for the case of protein adsorption, it can be concluded from a simple calculation that solute depletion was not a serious problem requiring correction.\(^14\).

**Gibbs’ Surface Excess:** Practical use of concentration-dependent contact angles as a measure of adsorption to the solid-liquid (SL) interface has been discussed at length elsewhere; see, for examples, refs. \(^1,2,3,4\) and citations therein. Briefly, for the purposes of this paper, the amount of
solute adsorbed to SV and SL interfaces is measured by the Gibbs’ surface excess quantities $\Gamma_{sv}$ and $\Gamma_{sl}$, respectively, in units of moles/area (the subscript “a” specifying advancing contact angles is not carried in $\Gamma$ symbology for the sake of notational compactness). The difference $[\Gamma_{sl} - \Gamma_{sv}]$ (but not separate excess parameters) can be computed from data comprising contact-angle isotherms using Eq. (1):

$$[\Gamma_{sl} - \Gamma_{sv}] = -\left[\frac{\gamma_{lv}\sin\theta_a}{RT}\left(\frac{d\theta_a}{d\ln C_B}\right) + \left[\Gamma_{lv}\cos\theta_a\right]\right]$$

where $d\theta_a/d\ln C_B$ is the slope of a contact-angle isotherm. $\Gamma_{lv} = -\frac{1}{RT}\left(\frac{d\gamma_{lv}}{d\ln C_B}\right)$ is the surface excess at the LV interface determined from separate measurement of concentration-dependent $\gamma_{lv}$ of the solute under study. This form of the Gibbs’ adsorption isotherm is appropriate for a single, isomerically-pure non-ionizing solute or a polyelectrolyte in swamping salt concentrations of buffer salts. It is also important to stress that $[\Gamma_{sl} - \Gamma_{sv}]$ and $\Gamma_{lv}$ values obtained without correcting concentration $C_B$ for solute activity are “apparent” surface excess values that can substantially deviate from actual surface excess calculated from $\left(\frac{d\theta_a}{d\mu}\right)$ and $\left(\frac{d\gamma_{lv}}{d\mu}\right)$; where $\mu$ is activity-corrected chemical potential. However, previous work suggests that the discrepancy between apparent and actual $\Gamma_{lv}$ is roughly constant for the proteins of this study and apparent surface excess was about 56X larger than actual surface excess. We thus assume that apparent $[\Gamma_{sl} - \Gamma_{sv}]$ is also ~56X larger than actual, activity-corrected surface excess because the ratio $\left[\frac{[\Gamma_{sl} - \Gamma_{sv}]}{\Gamma_{lv}}\right] \sim 1$ (see below). Comparison to instrumental measures of adsorption confirms this factor (see Table 3, Results section).
For relatively hydrophobic surfaces exhibiting $\theta_s > 60^\circ$ and under experimental conditions that avoid inadvertent mechanical deposition of solute at the (SV) interface, as through drop movement on the surface or evaporation for examples, it has been shown that $\Gamma_{sv} \sim 0$ and $[\Gamma_{sl} - \Gamma_{sv}] \rightarrow \Gamma_{sl}$. Under the additional restrictions that (i) solute activities at SL and LV interfaces are approximately equal and (ii) $\Gamma_{sl} \sim \Gamma_{lv}$, it can be expected that $\left\{ \frac{[\Gamma_{sl} - \Gamma_{sv}]}{\Gamma_{lv}} \right\} \sim 1$.

Experimental results confirm that these stringent physical conditions prevail and it is therefore concluded that apparent $[\Gamma_{sl} - \Gamma_{sv}] \sim \Gamma_{sl}$ for proteins reported herein.

**Theory of Protein Adsorption:** Previous work disclosed a theory of protein adsorption to the LV surface that appears to be directly applicable to adsorption to the SL surface with little-or-no modification because apparent $[\Gamma_{sl} - \Gamma_{sv}]$ can be directly interpreted in terms of $\Gamma_{sl}$, as discussed above. This protein-adsorption theory was based on two related experimental observations and implications thereof; namely, (i) a surprisingly slight variation in concentration dependence of $\gamma_{lv}$ among the same diverse globular proteins studied herein (Tables 1, 2) and (ii) a substantially constant, MW-independent value of the apparent Gibbs’ surface excess $\Gamma_{lv} = 179 \pm 27$ picomoles/cm$^2$. This work demonstrates parallel behavior at the hydrophobic SL surface with (i) only modest variation in $\Pi_a$ isotherms (Tables 1, 2) and (ii) a substantially constant value of the apparent Gibbs’ surface excess $[\Gamma_{sl} - \Gamma_{sv}] = 175 \pm 33$ picomoles/cm$^2$ (Table 3, Fig. 5). Protein adsorption theory asserts that these experimental observations are outcomes of a relatively constant partition coefficient $P$ that entrains protein within a 3D interphase separating surface regions from
bulk phases (bulk-solution from bulk-vapor for the LV surface or bulk-solution from bulk-solid for the SL surface). This “Guggenheim” interphase treatment, which is especially relevant to the adsorption of large solutes such as proteins, is to be contrasted with the more familiar 2D interface “Langmuir” paradigm in which the surface is construed to be a planar area with negligible thickness; see ref. 2 for more discussion. The 3D interphase is proposed to thicken with increasing protein size because volume occupied by adsorbed-protein molecules scales in proportion to MW according to the well-known relationships among MW, solvent-exposed area, volume, and packing density 39. As a consequence of these relationships, molar interphase concentrations $C_I$ of larger proteins are lower than that of smaller proteins at constant $P = \frac{C_I}{C_B}$. In fact, $C_I$ varies inversely with MW and this leads directly to the Traube-rule-like ordering for proteins mentioned in the Introduction. Protein size and repulsion between molecules within the 3D interphase place an upper bound on maximal interphase concentration denoted $C_{I,\text{max}}$. Interphase saturation occurs at $C_{I,\text{max}}$ and corresponds to the bulk concentration $C_{B,\text{max}}$ at which the limiting adhesion tension $\tau^*_a$ is achieved (i.e. the concentration at maximum spreading pressure $\Pi^*_a = \left(\tau^*_a - \tau^*_a\right)$). Calibration of theory to neutron-reflectometry 40 and quasi-electric light scattering 41,42 of albumin adsorbed to the LV surface at $C_{I,\text{max}}$ suggests that hydrated spheroidal protein molecules achieve nearly FCC densities or, equivalently, that core proteins pack with an efficiency factor $\varepsilon \sim 0.45$. $C_{B,\text{max}}$ is an experimental parameter that can be estimated from concentration-dependent $\theta_a$ curves (see Appendix 7.1) and is related to $C_{I,\text{max}}$ through the partition coefficient $P = \frac{C_{I,\text{max}}}{C_{B,\text{max}}}$. Eq. (2) states relationships among packing densities, molecular dimensions ($MW$), and $C_{B,\text{max}}$ in the form of a logarithmic expression that is convenient to apply to concentration-dependent $\theta_a$ data:
\[ \ln C_{B}^{\text{max}} = \ln \left( \frac{C_{i}^{\text{max}}}{P} \right) = \ln (9.68 \times 10^{11}) - \ln MW + \ln (\varepsilon / P) = - \ln MW + \left[ 27.6 + \ln (\varepsilon / P) \right] \]  

(2)

Assuming that \( \varepsilon / P \) is constant for all proteins within this study, Eq. (2) predicts a linear relationship between \( \ln C_{B}^{\text{max}} \) and \( \ln MW \) with a slope of \(-1\). A value for the unknown ratio \( \varepsilon / P \) can be extracted from the intercept (see Results section).

6.4. Results

SAM Stability: Pure PBS buffer contact angles on SAMs were observed to monotonically decrease with observation time while interfacial tension \( \gamma_{lv} \) (measured by PDT) remained constant, as shown in Fig. 2 (compare open and closed circles). Specifically, it was observed that \( \theta_{o}^{a} \) of a pure PBS droplet slowly decreased with time from the initial value of \( 108^\circ < \theta_{o}^{a} < 106^\circ \) at \( t = 0 \) to \( 104^\circ < \theta_{o}^{a} < 102^\circ \) at \( t = 1 \text{ hr} \); where \( \theta_{o}^{a} \) is the pure buffer contact angle. The range of reported results corresponds to all 17 methyl-terminated SAM surfaces analyzed during the course of this work. This phenomenon attributed to SAM “hydration” apparently affected time-dependent measurement of protein-solution contact angles because we observed that the whole contact angle isotherm (\( \theta_{o}^{a} \) vs. concentration) slowly shifted lower with time (see Fig. 1, annotation in Panel B). Steady-state spreading pressure \( \Pi_{a} \) isotherms effectively correct for the SAM hydration effect in the adsorption measurement by normalizing to final \( \tau_{a}^{o} \); that is \( \Pi_{a} = \left( \tau_{a} - \tau_{a}^{o} \right) \). A similar strategy was applied to analysis of protein adsorption kinetics, as further illustrated in Fig. 2 (compare closed triangles and open triangles). At any time \( t \), reduction in pure PBS contact angle due to hydration (closed circles, Fig. 2) was added to the recorded \( \theta_{a}^{o} \) for a protein-containing solution (closed triangles) to ‘correct’ observed \( \theta_{a}^{o} \) for the hydration effect (open triangles). This correction procedure typically eliminated the long-term drift in \( \theta_{a}^{o} \) observed for protein-containing solutions (see filled triangles, Fig. 2 for
example), suggesting that protein adsorption kinetics had, in fact, dampened within the 1 hr observation period; as had been generally observed for adsorption of these same proteins at the LV surface \(^{10,11}\).

**General Aspects of the Data:** Table 2 compiles quantitative results of this work. Replicate protein preparations were studied for Ub, FV HSA, FXII, IgM and \(\alpha_2\)-macroglobulin. Different vendors were used as a means of controlling for discrepancies that might arise from sourcing (Table 1). Contact angle parameters \(\theta_a^o\), \(\theta_a^l\), In \(\Theta_{B}^{\theta/2}\) and M listed in cols 2-5 of Table 2 are the mean fitted values corresponding to final 25 \(\theta_a\) curves recorded within the 60-minute time frame of the TPG experiment. Listed error is standard deviation of this mean. Corresponding adhesion tensions \(\tau_a^+\) and \(\tau_a^-\) (Columns 6, 7) were computed from \(\theta_a^o\) and \(\theta_a^l\) values, respectively, with uncertainty estimates computed by propagation of error in \(\theta_a\) and \(\gamma_{lv}\) measurements (Materials and Methods). Maximum “spreading pressure” \(\Pi_a^{\max} \equiv \left(\tau_a^- - \tau_a^+\right)\) (Column 8) was computed directly from aforementioned \(\tau_a\) values and associated uncertainty again estimated by propagation of error. Only computed estimates of \(\tau_a^+\), \(\tau_a^-\) and \(\Pi_a^{\max}\) parameters are provided for FXII since the required \(\gamma_{lv}\) values were graphical estimates \(^{10,11}\). Parameters for ubiquitin and \(\alpha_2\)-macroglobulin are also graphical estimates from the steady-state, concentration-dependent \(\theta_a\) curve since surface saturation was not reached within solubility limits for low-MW proteins at the SL interface (as discussed in Appendix 7.3). Therefore, firm values could not be ascertained by statistical-fitting procedures described in Materials and Methods section.
**Adsorption Reversibility:** Fully-reversible adsorption is technically challenging to unambiguously prove. Assumption of reversible adsorption, and hence achievement of thermodynamic equilibrium applied herein, is supported by the following experimental observations:

1. Concentration-dependent $\gamma_w$ and $\theta_a$ of proteins spanning 3 decades in MW (referred to as ‘protein’ or ‘proteins’ below) were like those obtained with small-molecule surfactants in that both followed expectations of Gibbs’ adsorption isotherm $^{23,24}$, with a linear-like decrease in $\gamma_w$ and $\theta_a$ as a function of concentration expressed on a logarithmic concentration axis. Surface excess values ($\Gamma_w$ and $[\Gamma_{sv} - \Gamma_w]$; see next section) computed from Gibbs’ isotherm for surfactant standards agreed with instrumental methods of analysis within experimental error. Surface excess values for proteins adsorbed to LV and SL surfaces were statistically identical.

2. Concentration-dependant $\gamma_w$ and $\theta_a$ continuously decreased as a function of solution concentration, well past the concentration required to fill the surface at theoretical monolayer coverage anticipated for irreversible adsorption.

3. Proteins were observed to be weak surfactants with a commensurately low partition coefficient deduced from concentration-dependent $\gamma_w$ and $\theta_a$ measurements. Free energy of protein adsorption to hydrophobic LV and SL surfaces calculated from partition coefficients agree with values measured by hydrophobic interaction chromatography $^9$.

4. Quantitative aspects of protein and surfactant standards adsorbed to hydrophobic LV and SL surfaces were identical within experimental error. Protein adsorption to hydrophobic LV and SL surfaces followed a ‘Traube-like’ ordering wherein the molar concentration required to achieve an arbitrary spreading pressure decreased in regular progression with MW.

5. Competitive-protein adsorption experiments at hydrophobic LV and SL surfaces demonstrate protein displacement that follows a simple mass-balance exchange.
These lines of evidence support our contention that protein adsorption was reversible under the experimental conditions applied herein and corroborate the conclusion drawn by other investigators employing very different experimental methods that irreversible adsorption is not an inherent property of proteins\textsuperscript{6,43-46}; see also \textsuperscript{2} for a review and citations therein.

**Contact-angle Isotherms:** Time-and-concentration-dependent $\theta_a$ for the nonionic surfactant Tween-20 (MW = 1226Da), and purified proteins, prothrombin (FII; MW = 72kDa) and IgM (MW = 1000kDa) are compared in Fig. 1 in both three-dimensional (3D, $\theta_a$ as a function of time and concentration) and two-dimensional (2D, $\theta_a$ as a function of concentration at specified times) representations. Note that the logarithmic-solute-concentration ordinate $\ln C$ in Fig. 1 is expressed in picomolarity units (pM, $10^{-12}$ moles solute/L solution; see Materials and Methods section for computational and data representation details). Examining first 3D and 2D representations of Tween-20 surfactancy (Fig. 1A) which serves as a reference compound, it was observed that the $\theta_a$ curve was sigmoidal in shape, with a well-defined low-concentration asymptote $\theta'_a$ and a high-concentration asymptote $\theta''_a$. In this latter regard, Tween-20 exhibited concentration-limiting behavior that is typically interpreted as achievement of a critical micelle concentration (CMC), at least for surfactants. This paper provides no evidence of micelles, for either proteins or surfactants, and so only acknowledges a limiting behavior at which further increase in solute concentration did not measurably change $\theta_a$. Smooth curves through the data of Fig. 1 result from least-squares fitting of the four-parameter logistic equation described in Materials and Methods.
Results for all proteins were similar to the surfactant standard Tween-20 (as illustrated for FII and IgM in Fig. 1B and 1C, respectively) in that sigmoidal-shaped $\theta_a$ isotherms connected low- and high-concentration asymptotes. Significantly more pronounced time dependence in $\theta_a$ was observed for proteins, however, especially for intermediate concentrations (in addition to the hydration effects mentioned above). These dynamics were undoubtedly due to rate-limiting, mass-transfer and adsorption steps that slowly brought large macromolecules to LV and SL interfaces relative to the small-molecule reference compound Tween-20 for which only limited dynamics were observed. Observation of time-dependence was important in this particular work only in so far as data demonstrate that $\theta_a$ dynamics dampen within the time frame of experimentation, achieving or approaching steady-state (equilibrium) within the 1 hr observation window. In fact, data collected in Table 2 refers only to steady-state measurements. The bulk-solution concentration at which the limiting $\theta_a$ occurs ($\ln C_B^{\text{max}}$) is of theoretical interest in this work and was estimated from fitted parameters compiled in Table 2, as described in the Appendix 7.1.

**Adhesion Tension and Spreading Pressure Isotherms:** Fig. 3 traces sequential interpretation of steady-state (1 hr drop age), concentration-dependent $\theta_a$ data (Panel A) in terms of concentration-dependent $\tau_a$ (Panel B) and spreading pressure $\Pi_a$ (Panel C) for human serum albumin (FV HSA). Steady-state (equilibrium) spreading pressure isotherms $\Pi_a$ were used as the basis of comparison of protein adsorption for the compounds listed in Table 1. Fig. 4 collects $\Pi_a$ isotherms for selected proteins spanning the molecular weight range $10 < MW < 1000$ kDa showing only smoothed curves for the sake of clarity, but representative $\theta_a$, $\tau_a$, and $\Pi_a$ isotherms with authentic data are amply illustrated in Figs. 1, 3. The dynamic range of $\Pi_a \sim 20$ mN/m was similar to that observed for these
proteins at the LV surface and $\Pi_a^{\text{max}}$ fell within a relatively narrow 5 mN/m band for the diverse set of proteins studied. Furthermore, the same ‘Traube-rule’ ordering of protein adsorption observed at the LV interface was repeated at the SL interface in that high-MW proteins reduce $\Pi_a$ to any arbitrary value at lower molarity than low-MW proteins, as suggested by the horizontal arrow annotation on Fig. 4.

**Apparent Gibbs’ Surface Excess:** Adsorption to the solid-liquid (SL) interface was measured through the apparent Gibbs’ excess parameter $[\Gamma_{sl} - \Gamma_{sv}]$ computed using Eq. (1) applied to contact-angle isotherms (see Appendix 7.2 for example calculations). As noted in the Theory Section, the term “apparent” alerts the reader to the fact that casual application of Gibbs’ adsorption isotherm using $C_B$ instead of activity treats solutes (proteins and surfactants) as isomerically-pure, non-ionized polyelectrolytes at infinite dilution with unit activity coefficients. Table 3 collects results for proteins and the small-molecule surfactant standards SDS and Tween-20. $\Gamma_{lv}$ used in calculation of $[\Gamma_{sl} - \Gamma_{sv}]$ and $\left\{\frac{[\Gamma_{sl} - \Gamma_{sv}]}{\Gamma_{lv}}\right\}$ for surfactant standards was 342±10 and 455±17 picomole/cm$^2$ for SDS and Tween-20, respectively, and were measured by PDT specifically for this work. $[\Gamma_{sl} - \Gamma_{sv}]$ for proteins were computed using the average $\Gamma_{lv} = 179 \pm 27$ picomoles/cm$^2$ previously reported to be characteristic of the proteins listed in Table 1. Table 3 also lists results of independent measures of adsorption, $C_{sl}$, for a few of the compounds listed in Table 3 to be compared to apparent $[\Gamma_{sl} - \Gamma_{sv}]$ measured by TPG. Note that results for small molecule surfactants SDS and Tween 20 were in good agreement with TPG (i.e. $\left\{\frac{[\Gamma_{sl} - \Gamma_{sv}]}{C_{sl}}\right\} ~0.99 \pm 0.01$; rows 10, 11.
column 6). However, results for proteins (rows 1-9, column 6) were in substantial disagreement (i.e. \[ C_{sl} \geq 62.5 \pm 14.9 \]). Fig. 5 shows that MW dependence of apparent \[ \Gamma_{sl} - \Gamma_{sv} \] (panel A) and \( \Gamma_{hv} \) (panel B) as well as the ratio \[ \frac{\Gamma_{sl} - \Gamma_{sv}}{\Gamma_{hv}} \] (panel C) was flat for proteins listed in Table 1 yielding \[ \frac{\Gamma_{sl} - \Gamma_{sv}}{\Gamma_{hv}} \sim 1 \] (see Theory section).

**A Traube-Rule-Analog for Protein Adsorption and Partition Coefficient:** Fig. 6 plots \( \ln C_{\text{max}} \) data compiled in Table 2 for proteins at the solid-liquid (SL) interface (panel A), and compares with results from the liquid-vapor (LV) interface collected in panel B \( ^{10,11} \) on natural logarithmic coordinates compatible with Eq. (2) of the Theory section (data corresponding to ubiquitin was estimated as described in the Appendix 7.3). Protein data fell within a monotonically-decreasing band generally consistent with the anticipation of a unit slope and positive intercept

\[
\ln C_{\text{max}} = (-1.3 \pm 0.2) \ln MW + (19.8 \pm 1.0); R^2 = 78% \]

Comparison to Eq. (2) revealed that \( \varepsilon / P \sim 4.1 \times 10^{-4} \) from the nominal intercept value and, by assuming \( \varepsilon \sim 0.45 \) (as discussed in the Theory section), estimated \( P \sim 1,100. \)

**Competitive Protein Adsorption:** We have observed that \( \tau_a \) for all of the diverse proteins studied herein fell within a relatively narrow 10 mN/m band. However, no two proteins were found to be identical in this regard, mirroring results obtained for these same proteins adsorbed to the hydrophobic LV surface \( ^{10-12} \). In fact, we found that this ‘interfacial signature’ could be used as a kind of tracer in competitive-adsorption experiments revealing the composition of the interphase
formed by adsorption from binary protein mixtures. These mixing experiments also demonstrate that one protein can displace another, strongly indicating that proteins were not irreversibly adsorbed to the surface. Fig. 7 examines time-dependent adhesion tension and spreading pressure of hIgM and FV HSA solutions mixed in various proportions at a fixed total protein concentration of 5 mg/mL; see ref. 11 for more details of HSA and IgM interfacial properties. Protein-adsorption kinetics led to time-dependent τ_a (corrected for SAM hydration, see above) wherein adhesion tension was observed to quickly rise from τ_a^0 ∼ −20 mN/m characteristic of pure PBS on the SAM surface to a steady-state (equilibrium) τ_a characteristic of that protein solution, as illustrated in Fig. 7A for 100% albumin (circles), 50:50 albumin:IgM (diamonds), and 100% IgM (squares). Fig. 7B plots observed steady-state (1 hr) spreading pressure Π_{obs} at varying weight-fraction albumin compositions f_{alb} in hIgM (expressed as per-cent of 5 mg/mL total protein). These results strongly suggest that competitive adsorption between proteins leads to displacement of hIgM by albumin through a process that strictly follows the wt/v concentration of competing proteins and clearly indicate that IgM was not irreversibly adsorbed.

6.5. Discussion

Adsorption Isotherms: Adsorption isotherms constructed from concentration-dependent contact angles (θ_a, τ_a, and Π_a, see Figs. 1, 3 and 4) for the proteins studied herein exhibited many similarities to concentration-dependent γ_{lv} reported previously 10. Maximum spreading pressure, Π_{a}^{max}, fell within a relatively narrow 10 < Π_{a}^{max} < 20 mN/m band characteristic of all proteins studied, just as observed at the LV surface. Furthermore, Π_a isotherms exhibited the ‘Traube-rule-like’ progression in MW observed at the LV surface wherein the molar concentration required to
reach a specified $\Pi_a$ value decreased with increasing MW. Bearing in mind the great range in MW spanned by proteins in Fig. 4, it is reasonable to conclude that commensurate variability in protein composition did not confer widely-varying SL interfacial activity; at least not in comparison to the full range available to ordinary surfactants. The inference taken from the Traube-rule-like progression is that protein concentration required to reduce $\Pi_a$ to a specified value decreases with MW in a manner loosely consistent with the addition of a generic amino-acid-building-block having an “average amphilicity” that increases MW but does not radically change protein amphilicity. Otherwise, if MW increased by addition of amino-acid-building-blocks with highly-variable amphilicity, then $\Pi_a$ would be expected to be a much stronger function of protein MW than is observed. Thus, it appears that molar variability in $\Pi_a$ is achieved by aggregating greater mass of similar amphiphilic character, as opposed to accumulating greater amphilicity with increasing MW.

**Apparent Gibbs’ Surface Excess:** Adsorption measurements by concentration-dependent contact angles were in good agreement with literature values for the surfactant standards SDS and Tween-20, as listed in Table 3. Close agreement between apparent $[\Gamma_{sl} - \Gamma_{sv}]$ and $C_{sl}$ from alternative methods suggests that (i) assumptions of purity and unitary activity coefficients were reasonable for these small molecules and (ii) solute deposition at the SV interface was negligible (see Theory section). However, $[\Gamma_{sl} - \Gamma_{sv}]$ for proteins were quite different than values drawn from comparable literature sources, as was observed to be the case for apparent $\Gamma_{lv}^{10}$. No doubt proteins violate assumptions of ideality and unitary activity coefficients$^{47,48}$, causing apparent $[\Gamma_{sl} - \Gamma_{sv}]$ to deviate substantially from real, activity-corrected surface excess. Previous work showed that apparent and real $\Gamma_{lv}$ for proteins were different by a factor of about 56 and that apparent $\Gamma_{lv}$ was
approximately constant across the span of protein MW studied\textsuperscript{10}. Apparent $[\Gamma_{sl} - \Gamma_{sv}]$ was found to differ from independent measures by a factor of 62.5±14.9, as inferred from the mean $\left\{\frac{[\Gamma_{sl} - \Gamma_{sv}]}{C_{sl}}\right\}$ ratio for proteins (see column 6, rows 1-9, Table 3), consistent with estimates from the LV interface above. Fig. 5 plots apparent $[\Gamma_{sl} - \Gamma_{sv}]$ and $\Gamma_{lv}$, and the ratio $\left\{\frac{[\Gamma_{sl} - \Gamma_{sv}]}{\Gamma_{lv}}\right\}$ as a function of MW showing that $\Gamma_{lv} \sim \left[\Gamma_{sl} - \Gamma_{sv}\right]$ and that, as a consequence, $\left\{\frac{[\Gamma_{sl} - \Gamma_{sv}]}{\Gamma_{lv}}\right\} \sim 1$. We thus conclude that $\left[\Gamma_{sl} - \Gamma_{sv}\right] \sim \Gamma_{sl} \sim \Gamma_{lv}$ for the globular proteins studied herein. By contrast, $\left\{\frac{[\Gamma_{sl} - \Gamma_{sv}]}{\Gamma_{lv}}\right\} = 3.8±0.1$ for Tween 20 (row 11, column 4, Table 3) suggesting nearly 4X concentration at the SL interface over LV, consistent with results reported for Tween-80 at silanated glass surfaces\textsuperscript{1}.

**A Traube-Rule-Analog for Protein Adsorption and Partition Coefficient:** A flat trend in $\Gamma_{lv}$ and $\Gamma_{sl}$ with MW is consistent with an interphase concentration $C_t$ (in units of moles/cm\textsuperscript{3}) scaling inversely with MW and an interphase thickness $\Omega$ (in units of cm) that scales directly with MW. This is because $\Gamma = C_t \Omega$ (when the partition coefficient $P >> 1$) and MW dependence cancels\textsuperscript{10}. In other words, the interphase thickens as adsorbed proteins become larger and Gibbs’ dividing plane descends deeper into the surface region\textsuperscript{1,23,24}. Interpreted in terms of the theory of protein adsorption briefly outlined in the Theory section, hydrated spheroidal protein molecules with net radius $R$ scaling as a function of $MW^{1/3}$ pack into the interphase to a concentration $C_t^{max}$ limited by osmotic repulsion between molecules. Or stated another way, $C_t^{max}$ is limited by the extent to which the interphase can be dehydrated by protein displacement of interfacial water. Interphase
dehydration is more related to the properties of water than the proteins themselves and so the partition coefficient \( P \equiv \frac{C_I}{C_B} \) is observed to be approximately constant among the proteins investigated.

Fig. 6A plots \( C_B^{\text{max}} \) data compiled in Table 2 on logarithmic coordinates compatible with Eq. (2) of the Theory section. Proteins fell within a monotonically-decreasing band roughly consistent with the anticipation of a unit slope and positive intercept

\[
\ln C_B^{\text{max}} = (-1.3 \pm 0.2) \ln MW + (19.8 \pm 1.0); R^2 = 78\%.
\]

A similar trend was observed for protein adsorption at the LV surface, shown in Fig. 6B. Interpretation of these results must take into account that the highly-simplified model of adsorption treats proteins as uniform hard spheres and does not attempt to account for structural complexities of real molecules, or unfolding (denaturation) that may occur upon packing within the surface region. Hence failure of data to quantitatively adhere to Eq. (2) is hardly surprising. Even so, results for Ub were significantly off the trend obtained at the LV surface (compare to Figs 6A, B), possibly signaling that this small protein does not retain a spherical geometry at the SL surface. Clearly, more work is required to further test such speculation and expand the range of proteins explored. However, even in light of scatter in the data of Fig. 6A, it is of interest to estimate \( \epsilon/P \sim 4.1 \times 10^{-4} \) from the nominal intercept value and, by assuming \( \epsilon \sim 0.45 \) (see Theory section), estimate \( P \sim 1,100 \); which is within an order-of-magnitude of the \( P \sim 150 \) estimate from analysis of protein adsorption to the LV surface and \( P \sim 5,000 \) from neutron reflectometry of albumin adsorption to the LV surface \(^{10} \). Clearly, goniometry is not a good method for deducing partition coefficients, but it is of continued interest to compute protein adsorption energetics based on these rough estimates. With \( 10^2 < P < 10^3 \), the free energy of protein adsorption
to the hydrophobic surface $\Delta G_{\text{ads}}^o = -RT \ln P$ is very modest, lying within the range $-7RT < \Delta G_{\text{ads}}^o < -4RT$. This is consistent with estimates for lysozyme, myoglobin, and $\alpha$-amylase adsorption to hydrophobic surfaces ($\Delta G_{\text{ads}}^o \sim -5RT$) measured by hydrophobic interaction chromatography. Thus, a conclusion that can be drawn, in spite of rather poor estimates of $P$, is that adsorption of proteins to a hydrophobic surface is energetically favorable by only small multiples of thermal energy $RT$ and apparently does not vary significantly among proteins.

According to Eq. (2) and Fig. 6, low-MW proteins require greater bulk-phase concentrations to saturate the SL (or LV) interphase than higher-MW proteins. Given that $C_{\text{b}}^{\text{max}}$ values plotted in Fig. 6 approach 1% w/v, it is reasonable to anticipate that extrapolated $C_{\text{b}}^{\text{max}}$ values for yet-lower-MW proteins must equal or exceed protein-solubility limits. As a consequence, surface saturation and the related limiting $\Pi_a^{\text{max}}$ is not expected for low-MW proteins at fixed $P$. In this regard, it is noteworthy that $\Pi_a$ isotherms for low-MW proteins such as ubiquitin (10.7 kDa) fail to achieve a limiting $\Pi_a^{\text{max}}$ at any concentration below the solubility limit, as was observed for concentration-dependent $\gamma_{lv}$.

**Competitive Protein Adsorption:** Fig. 7 is strong evidence that there is ready exchange of albumin and IgM at the SAM surface, with relative amounts of adsorbed protein following a simple linear combining rule expressed in weight-fraction protein in the bulk phase. Taken together with related observations summarized in the Theory section, we are led to conclude that protein adsorption to hydrophobic SAM surfaces was substantially reversible under the experimental conditions employed in this work. The word ‘substantially’ is purposely used here because evidence at hand does not
guarantee that every adsorbed protein molecule was reversibly bound to the surface (or within the surface region). Indeed, some unknown fraction of adsorbed protein could be irreversibly bound to surface defects which are undetected by tensiometric methods applied herein. However, given the exquisite quality of SAM surfaces and similarity of results obtained at molecularly-smooth LV surfaces, this putative fraction of irreversibly-bound protein must be vanishingly small.

### 6.6. Conclusions

Interfacial energetics of protein adsorption from aqueous-buffer solutions to hydrophobic methyl-terminated SAM surfaces are strikingly similar to the interfacial energetics of protein adsorption to the hydrophobic air-water surface. The observed ‘Traube-rule-like’ progression in interfacial-tension reduction ($\gamma_{lv}$ and $\tau_s$), conserved partition coefficient $P$, and constant Gibbs’ surface excess ($\Gamma_{lv}$ and $\Gamma_{sv}$) for globular proteins spanning 3 decades in MW all occur because water controls the energetics of the adsorption process. Hence, protein adsorption to hydrophobic surfaces has more to do with water than the proteins themselves. A relatively straightforward theory of protein adsorption predicated on the interfacial packing of hydrated spherical molecules with dimensions scaling as a function of MW accounts for the essential physical chemistry of protein adsorption and rationalizes significant experimental observations. From this theory it is evident that displacement of interfacial water by hydrated proteins adsorbing from solution places an energetic cap on protein adsorption to hydrophobic surfaces ($-7RT < \Delta G_{ads}^o < -4RT$). This phenomenon is generic to all proteins. Thus, globular-blood protein adsorption to hydrophobic surfaces is not found to significantly vary among diverse protein types.
Variations from general trends discussed above may reflect deviations in protein geometry from simple spheres and/or tendency of some proteins to adopt a more spread/compact configuration (denature) in the adsorbed state. Indeed, there is the expectation from a burgeoning literature base that proteins ‘denature’ over time \(^{49}\). Denaturation can include changes in molar free volume/interfacial area, loss of higher-order structure with concomitant change in specific bioactivity, and irreversible adsorption. Of course, tensiometric methods are effectively blind to these molecular processes, except insofar as denaturation may lead to time-varying interfacial tensions and contact angles. Our measurements achieved, or asymptotically approached, a well-defined steady-state within the hour observation window applied, suggesting that putative ‘denaturation processes’ either had an insignificant impact on results or occurred significantly faster/slower than the time frame of experimentation. Given the similarity in adsorption energetics to hydrophobic LV and SL surfaces among the broad array of proteins studied and the general expectation that denaturation is a slow process, we are inclined to conclude that either denaturation did not significantly affect results (perhaps accounting for small-but-measurable differences among proteins) or the denaturation effect was astonishingly similar among very different proteins. With regard to irreversible adsorption, we note that experiments examining competitive adsorption between albumin and IgM at the LV surface demonstrated protein displacement (Vroman effect) that followed a simple mass-balance exchange \(^{12}\), strongly suggesting that neither albumin nor IgM was irreversibly adsorbed to this surface.
6.7. Appendix

6.7.1. Estimation of $C_B^{\text{max}}$

$C_B^{\text{max}}$ was calculated from the slope of an advancing contact angle $\theta_a$ isotherm $\frac{\Delta \theta_a}{\Delta \ln C_B}$ and fitted data (Table 2) by evaluating Eq. A1 at half-maximal change in $\theta_a$, which occurs at a bulk-phase composition $\ln C_B^{\Theta/2}$ (where $\Theta/2 = 1/2 \Theta^{\text{max}}$ and $\Theta^{\text{max}} \equiv \theta_a^* - \theta_a^*$):

$$\frac{\Delta \theta_a}{\Delta \ln C_B} = -RTS = \left( \frac{\theta_a^* - \theta_a^*}{\ln C_B^{\text{max}} - \ln C_B^{\Theta/2}} \right) = \left( \frac{\theta_a - \left( \frac{\theta_a^* + \theta_a^*}{2} \right)}{\ln C_B^{\text{max}} - \ln C_B^{\Theta/2}} \right)$$

............... Eq. A1

$$\Rightarrow \ln C_B^{\text{max}} = \frac{\Theta^{\text{max}}}{2RTS} + \ln C_B^{\Theta/2}$$

where the terms $S = -\left( \frac{1}{RT} \right) \frac{\Delta \theta_a}{\Delta \ln C_B}$, $\theta_a^*$ and $\theta_a^*$ measured at $\ln C_B^{\Theta/2}$ and $\Theta^{\text{max}} \equiv \theta_a^* - \theta_a^*$. All of the parameters in the RHS of Eq. A1 are derived from non-linear, least-squares fitting of $\theta_a$ isotherms to the four-parameter logistic equation described in the Materials and Methods section. Confidence in $C_B^{\text{max}}$ values listed in Table 2 and plotted in Fig. 6 was computed by propagation of the standard errors in best-fit parameters through Eq. A1, as given by Eq. A2. In consideration of all sources of experimental error, we conclude that $\ln C_B^{\text{max}}$ estimates are no better than about 20%. 

$$\sigma^2_{\ln C_B^{\text{max}}} = \sigma^2_{\ln C_B^{\Theta/2}} + \frac{1}{4(RTS)^2} \left[ \sigma^2_{\theta_a^*} - \sigma^2_{\theta_a^*} - \left( \frac{\theta_a^* - \theta_a^*}{S^2} \right)^2 \sigma^2_S \right]$$

...............Eq. A2

where $\sigma$’s represent standard errors in $\ln C_B^{\text{max}}$ and the best-fit parameters $\ln C_B^{\Theta/2}$, $\theta_a^*$, $\theta_a^*$ and $S$ as denoted by subscripts.
6.7.2. Estimation of \( [\Gamma_{sl} - \Gamma_{sv}] \)

The apparent Gibbs’ surface excess \( [\Gamma_{sl} - \Gamma_{sv}] \) was computed from Eq. 1 of the Theory section for each of the proteins and surfactants listed in Table 3. The following steps illustrate surface excess calculations for FV HSA (preparation 1, Table 2) at the solid-liquid (SL) interface. Fit of \( \theta_a \) isotherm data plotted in Fig. 3 yielded \( \theta_a^r = 103.3 \pm 0.8, \theta_a^l = 88.3 \pm 0.8, \ln C_B^{\Theta/2} = 15.9 \pm 0.3 \), and \( M = -14.1 \pm 5.7 \). Inflections in the \( \theta_a \) curve were located at \( X_1 = 13.7 \) and \( X_2 = 10.9 \) (dimensionless), yielding a slope estimate \( \Delta' \) from the finite difference with calculated uncertainty as

\[ \Delta' \equiv \frac{\Delta \theta_a}{\Delta X} = -2.95 \pm 0.04 \text{deg} = -0.050 \pm 0.007 \text{rad} ; \]

where \( \Delta \theta_a = \theta_a\big|_{X_2} - \theta_a\big|_{X_1} \) and \( \Delta X = X_2 - X_1 \). Values for \( \theta_a \) were calculated from the characteristic parameters above, conveniently evaluated at \( \ln C_B = \ln C_B^{\Theta/2} \) where the logistic equation simplifies to

\[ \theta_a = \theta_a^r = \left( \frac{\theta_a^r + \theta_a^l}{2} \right) \].

Thus, \( \theta_a^r = \frac{103.3 + 88.3}{2} = 95.8^\circ; \sin \theta_a^r = 0.99; \cos \theta_a^r = -0.10 \).

The required term \( \gamma_{lv} \) was calculated from a comparable logistic equation for \( \gamma_{lv} \) isotherms, using LV fitted parameters,\(^ {10,11} \) but evaluated at \( \ln C_B = \ln C_B^{\Theta/2} = 11.7 \) as

\[ \gamma_{lv} = \frac{70.8 - 46.2}{1 + (12.4/11.7)^{1.3}} + 46.2 = 61.1 \text{mN/m} . \]

Using \( \Gamma_{lv} = 179 \text{pmol/cm}^2 \) determined from ref.\(^ {10} \), Eq. 1 was computed as

\[ [\Gamma_{sl} - \Gamma_{sv}] = - \left( \left[ 61.1 \sin 95.8 \right] \text{ergs/cm}^2 + \left[ 179 \cos 95.8 \right] \right) = 145 \text{ pmol/cm}^2 \]

Uncertainty in \( [\Gamma_{sl} - \Gamma_{sv}] \) was computed by propagation of error into \( \Delta \Gamma = [\Gamma_{sl} - \Gamma_{sv}] \) as:

\[ \sigma^2_{\Delta \Gamma} = \left[ \frac{\sin \theta_a}{RT} \frac{d \theta_a}{d \ln C_B} \right]^2 \sigma_{\theta_a}^2 + \left[ \frac{\gamma_{lv}}{RT} \frac{d \theta_a}{d \ln C_B} \cos \theta_a + \Gamma_{lv} \sin \theta_a \right]^2 \sigma_{\gamma_{lv}}^2 + \left[ \frac{\gamma_{lv} \sin \theta_a}{RT} \right]^2 \sigma_{\gamma_{lv}}^2 \cos^2 \theta_a \]

where $S' \equiv \frac{d\theta_a}{d \ln C_B}$. The $\sigma$ terms for $\gamma_{iv}, \theta_a$ and $S'$ were computed from

$$
\sigma_{\gamma_{iv}}^2 = \frac{\sigma_{IV}^2 + \sigma_{\gamma'}^2}{4} \quad \text{and} \quad \sigma_{\theta_a}^2 = \frac{\sigma_{\theta'}^2 + \sigma_{\theta a}^2}{4};
$$

where $\gamma_{iv}, \gamma_{iv}'$ and $\theta_a, \theta_a'$ are fitted parameters from $\gamma_{iv}$ and $\theta_a$ isotherms; as described above. Uncertainty in slope $\sigma_S^2 = \frac{\sigma_{\gamma_{iv}}^2 + \sigma_{\gamma'}^2}{\Delta \Lambda^2}$. Thus, uncertainty in $\Delta \Gamma$ is given by:

$$
\sigma_{\Delta \Gamma} = \sqrt{\left[\sin 95.8 \left( \frac{61.1 \sin 95.8}{2.48 \times 10^{-2}} \right) (0.05) \right]^2 + \left[61.1 \cos 95.8 \cos 95.8 + 179 \sin 95.8 \cos 95.8 (9.8 \times 10^{-5}) \right]^2}
\sigma_{\Delta \Gamma} = 18.02
$$

where $RT = (8.31 \times 10^7)(298.15)(10^{-12}) = 2.48 \times 10^{-2}$ ergs / pmol.

Thus, $[\Gamma_{sl} - \Gamma_{iv}] = 145 \pm 18$ pmol / cm$^2$ as reported in Table 3 (row 4 column 2).

### 6.7.3. Estimation of parameters of Ubiquitin and $\alpha_2$-macroglobulin

Parameters for ubiquitin and $\alpha_2$-macroglobulin listed in Tables 2-3 and shown in Figs. 4-6 were graphical estimates from the steady-state, concentration-dependent $\theta_a$ curve. Firm values could not be ascertained by statistical-fitting procedures described in Materials and Methods because surface saturation was not reached within solubility limits for this protein. Thus, well-defined high concentration asymptotes, $\theta_a'$ were not achieved at physically-realizable concentrations. Hence, $\theta_a$ measured at the highest-concentration studied was used as an estimate for $\theta_a'$. Adhesion tensions were computed accordingly, with graphical estimates from $\gamma_{iv}$ isotherm as $\tau_a^o = \gamma_{iv}^o \cos \theta_a^o$ and
\[ \tau_a' = \gamma' \cos \theta_a' \]  

in \( C_B^{\alpha/2} \) and \( \frac{d\theta_a}{d \ln C_B} \) parameters were estimated by graphical location of inflection points on the \( \theta_a \) curve. These estimates were used in the calculation of \( C_B^{\text{max}} \) and \( [\Gamma_{sl} - \Gamma_{sv}] \) parameters, as described in Appendix 7.1 and 7.2.


(18) Garcia de la Torre, J. Hydration from hydrodynamics. General considerations and applications of bead modelling to globular proteins, Biophysical Chemistry 2001, 93, 159.


(20) Durchschlag, H.; Zipper, P. Comparative investigations of biopolymer hydration by physicochemical and modeling techniques, Biophys. Chem. 2001, 93, 141.


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<td>160 Powder</td>
<td></td>
<td>97%</td>
<td>800-1800 (1300)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>FIBRINOGEN (Fb)</td>
<td>340 Powder</td>
<td></td>
<td>70% clottable protein</td>
<td>200-450 (325)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>COMPLEMENT COMPONENT C1q (C1q)</td>
<td>400 Solution (1.1)</td>
<td></td>
<td>Single band by immunoelectrophoresis</td>
<td>10-25 (17.5)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td></td>
<td>Preps 98%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2-MACROGLOBULIN (αmac)</td>
<td>Prep 1: 725 Powder</td>
<td></td>
<td>98%</td>
<td>150-350 (250)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td></td>
<td>Prep 2: 98%</td>
<td></td>
<td></td>
<td></td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td></td>
<td>Prep 3: 98%</td>
<td></td>
<td></td>
<td></td>
<td>MP Biomedicals</td>
</tr>
<tr>
<td>HUMAN IgM (IgM)</td>
<td>Prep 1: Solution (0.8)</td>
<td></td>
<td>98%</td>
<td>60-250 (155)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td></td>
<td>Prep 2: Solution (5.1)</td>
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<td>Single band by immunoelectrophoresis</td>
<td></td>
<td>MP Biomedicals</td>
</tr>
<tr>
<td>SODIUM DODECYL SULFATE (SDS)</td>
<td>0.28 Powder</td>
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<td>N/A</td>
<td>N/A</td>
<td>Sigma Aldrich</td>
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<tr>
<td>TWEEN 20 (TWN20)</td>
<td>1.23 Neat</td>
<td></td>
<td>N/A</td>
<td>N/A</td>
<td>Sigma Aldrich</td>
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### TABLE 2: STEADY-STATE PROTEIN ADSORPTION PARAMETERS

<table>
<thead>
<tr>
<th>NAME OF PROTEIN/SURFACANT (acronym)</th>
<th>$\theta_a^\circ$ (degrees)</th>
<th>$\theta_a^\circ$ (degrees)</th>
<th>$\ln C_a^{9/2}$ PPT (pM)</th>
<th>$M$ (dimensionless)</th>
<th>$\tau_a^\circ$ (mN/m)</th>
<th>$\tau_a^\circ$ (mN/m)</th>
<th>$\Pi_a^{\max}$ (mN/m)</th>
<th>$\ln C_a^{\max}$ (pM)</th>
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<tr>
<td>UBIQUITIN (Ub)</td>
<td></td>
<td>75</td>
<td>19 (17)</td>
<td>-</td>
<td>-14</td>
<td>7</td>
<td>21</td>
<td>19</td>
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<tr>
<td>Prep 1</td>
<td>100.9±0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prep 2</td>
<td>102.2±0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>THROMBIN (FIIa)</td>
<td>99.8±0.5</td>
<td>84.6±0.9</td>
<td>17.5±0.2 (13.9±0.2)</td>
<td>-25.0±8.5</td>
<td>-12.3±0.6</td>
<td>4.5±0.7</td>
<td>16.7±0.9</td>
<td>15.1±0.2</td>
</tr>
<tr>
<td>HUMAN SERUM ALBUMIN (HSA)</td>
<td>103.3±0.8</td>
<td>88.3±0.8</td>
<td>15.9±0.3 (11.7±0.3)</td>
<td>-14.1±5.7</td>
<td>-16.3±0.9</td>
<td>1.4±0.6</td>
<td>17.7±1.2</td>
<td>13.6±0.3</td>
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<tr>
<td>Prep 1</td>
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<td></td>
<td></td>
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<tr>
<td>Prep 2</td>
<td>104.5±0.8</td>
<td>88.5±0.6</td>
<td>15.7±0.3 (11.5±0.3)</td>
<td>-11.6±3.0</td>
<td>-17.7±0.9</td>
<td>1.2±.5</td>
<td>18.9±1.1</td>
<td>13.7±0.3</td>
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<td>PROTHROMBIN (FII)</td>
<td>100.6±0.5</td>
<td>86.5±0.9</td>
<td>15.1±0.4 (10.8±0.4)</td>
<td>-10.1±2.7</td>
<td>-12.9±0.6</td>
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<td>15.6±0.9</td>
<td>13.2±0.4</td>
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<td>Factor XII</td>
<td>102.9±0.5</td>
<td>94.8±1.0</td>
<td>15.6±0.5 (11.3±0.5)</td>
<td>-17.9±1.2</td>
<td>-15.6</td>
<td>-3.1</td>
<td>12.5</td>
<td>12.7±0.5</td>
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<tr>
<td>Prep 1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prep 2</td>
<td>102.0±0.4</td>
<td>88.2±0.8</td>
<td>15.7±0.4 (11.3±0.4)</td>
<td>-10.9±3.3</td>
<td>-14.6</td>
<td>1.2</td>
<td>15.8</td>
<td>13.6±0.4</td>
</tr>
<tr>
<td>HUMAN IgG (IgG)</td>
<td>103.7±0.7</td>
<td>94.9±1.4</td>
<td>15.1±0.9 (10.1±0.9)</td>
<td>-6.9±1.3</td>
<td>-16.8±0.9</td>
<td>-4.4±1.3</td>
<td>12.4±1.5</td>
<td>13.3±0.9</td>
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<tr>
<td>FIBRINOGEN</td>
<td>103.9±0.6</td>
<td>85.3±.9</td>
<td>15.5±0.3 (9.8±0.3)</td>
<td>-6.4±1.3</td>
<td>-16.8±0.8</td>
<td>-3.8±0.8</td>
<td>20.7±1.1</td>
<td>13.2±0.3</td>
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<tr>
<td>COMPLEMENT COMPONENT C1q (C1q)</td>
<td>102.6±0.4</td>
<td>95.3±0.7</td>
<td>15.6±0.4 (9.6±0.4)</td>
<td>-12.1±5.6</td>
<td>-15.6±0.5</td>
<td>-5.0±0.7</td>
<td>10.6±0.8</td>
<td>11.4±0.4</td>
</tr>
<tr>
<td>$\alpha_2$-MACROGLOBULIN$^*$ ($\alpha$ mac)</td>
<td>101.9±0.5</td>
<td>86</td>
<td>19 (13)</td>
<td>-</td>
<td>-15</td>
<td>4</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>Prep 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prep 2</td>
<td>100.2±0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prep 3</td>
<td>103.2±0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUMAN IgM (IgM)</td>
<td>102.7±0.6</td>
<td>91.3±1.6</td>
<td>15.5±0.5 (8.7±0.5)</td>
<td>-7.4±2.9</td>
<td>-15.7±0.7</td>
<td>-1.1±1.4</td>
<td>14.6±1.6</td>
<td>11.3±0.5</td>
</tr>
<tr>
<td>Prep 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prep 2</td>
<td>102.4±0.6</td>
<td>87.8±2.0</td>
<td>15.9±0.6 (9.2±0.7)</td>
<td>-4.9±1.6</td>
<td>-15.4±0.7</td>
<td>1.9±1.7</td>
<td>17.3±1.9</td>
<td>12.6±0.8</td>
</tr>
<tr>
<td>SODIUM DODECYL SULFATE (SDS)</td>
<td>100.1±1.9</td>
<td>56.0±2.3</td>
<td>17.7±0.4 (18.9±0.4)</td>
<td>-17.3±4.6</td>
<td>-12.5±2.3</td>
<td>18.7±1.1</td>
<td>31.2±2.6</td>
<td>21.4±0.4</td>
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<tr>
<td>TWEEN 20 (TWN20)</td>
<td>97.1±0.6</td>
<td>65.1±0.7</td>
<td>16.4±0.3 (16.2±0.1)</td>
<td>-23.4±3.3</td>
<td>-8.9±0.8</td>
<td>14.6±0.5</td>
<td>23.5±0.6</td>
<td>17.8±0.1</td>
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</table>

Notes: * Parameters are graphical estimates of fitted parameters. See Results section.
<table>
<thead>
<tr>
<th>NAME OF PROTEIN/SURFACTANT (acronym)</th>
<th>Apparent Surface Excess † (picomoles/cm²)</th>
<th>Comparison to Literature values</th>
<th>Technique (Citation)</th>
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<td></td>
<td>$\left[ \Gamma_{v_s} - \Gamma_{v_i} \right]$</td>
<td>$\Gamma_{v_i} \left{ \frac{\left[ \Gamma_{v_s} - \Gamma_{v_i} \right]}{\Gamma_{v_i}} \right}$</td>
<td>$C_{SL}$ (picomoles/cm²)</td>
</tr>
<tr>
<td></td>
<td>$\left[ \Gamma_{v_s} - \Gamma_{v_i} \right]$</td>
<td>$\Gamma_{v_i} \left{ \frac{\left[ \Gamma_{v_s} - \Gamma_{v_i} \right]}{\Gamma_{v_i}} \right}$</td>
<td>$C_{SL}$ (picomoles/cm²)</td>
</tr>
<tr>
<td>UBIQUITIN (Ub)*</td>
<td>Prep 1  224</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prep 2  193</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>THROMBIN (FIIa)</td>
<td>308±34</td>
<td>1.7±0.3</td>
<td>-</td>
</tr>
<tr>
<td>HUMAN SERUM ALBUMIN</td>
<td>FV HSA Prep 1  145±18</td>
<td>0.8±0.2</td>
<td>2.4</td>
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<td>Prep 2  196±21</td>
<td>1.1±0.2</td>
<td>80</td>
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<td>PROTHROMBIN (FII)</td>
<td>146±17</td>
<td>0.8±0.2</td>
<td>-</td>
</tr>
<tr>
<td>FACTOR XII†</td>
<td>Prep 1  136</td>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Prep 2  153</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>HUMAN IgG (IgG)</td>
<td>198±37</td>
<td>4.5</td>
<td>44</td>
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<td></td>
<td></td>
<td>1.1±0.3</td>
<td>2.9</td>
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<td>COMPLEMENT COMPONENT C1q (C1q)</td>
<td>117±28</td>
<td>0.7±0.2</td>
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<tr>
<td>MACROGLOBULIN* (αmac)</td>
<td>Prep 1  130</td>
<td>0.7</td>
<td>-</td>
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<tr>
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<td>Prep 2  130</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Prep 3  130</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>HUMAN IgM (IgM)</td>
<td>Prep 1  222±42</td>
<td>1.2±0.3</td>
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<td></td>
<td>Prep 2  101±27</td>
<td>0.6±0.2</td>
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<tr>
<td>SODIUM DODECYL SULFATE (SDS)</td>
<td>276±14</td>
<td>342±10</td>
<td>1.2±0.2</td>
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<td>TWEEN 20 (TWEEN-20)</td>
<td>120±16</td>
<td>455±17</td>
<td>3.8±0.1</td>
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Notes: * Parameters are graphical estimates of fitted parameters. See Results section
† Apparent $\left[ \Gamma_{v_s} - \Gamma_{v_i} \right]$ or $\Gamma_{v_i}$ is computed without activity correction (see Theory section).
Figure 1: Advancing contact angle isotherms in 3D ($\theta_a$ as a function of analysis time (drop age) and logarithmic (natural) solution concentration $C_B$) and 2D ($\theta_a$ as a function of logarithmic solution concentration $C_B$ at selected times) formats comparing Tween-20 (Panel A, TWEEN-20, Table 2), prothrombin (Panel B, FII, Table 2), and immunoglobulin-M (Panel C, IgM, preparation 2, Table 2) adsorption to a methyl-terminated SAM surface. In each case, solute concentration $C_B$ is expressed in picomoles/L (pM) on a natural logarithmic scale. Symbols in 2D panels represent time slices through 3D representations (filled circle = 0.25 sec, open circle = 900 sec, filled triangles = 1800 sec, and open triangles = 3594 sec; annotations in Panel A indicate maximum and half-maximum contact angle reduction, $\theta_a^{\text{max}}$ and $\frac{1}{2} \theta_a^{\text{max}}$ respectively. Notice that adsorption kinetics dominated IgM adsorption whereas steady-state was achieved within about 1000 sec for FII, and nearly no adsorption kinetics is detected for Tween-20. Note also decrease in $\theta_a$ with time, attributed to slow hydration of the SAM surface (Panel B, arrow annotation; see Results section for more discussion).
Figure 2: Advancing PBS contact angles $\theta_a^o$ (left axis, closed circles) on 1-hexadecane thiol SAMs on gold decrease monotonically with observation time while liquid-vapor interfacial tension, $\gamma_{lv}$ (right axis, open circles) remains constant, suggesting time-dependent “hydration” of the SAM surface. SAM hydration also affects $\theta_a$ adsorption isotherms shown in Fig. 1 (arrow annotation, Panel B). SAM hydration dynamics were separated from protein adsorption kinetics by ‘correcting’ observed change in $\theta_a$ (closed triangles, corresponding to 40 mg/mL albumin in PBS) for the decrease in $\theta_a^o$ observed in control experiments with pure buffer (yielding open triangles).
Figure 3: Sequential interpretation of a steady-state (3600 sec drop age) contact angle adsorption isotherm for human serum albumin (FV HSA, preparation 2, Table 2); Panel A, advancing contact angles, $\theta_a$; Panel B, advancing adhesion tension, $\tau_a$; Panel C, advancing spreading pressure $\Pi_a$. Smoothed curves through the data serve as guides to the eye. Annotations identify low- and high-concentration asymptotes for contact angles ($\theta_a^+$, $\theta_a^-$), adhesion tensions ($\tau_a^+$, $\tau_a^-$) and maximum spreading pressure $\Pi_a^{\text{max}}$ that are used to characterize isotherms (Table 2).
Figure 4: Comparison of steady-state spreading pressure $\Pi_a$ isotherms for selected proteins spanning three decades in molecular weight (Table 1). Smooth curves are guides to the eye (see Figs. 1 and 3 for similar plots including authentic data and Table 2 for statistics of fit). Molar scaling reveals an ordering among diverse proteins, similar to the ‘Traube-rule’ observed for proteins at the liquid-vapor interface wherein molar concentration required to reach a specified $\Pi_a$ value decreased with increasing MW (arrow).
The graph shows the relationship between \( \Pi_a \) (mN/m) and \( \ln C_B \) (\( C_B \) in pM) for different proteins: IgM, IgG, C1q, FII, FV HSA, and Ub. The curves indicate the titration curves for each protein, with Ub being the highest at the top right.
**Figure 5:** Apparent Gibbs’ surface excess scaled by protein MW at the solid-liquid (SL) ([\(\Gamma_{sl} - \Gamma_{sv}\)], panel A) and the liquid-vapor (LV) interfaces (\(\Gamma_{lv}\), panel B) for multiple protein preparations (open circle = preparation 1, filled circle = preparation 2, filled triangle = preparation 3; see Tables 1, 3). Panel C plots the ratio of the surface excess parameters yielding \(\frac{[\Gamma_{sl} - \Gamma_{sv}]}{\Gamma_{lv}} \sim 1\).

Insets expand low-MW region and dashed lines represent arithmetic mean of the respective surface excess values listed in Table 3 (see Appendix 7.2 for sample calculations). Apparent \(\Gamma_{lv}\) (panel B) is reproduced \(^{10}\) for comparison to \([\Gamma_{sl} - \Gamma_{sv}]\). Apparent surface excess \([\Gamma_{sl} - \Gamma_{sv}]\) and \(\Gamma_{lv}\), as well as the ratio \(\frac{[\Gamma_{sl} - \Gamma_{sv}]}{\Gamma_{lv}}\), were found to be independent of protein MW (see Theory and Discussion sections).
Figure 6: Relationship between the surface-saturating bulk solution concentration $C_B^{\text{max}}$ and protein MW (natural logarithmic scale) at the solid-liquid (SL, panel A) and liquid-vapor interfaces (LV, panel B) for multiple protein preparations (open circle = preparation 1, filled circle = preparation 2, filled triangle = preparation 3). Error bars represent uncertainty computed by propagation of experimental errors into compiled $\ln C_B^{\text{max}}$ values (see Table 2 and Appendix 7.1 for representative calculations). Panel B is reproduced from 10 for the purpose of comparing the LV and SL interfaces. Linear regression through the SL data yielded $[\ln C_B^{\text{max}} = (-1.3 \pm 0.2) \ln MW + (19.8 \pm 1.0); R^2 = 78\%]$ compared to $[\ln C_B^{\text{max}} = (-1.4 \pm 0.2) \ln MW + (21.8 \pm 1.3); R^2 = 72\%]$ for the LV interface, consistent with the expectation of unit slope and a positive intercept (see Theory and Discussion sections). Note that low-MW proteins require greater bulk-phase concentrations to saturate the interphase than higher-MW proteins.
**Figure 7:** Time-dependent adhesion tension $\tau_a$ (Panel A) of pure albumin (circles), pure hIgM (squares), and a 50:50 mixture of albumin in hIgM (diamonds) at constant 5mg/mL total protein. Note that $\tau_a$ of the 50:50 mixture fell between the pure protein solutions. Observed spreading pressure $\Pi_{obs}$ (Panel B) followed a simple linear combining rule expressed in weight-fraction protein in the bulk phase $\Pi_{obs} = \Pi_{alb} - f_{IgM}(\Delta\Pi)$; where $\Delta\Pi = (\Pi_{alb} - \Pi_{IgM})$, $\Pi_{alb}$ or $\Pi_{IgM}$ refer to $\Pi_{obs}$ at 100% albumin ($f_{IgM} = 0$) or 100% IgM ($f_{IgM} = 1$), respectively. Error bars represent standard deviation of the mean of the final 25 $\Pi_{obs}$ values observed at 1hr equilibration time.
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<td>$C_B$</td>
<td>Bulk solution concentration (moles/volume)</td>
</tr>
<tr>
<td>$C_B^{\text{max}}$</td>
<td>Bulk solution concentration at limiting interfacial tension or contact angle (moles/volume)</td>
</tr>
<tr>
<td>$C_I$</td>
<td>Interphase concentration (moles/volume)</td>
</tr>
<tr>
<td>$C_I^{\text{max}}$</td>
<td>Maximal interphase concentration (moles/volume)</td>
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<td>$C_B^{\theta/2}$</td>
<td>Bulk solution concentration at half-maximal-change in contact angle (moles/volume)</td>
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<tr>
<td>$C_{sl}$</td>
<td>Independent measure of protein adsorption</td>
</tr>
<tr>
<td>$\chi$</td>
<td>Proportionality constant, $\chi \equiv R / r_v$</td>
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<tr>
<td>$\varepsilon$</td>
<td>Packing efficiency</td>
</tr>
<tr>
<td>$\Delta G_{\text{ads}}^o$</td>
<td>Free energy of protein adsorption</td>
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<td>$\gamma_{lv}$</td>
<td>Liquid-vapor (LV) interfacial tension (mN/m)</td>
</tr>
<tr>
<td>$\gamma_{sl}$</td>
<td>Solid-liquid (SL) interfacial tension (mN/m)</td>
</tr>
<tr>
<td>$\gamma_{sv}$</td>
<td>Solid-vapor (SV) interfacial tension (mN/m)</td>
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<td>$\gamma_{lv}^o$</td>
<td>Low-concentration asymptote of a concentration-dependent $\gamma_{lv}$ curve (mN/m)</td>
</tr>
<tr>
<td>$\gamma_{lv}^*$</td>
<td>High-concentration asymptote of a concentration-dependent $\gamma_{lv}$ curve (mN/m)</td>
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<tr>
<td>$\Gamma_{lv}$</td>
<td>Apparent Gibbs’ surface excess calculated at the liquid-vapor (LV) interface (moles/area)</td>
</tr>
<tr>
<td>$[\Gamma_{sl} - \Gamma_{sv}]$</td>
<td>Apparent Gibbs’ surface excess calculated at the solid-liquid (SL) interface (moles/area)</td>
</tr>
<tr>
<td>$M$</td>
<td>Parameter fitted to concentration-dependent $\gamma_{lv}$ or $\theta_a$ curve</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Activity-corrected chemical potential</td>
</tr>
<tr>
<td>$P$</td>
<td>Partition coefficient, $P \equiv \frac{C_I}{C_B}$</td>
</tr>
<tr>
<td>$\Pi_a$</td>
<td>Advancing spreading pressure (mN/m)</td>
</tr>
<tr>
<td>$\Pi_a^{\text{max}}$</td>
<td>Maximum advancing spreading pressure (mN/m)</td>
</tr>
<tr>
<td>$r_v$</td>
<td>Protein radius (cm)</td>
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<tr>
<td>$R$</td>
<td>Effective radius (cm), $R \equiv \chi r_v$</td>
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<tr>
<td>$RT$</td>
<td>Product of Universal gas constant and Kelvin temperature (ergs/mol)</td>
</tr>
<tr>
<td>$S$</td>
<td>Parameter computed from slope of $\theta_a$ isotherm $S = -\frac{1}{RT} \frac{\Delta \theta_a}{\Delta \ln C_B}$ (moles/area)</td>
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<tr>
<td>$\tau_a$</td>
<td>Advancing adhesion tension (mN/m)</td>
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<tr>
<td>$\tau_a^o$</td>
<td>Low-concentration asymptote of a concentration-dependent $\tau_a$ curve (mN/m); $\tau_a^o = \gamma_{lv}^o \cos \theta_a^o$</td>
</tr>
<tr>
<td>$\tau_a^*$</td>
<td>High-concentration asymptote of a concentration-dependent $\tau_a$ curve (mN/m); $\tau_a^* = \gamma_{lv}^* \cos \theta_a^*$</td>
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<tr>
<td>$\theta_a$</td>
<td>Advancing contact angle (degrees)</td>
</tr>
<tr>
<td>$\theta_a^o$</td>
<td>Low-concentration asymptote of a concentration-dependent $\theta_a$ curve (degrees)</td>
</tr>
<tr>
<td>$\theta_a^*$</td>
<td>High-concentration asymptote of a concentration-dependent $\theta_a$ curve (degrees)</td>
</tr>
<tr>
<td>$\theta_a^*$</td>
<td>Advancing contact angle at half-maximal change in $\theta_a$ isotherm $\theta_a^* = \left(\frac{\theta_a + \theta_a^o}{2}\right)$ (degrees)</td>
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<td>$\Omega$</td>
<td>Total interphase thickness (cm)</td>
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Chapter 7

An Evaluation of Methods for Contact Angle Measurement

Abstract

A systematic comparison of Wilhelmy-balance tensiometry (WBT), tilting-plate goniometry (TPG) and captive-drop goniometry (CDG), using a variety of silane-treated glass surfaces of variable wetting characteristics is reported. WBT was assumed to be the benchmark of comparison (gold standard). Advancing angles measured by TPG and CDG were in statistical agreement with corresponding WBT measurements. In contrast, receding angles from both goniometric techniques were systematically higher than WBT.
7.1. Introduction

Investigators seeking to apply standard tools of contact angle and wettability (tensiometry) in studies of surface phenomena are frequently confronted with a hard choice among a variety of techniques and instrumentation vendors. Among the many tensiometric methods that have been developed over the years, contact angle goniometry and Wilhelmy balance tensiometry have become the standard and most popular methods (see refs. 1-3 and citations therein). In goniometry, a back-lit drop (silhouette) is optically imaged and the angle subtended by the drop at the point of solid-liquid contact (on left and right sides) estimated with an optical goniometer (manual) or by image analysis (computerized). Wilhelmy balance tensiometry measures wetting forces along the perimeter of a regularly-shaped object (typically a plate or cylinder) as it is immersed into or emersed from the wetting liquid (see ref. 2 for a brief tutorial). Modern computerized instrumentation has greatly reduced labor and subjectivity of goniometric and balance techniques, but there are a number of analytical subtleties that can be easily overlooked in instrument setup and operation. For example, in goniometry, it is essential to establish an accurate baseline between the drop and surface and choose the correct point of contact. These two aspects are intertwined and both require that the observer must be looking down on the drop at a slight angle above (not below) the horizon. Otherwise, the base of the drop can be cropped by the horizon and finding the maximum profile width becomes highly ambiguous. In Wilhelmy balance tensiometry (WBT), buoyancy correction requires accurate knowledge of the wetted perimeter and point of liquid-plate contact; both of which insist that the test plate or cylinder enter and exit the fluid perpendicularly.
Measurement of advancing and receding contact angles, $\theta_a$ and $\theta_r$ respectively, is an essential aspect of tensiometry because these are the two reproducible angles that characterize wetting.

No doubt WBT is the most accurate and least subjective approach to measuring hysteresis ($\Delta = \theta_a - \theta_r$) because the three-phase (solid-liquid-vapor) line is in wholesale motion, assuring achievement of maximal $\theta_a$ and minimum $\theta_r$. As a consequence, WBT is a reasonable choice as a benchmark of comparison for goniometric methods. Two goniometric methods widely applied in commercial instruments to measure $\theta_a$ and $\theta_r$ are the captive-drop (CDG) and tilting plate (TPG) goniometric techniques. In CDG, a drop is held in place on a surface under study with a fine needle connected to a syringe. Advancing angles $\theta_a$ are read by slightly filling the drop, advancing liquid over the surface. Receding angles $\theta_r$ are read by removing liquid from the drop, receding liquid from the surface. Maximum $\theta_a$ and minimum $\theta_r$ is assured by incrementally increasing volume added or removed, respectively, until no change in angles is observed. Care must be taken with CDG to use a needle diameter that is a very small portion of the drop diameter. We have found that if the needle diameter exceeds a few tens of microns, drop shape can be significantly affected and introduce serious errors into the estimation of $\theta_a$ and $\theta_r$. TPG measures left and right sides of a drop as the surface under study is tilted with respect to the optical axis so fluid accumulates in the leading (advancing) edge of the drop and drains from the trailing (receding) edge. Maximum $\theta_a$ and minimum $\theta_r$ are attained when the drop is at a point of ‘incipient motion’; that is, just at the point when the drop rolls out of the observation window. Needless to say, finding the point of incipient motion is experimentally inconvenient. A typical remedy for a particular surface under investigation is to plot observed contact angle against tilt angle to find a tilt smaller than required to induce drop rolling yet large
enough that an incremental increase or decrease in tilt does not observably change left-and-right angles. Thus, finding maximal $\theta_a$ and minimum $\theta_r$ is yet another subtlety of the goniometric methods that warrants verification against WBT for stringent work. Lander et. al.\textsuperscript{3} systematically compared hysteresis measured by CDG and TPG to WBT using a hexadecylsilane-coated glass and silicon wafers as a model surface. Using a multiplicity of similarly-prepared surfaces and hundreds of contact angle measurements, Lander found that WBT and TPG gave statistically-identical results whereas CDG consistently underestimated $\theta_a$ and over estimated $\theta_r$.

Primary objective of this paper is to expand on the work of Lander et. al. by comparing goniometric technique (CDG and TPG) hysteresis measurements to Wilhelmy-balance tensiometry (WBT) using a variety of surfaces spanning a broad range of water wettability. We find good agreement among CDG, TPG, and WBT in measured advancing angles $\theta_a$, corroborating Lander’s results. However, in contrast to Lander’s findings, receding contact angles $\theta_r$ measured by both CDG and TPG were found to be systematically offset from that of WBT and were highly variable relative to $\theta_a$ measurements. Thus, this work recommends neither CDG nor TPG for accurate measurement of receding contact angles or contact angle hysteresis.

7.2. Materials and Methods

**Surfaces:** Glass cover slips (Fisher Brand 22 x 30 x 0.1 mm) were used as substrata for the comparative analysis of contact angle measurements between tilting-plate goniometry (TPG), captive-drop goniometry (CDG) and Wilhelmy-balance tensiometry (WBT). As-received slides
were cleaned by 3X sequential rinses in each of water, isopropanol and chloroform, and plasma-discharge treated for ~5min in a Harrick Plasma cleaner (Ossining, NY) at 100 mTorr air. Distilled-deionized (18.2 MΩ·cm) water was used as test-solution for contact angle measurements. Surfaces of varying water wettability (50°< \( \theta_a <120° \)) were prepared by silanization of clean glass cover slips. Three kinds of hydrophobic surfaces were prepared using (i) octadecyltrichlorosilane (OTS; \( \theta_a \sim110° \)), (ii) aminopropyltriethoxysilane (APTES; \( \theta_a \sim70° \)) and (iii) 0.2% solution of 1, 1, pentadecafluoroctylmethacrylate in trichlorotrifluoroethane (NYEBAR; \( \theta_a \sim120° \); commercial fluorocarbon polymer coating fluid; Nye Lubricants, Fairhaven MA). Clean glass coverslips were silanated by a 2 hr reaction with 5% OTS in chloroform or 5 min reaction with 2% APTES in acetone. Silanated glass slides were 3X rinsed in chloroform (OTS) or acetone (APTES) before being cured in a vacuum oven at 110°C for 24 hr to ensure stable surface chemistry. NYEBAR surfaces were prepared by immersing OTS surfaces in NYEBAR solution for about 10 min with subsequent air-drying. Surfaces with incrementally-increasing wettability (90°>\( \theta_a >45° \)) were prepared by chemical oxidation of OTS surfaces. Cured OTS surfaces were immersed at 5-minute intervals in 50% solution of H\(_2\)SO\(_4\)/Cr\(_2\)O\(_3\) in water, followed by 3X sequential wash in ethanol, and air drying.

**Tensiometry:** Wilhelmy-balance tensiometry (WBT) was performed using a commercial computer-controlled instrument (Camtel CDCA 100, Royston UK) using solvent-and-plasma-discharge-cleaned glass coverslips as the plate. The balance was calibrated with standard weights thereby accounting for local variation in the force of gravity. No attempt was made to thermostat the balance and all reported measurements were made at ambient laboratory temperature. Also, no attempt was made to correct for the (presumably small but not measured)
variation in the perimeter of the glass coverslips. Solutions (approximately 10 mL) were contained in disposable polystyrene beakers (Fisher) previously determined not to measurably affect interfacial tension of water contained therein. Advancing and receding contact angles were calculated from the last of three immersion and emersion force measurements respectively, using a force-balance equation corrected for buoyancy (by extrapolation to zero volume);

\[ f = P \gamma \cos \theta \]

where \( f \) is the force in mN, \( \gamma \) is the surface tension of water at 71.9 mN/m at 25 °C, \( P \) is the perimeter (wetted length) of the glass coverslip (44.2mm, for a thickness of 0.1mm) and \( \theta \) is either advancing (\( \theta_a \)) or receding (\( \theta_r \)) contact angle.

**Goniometry:** Tilting-plate goniometry (TPG) was performed using a commercial-automated goniometer (First Ten Angstroms Inc., Portsmouth, VA). The tilting-plate goniometer (TPG) employed a Tecan liquid-handling robot to aspirate 12 µL of water contained in a 96-well microtiter plate. The robot was used to reproducibly transfer the tip with fluid contents into a humidified (99+ % RH) analysis chamber and dispense 10 µL drops onto the surface of test substrata (see below) held within the focal plane of a magnifying camera. These and all other aspects of TPG were performed under computer control. Proprietary algorithms supplied by the vendor were used to deduce contact angles from drop images captured at a programmed rate by a frame grabber. Typically, 300 images were captured at a rate of 1 image every 6 sec following 0.25 sec delay to permit vibrations of the expelled drop to dampen. Drop evaporation rates within the humidified chamber deduced from computed-drop volumes (based on image analysis) were approximately 25 nL/min for pure water. Precision of \( \theta_a \) was about 0.5° based on repeated measurement of the same drop. The analysis chamber was thermostated to a lower-limit of 25±1 °C by means of a computer-controlled resistive heater. Upper-temperature limit was not
controlled but rather floated with laboratory temperature, which occasionally drifted as high as
29 °C during summer months. Thus, reported $\theta_a$ values were probably not more accurate than
about 1° on an inter-sample basis considering the small, but measurable, variation of water
interfacial tension with temperature. Test substrata were held on a rotating, tilting-plate platform
driven by stepper motors under computer control. Substrata were allowed to come to
equilibrium within the sample-chamber environment for no less than 30 min before contact angle
measurements were initiated. The platform was programmed to tilt at 1°/sec from horizontal to
25° after the drop was deposited on the surface by the robot. The first 120 sec (20 images)
monitored evolution of the advancing angle.

Captive-drop goniometry (CDG) was implemented using a home-built goniometer, as described
elsewhere.4 Briefly, CDG involved capturing the droplet on the test surface with a fine needle
connected to a 50μL syringe. $\theta_a$ or $\theta_r$ was read by adding or withdrawing water from the drop,
respectively. Contact angles were measured from images captured by a CCD camera when
observable motion had ceased.

7.3. Results and Discussion

Table 1 collects advancing ($\theta_a$) and receding ($\theta_r$) contact angles of water on silane-treated and
surface-modified, silane-treated glass coverslips with varying water-wettability created for the
purpose of comparing goniometric techniques (see Materials and Methods). Fig. 1 compares
goniometry (TPG and CDG) to WBT where data falling along the diagonal corresponds to
perfect agreement among techniques. Inspection of the data trends reveal that $\theta_a$ measured by
goniometric methods were in close agreement with WBT (and therefore regarded accurate) but \( \theta_r \) was more noisy and systematically offset from WBT (and therefore regarded not accurate).

We attribute failure to achieve a stable lower-bound \( \theta_r \) to a three-phase-line-pinning phenomenon\(^5\)\(^-\)\(^7\) that requires wholesale drop motion to overcome energetic barriers to formation of a uniform drop perimeter.

### 7.4. Conclusions

No single contact angle adequately characterizes wettability of a surface. Instead, a maximal advancing angle \( \theta_a \) and minimum receding angle \( \theta_r \) are required, with a range of metastable contact angles observable between these two bounds. An important question that arises in detailed analysis of contact angles and in choice among contact angle methods asks which technique offers the most accurate and precise measures of contact angle hysteresis.

Comparison of \( \theta_a \) measured by tilting-plate goniometry (TPG) and the popular captive drop goniometry (CDG) to Wilhelmy balance tensiometry (WBT) confirms statistical agreement among methods for \( \theta_a \). However, \( \theta_r \) measurements by TPG and CDG were systematically offset from the benchmark WBT and exhibited greater variability.
Citations


<table>
<thead>
<tr>
<th>Surface</th>
<th>Method</th>
<th>( \theta_a ) WBT</th>
<th>( \theta_r ) WBT</th>
<th>( \theta_a ) TPG (%) diff from ( \theta_a ) WBT</th>
<th>( \theta_r ) TPG (%) diff from ( \theta_r ) WBT</th>
<th>( \theta_a ) CDG (%) diff from ( \theta_a ) WBT</th>
<th>( \theta_r ) CDG (%) diff from ( \theta_r ) WBT</th>
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</thead>
<tbody>
<tr>
<td>1a</td>
<td>Nyebar* on OTS</td>
<td>120.1</td>
<td>84.1</td>
<td>120.2 (-0.1)</td>
<td>116.5 (38.5)</td>
<td>112.6 (-6.3)</td>
<td>81.4 (-3.1)</td>
</tr>
<tr>
<td>2a</td>
<td>OTS</td>
<td>100.0</td>
<td>71.9</td>
<td>99.1 (-0.9)</td>
<td>96.0 (33.5)</td>
<td>96.1 (-3.9)</td>
<td>91.0 (26.6)</td>
</tr>
<tr>
<td>2b</td>
<td>OTS</td>
<td>109.1</td>
<td>75.7</td>
<td>102.9 (-5.6)</td>
<td>95.1 (25.6)</td>
<td>105.4 (-3.5)</td>
<td>87.7 (15.9)</td>
</tr>
<tr>
<td>2c</td>
<td>OTS</td>
<td>101.8</td>
<td>78.8</td>
<td>102.6 (0.8)</td>
<td>91.5 (16.1)</td>
<td>100.4 (-1.3)</td>
<td>91.5 (16.2)</td>
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<tr>
<td>2d</td>
<td>OTS</td>
<td>101.2</td>
<td>79.8</td>
<td>99.6 (-1.5)</td>
<td>91.1 (14.1)</td>
<td>98.5 (-2.6)</td>
<td>92.2 (15.4)</td>
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<tr>
<td>3a</td>
<td>APTES</td>
<td>76.4</td>
<td>44.3</td>
<td>73.4 (-3.9)</td>
<td>60.9 (37.5)</td>
<td>85.7 (12.2)</td>
<td>63.2 (42.6)</td>
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<tr>
<td>3b</td>
<td>APTES</td>
<td>74.9</td>
<td>34.9</td>
<td>72.7 (-2.9)</td>
<td>63.3 (81.1)</td>
<td>69.7 (-7.1)</td>
<td>44.4 (26.9)</td>
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<tr>
<td>3c</td>
<td>APTES</td>
<td>76.3</td>
<td>44.2</td>
<td>71.6 (-6.1)</td>
<td>70.1 (58.6)</td>
<td>72.1 (-5.6)</td>
<td>61.8 (39.7)</td>
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<tr>
<td>4a</td>
<td>Oxidized OTS† 5min</td>
<td>77.3</td>
<td>53.5</td>
<td>70.3 (-9.0)</td>
<td>61.4 (14.9)</td>
<td>75.2 (-2.75)</td>
<td>54.1 (1.2)</td>
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<td>4b</td>
<td>Oxidized OTS† 5min</td>
<td>93.1</td>
<td>56.1</td>
<td>92.6 (-0.5)</td>
<td>82.1 (46.3)</td>
<td>87.4 (-6.2)</td>
<td>80.1 (42.8)</td>
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<td>Oxidized OTS† 10min</td>
<td>58.5</td>
<td>30.0</td>
<td>56.5 (-3.4)</td>
<td>33.0 (10.1)</td>
<td>58.3 (-0.4)</td>
<td>42.6 (42.2)</td>
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<tr>
<td>5b</td>
<td>Oxidized OTS† 10min</td>
<td>82.1</td>
<td>58.5</td>
<td>76.4 (-6.9)</td>
<td>65.4 (11.8)</td>
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<td>54.6 (-6.6)</td>
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<td>6a</td>
<td>Oxidized OTS† 15min</td>
<td>51.6</td>
<td>28.2</td>
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<td>21.2 (-24.6)</td>
<td>48.6 (-5.7)</td>
<td>22.4 (-20.7)</td>
</tr>
<tr>
<td>6b</td>
<td>Oxidized OTS† 15min</td>
<td>76.1</td>
<td>59.4</td>
<td>72.0 (-5.4)</td>
<td>68.4 (15.0)</td>
<td>66.5 (-12.7)</td>
<td>53.4 (-10.1)</td>
</tr>
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<td>7a</td>
<td>Oxidized OTS† 20min</td>
<td>50.2</td>
<td>25.2</td>
<td>51.0 (1.6)</td>
<td>34.0 (36.0)</td>
<td>49.7 (-0.8)</td>
<td>32.2 (27.8)</td>
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<tr>
<td>7b</td>
<td>Oxidized OTS† 20min</td>
<td>70.3</td>
<td>44.0</td>
<td>71.1 (1.1)</td>
<td>66.0 (49.9)</td>
<td>79.5 (12.9)</td>
<td>69.0 (56.8)</td>
</tr>
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<td>8a</td>
<td>Oxidized OTS† 25min</td>
<td>48.4</td>
<td>23.1</td>
<td>48.0 (-0.8)</td>
<td>31.3 (35.5)</td>
<td>45.6 (-5.8)</td>
<td>35.7 (54.7)</td>
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<tr>
<td>8b</td>
<td>Oxidized OTS† 25min</td>
<td>71.8</td>
<td>39.8</td>
<td>62.9 (-12.4)</td>
<td>59.9 (50.4)</td>
<td>64.9 (-9.5)</td>
<td>46.1 (15.6)</td>
</tr>
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**Notes:**  
* 1, 1, pentadecafluoroctylmethacrylate deposited on OTS-treated glass.  
† Octadecyltrichlorosilane (OTS) treated glass-slide dipped in CrO4/H2SO4 solution for specified times.
**Figure 1:** Comparison of advancing ($\theta_a$, panel A) and receding ($\theta_r$, panel B) contact angles from goniometric techniques – tilting-plate (TPG, closed circles) and captive-drop (CDG, open circles), to Wilhelmy-balance tensiometry (WBT). Diagonal lines correspond to ideal 1:1 correlation between the techniques. $\theta_a$ measured by goniometric methods (TPG and CDG) were in statistical agreement with WBT, whereas $\theta_r$ measurements were systematically offset from WBT. Linear regression through advancing angles of TPG and CDG vs. WBT (panel A) yielded

$$[\theta_a^{TPG} = (0.99 \pm 0.03)\theta_a^{WBT} + (-2.3 \pm 2.9); R^2 = 98\%]$$ and

$$[\theta_a^{CDG} = (0.93 \pm 0.06)\theta_a^{WBT} + (2.2 \pm 5.0); R^2 = 94\%]$$ respectively. Corresponding results for receding angles (panel B) of TPG and CDG vs. WBT yielded $[\theta_r^{TPG} = (1.2 \pm 0.1)\theta_r^{WBT} + (5.3 \pm 6.5); R^2 = 83\%]$ and $[\theta_r^{CDG} = (0.9 \pm 0.1)\theta_r^{WBT} + (10.1 \pm 6.7); R^2 = 78\%]$ respectively.
Chapter 8

Interfacial Energetics of Blood Plasma and Serum at a Hydrophobic Solid/Aqueous-Buffer Interface

Abstract

Adsorption isotherms of blood plasma and serum derived from four different mammalian species (human, bovine, ovine and equine) confirm that interfacial energetics of protein adsorption to a hydrophobic solid-water (solid-liquid, SL) interface are not fundamentally different than adsorption to the water-air (liquid-vapor, LV) interface. Comparison of results to a previously-published survey of purified human-blood proteins at the two surfaces reveals that interfacial energetics of plasma and serum is strikingly similar to that of purified protein constituents at both LV and SL surfaces. Adsorption isotherms constructed from concentration-dependent advancing contact angles $\theta_a$ and adhesion tensions $\tau_a$ of buffered-plasma and -serum solutions on methyl-terminated, self-assembled monolayer surfaces show that maximum advancing spreading pressure, $\Pi^\text{max}_a$, falls within a relatively narrow $15 < \Pi^\text{max}_a < 25$ mN/m band, mirroring results obtained at the LV surface. It is thus concluded that any combination of blood-protein constituents will be substantially similar because concentration-dependence $\frac{d\tau_a}{d\ln C^*_a}$ of individual proteins are very similar.

Experimental results are further interpreted in terms of a recently-developed theory emphasizing the controlling role of water in protein adsorption. Accordingly, a hydrophobic interphase saturates with protein adsorbed from bulk solution at a fixed weight-volume concentration (~436 mg/mL) independent of protein identity or mixture. As a direct consequence, $\frac{d\tau_a}{d\ln C^*_a}$ of purified proteins closely resembles that of mixed solutions and does not depend on the relative proportions of individual proteins comprising a mixture. Thus variations in the plasma proteome between species are not reflected in $\frac{d\tau_a}{d\ln C^*_a}$ nor is serum different from plasma in this regard, despite being depleted of coagulation proteins (e.g., fibrinogen).
8.1. Introduction

Significant challenge of work outlined in this thesis has been to understand and interpret protein adsorption from multi-component solutions onto solid surfaces of more practical relevance to biomaterials. A common theme in studies of competitive nature of protein adsorption has been the need to elucidate specific interactions between diverse surfaces and different plasma proteins. Early observations include the “Vroman effect” (see refs. 1-21 and citations therein) wherein Leo Vroman observed that adsorption from plasma or serum occurred through a complex series of adsorption-displacement steps in which low-molecular-weight (MW) proteins arriving first at a surface are displaced by relatively higher MW proteins arriving later. Bagnall and coworkers\textsuperscript{22-24} preceded Vroman with both \textit{in vitro} and \textit{in vivo} measurements of interfacial tensions (solid-liquid, liquid-vapor) of plasma proteins (purified albumin, IgG and binary mixtures thereof) reporting constant free energies of adsorption at model hydrophobic surfaces including implant polymers. Even clinical interest in the water-air (liquid-vapor, LV) interfacial tension $\gamma_{lv}$ of blood plasma and serum can be traced back at least eight decades to comprehensive studies from Morgan and Woodward\textsuperscript{25} on human serum and later, by Harkins\textsuperscript{26,27} and DuNouy\textsuperscript{28,29} on appropriate methods for equilibrium $\gamma_{lv}$ measurements of biological fluids. A detailed review of early literature has already been outlined in our recent work\textsuperscript{30} on water-air (liquid-vapor, LV) interfacial tensions of blood plasma and serum from four different species. Biomaterials research has since explored protein adsorption from plasma/serum solutions at varying levels of molecular complexity using a multitude of techniques including ellipsometry\textsuperscript{31}, internal reflection spectroscopy\textsuperscript{21} and surface plasmon resonance\textsuperscript{32}. 
What is apparently lacking in this expansive literature base is a unifying theory applicable to both mass and energy inventories of protein adsorption from purified protein solutions as well as whole-blood plasma and serum. Our recent investigations on adsorption energetics of nine purified proteins (spanning three-orders-of-magnitude) revealed remarkable scaling relationships at two hydrophobic surfaces (liquid-vapor, LV and solid-liquid, SL). Significant experimental outcomes from both single protein solutions\textsuperscript{33,34} as well as complex protein mixtures\textsuperscript{30,35} were rationalized using a tractable quasi-thermodynamic theory of protein adsorption based on simple close-packing of protein molecules within a three-dimensional interphase.

This paper supplements previous work with adsorption energetics of whole-blood plasma and serum from human and animal species to a well-defined, hydrophobic solid-water (solid-liquid, SL) interface. Time-and-concentration-dependent contact angles are used to compare interfacial energetics of plasma and serum with purified proteins, in a manner that parallels previous studies\textsuperscript{30,33-37} of protein adsorption to the LV surface. Similarity in adsorption energetics revealed in this and previous work, not only among purified proteins\textsuperscript{33,34,36,37} but also for multi-protein mixtures\textsuperscript{30,35} such as plasma and serum at two hydrophobic surfaces, is remarkable in view of the substantial differences in plasma proteome across species.\textsuperscript{38} Furthermore, work outlined in this paper accommodates principal outcomes from our theory of competitive protein adsorption\textsuperscript{33,35} previously confirmed at the LV surface.
8.2. Materials and Methods

Plasma/Serum: Human platelet-poor plasma (citrated) was prepared from outdated (within 2 days of expiration) lots obtained from the Hershey Medical Center Blood Bank. Human serum was prepared in 15 mL batches by recalcification with 0.1 M CaCl₂ at 5:1 v/v plasma:calcium ratio in clean glass scintillation vials for about 15 min. Bovine, ovine and equine plasma and serum were used as received from Hemaresource and Supply Inc. (Aurora, OR) and were not subject to any additional fractionation/purification steps. Ref. 34 discloses details of protein purity and protein-solution preparation including serial dilutions of protein stock solutions (usually 10 mg/mL) that were performed in 96-well microtiter plates by (typically) 50:50 dilution in phosphate buffered saline solution (PBS) prepared from powder (Sigma Aldrich) in distilled-deionized (18 MΩ) water (interfacial tension of PBS and water was checked periodically by Wilhelmy-balance tensiometry).

Surfaces: Methyl-terminated self assembled monolayer surfaces (SAMs) were prepared according to standard methods of surface engineering. Details involved have been reported elsewhere. 39-43 Alkanethiol (Aldrich Chemical Co., Milwaukee, WI) and ethanol (commercial reagent-grade) were used as-received, without further purification. Samples were stored in the thiol solution until use, and were rinsed with ethanol just prior to an experiment.

Tensiometry & Goniometry: Liquid-vapor interfacial tensions required by this work were measured by Pendant Drop Tensiometry (PDT) as described in refs. 34,35,44,45. Contact angle and wettability methods applied in this work have been disclosed in detail elsewhere, including verification that measured advancing angles (θₐ) were in statistical agreement with that obtained
by Wilhelmy balance tensiometry. Receding angles ($\theta_r$) were shown to be not as reliable as $\theta_a$. Consequently, only $\theta_a$ was analyzed in this work. Briefly, for the purposes of this paper, $\theta_a$ measurements were made using a commercial automated tilting-plate goniometer (TPG, First Ten Angstroms Inc., Portsmouth VA). The goniometer employed a Tecan liquid-handling robot to aspirate 12 µL of solutions contained in a 96-well microtiter plate prepared by the serial-dilution protocol mentioned above. The robot was used to reproducibly transfer the tip with fluid contents into a humidified (99+ % RH) analysis chamber and dispense 10 µL drops of protein solution onto the surface of test substrata held within the focal plane of a magnifying camera. These and all other aspects of tilting-plate goniometry were performed under computer control. Proprietary algorithms supplied by the vendor were used to deduce contact angles from drop images captured at a programmed rate by a frame grabber. Typically, 600 images were captured at a rate of 1 image every 6 sec following 20 sec delay to permit vibrations of the expelled drop to dampen. Precision of $\theta_a$ was about 0.5° based on repeated measurement of the same drop.

The analysis chamber was thermostated to a lower-limit of 25±1 °C by means of a computer-controlled resistive heater. Thus, reported $\theta_a$ values were probably not more accurate than about 1° on an inter-sample basis considering the small, but measurable, variation of water interfacial tension with temperature. This range of accuracy was deemed adequate to the conclusions of this report which do not strongly depend on more highly accurate $\theta_a$ that is difficult to achieve on a routine basis. Instead, veracity of arguments raised herein depend more on a breadth of reliable measurements made across the general family of human proteins.
Test substrata were held on a rotating, tilting-plate platform driven by stepper motors under computer control. Substrata were allowed to come to equilibrium within the sample-chamber environment for no less than 30 min before contact angle measurements were initiated. The platform was programmed to tilt at 1°/sec from horizontal to 25° after the drop was deposited on the surface by the robot. The first 120 sec (20 images) monitored evolution of the advancing angle. At the end of the 1 hr $\theta_a$ measurement period, the platform was programmed to return to horizontal and rotate 15° to the next analysis position along the periphery of the semiconductor wafer. This process was repeated for all dilutions of the protein solution under study so that results reported for each plasma/serum were obtained on a single test surface, eliminating the possibility of substratum-to-substratum variation within reported results. We observed that the contact angle of a pure PBS droplet slowly decreased with time from the initial value of $108^\circ < \theta_a^o < 106^\circ$ at $t = 0$ to $104^\circ < \theta_a^o < 102^\circ$ at $t = 1$ hr; where $\theta_a^o$ is the pure buffer contact angle and the range of reported results corresponds to all of the 8 methyl-terminated SAM surfaces analyzed during the course of this work. We attribute this systematically-varying wettability to slow “hydration” of SAM surfaces, which may include dissolution of thiol from the surface into solution, but have no specific proof this was the cause. We do not believe this slight but apparently unavoidable attribute of thiol-SAMs on silicon wafers negatively affects the veracity of conclusions drawn herein which were based on final, steady-state measurements made at $\sim 1$ hr analysis time.

**Computation and Data Representation:** Computational, statistical, and theoretical methods used in this work have been discussed in detail elsewhere.$^{47-49}$ In brief, time-dependent $\theta_a$ data corresponding to protein dilutions (see above) were recovered from TPG files and correlated
with concentrations, leading to a matrix of results with row values representing concentration and time (in sec) as column values. It was generally observed that $\theta_a$ isotherms were sigmoidal in shape when plotted on logarithmic-concentration axes,\(^47,49\) with well-defined low-concentration asymptotes $\theta_a^o$ and high-concentration asymptotes $\theta_a'$ (see Fig. 1 for examples). Successive non-linear least-squares fitting of a four-parameter logistic equation

$$\theta_a = \frac{\theta_a^o - \theta_a'}{1 + (\ln C_B^{\Theta/2} / \ln C_B)^M} + \theta_a'$$


to contact angle isotherms data for each time within the observation interval quantified $\theta_a^o$ and $\theta_a'$ parameters with a measure of statistical uncertainty. Fitting also recovered a parameter measuring concentration-at-half-maximal-change in $\theta_a$, $\ln C_B^{\Theta/2}$ (where $\Theta / 2 = 1/2 \Theta_{\text{max}}$ and $\Theta_{\text{max}} \equiv \theta_a^o - \theta_a'$), as well as a parameter $M$ that measured steepness of the sigmoidal curve. This multi-parameter fitting to concentration-dependent $\theta_a$ data was a purely pragmatic strategy that permitted quantification of best-fit protein and surfactant characteristics but is not a theory-based analysis.\(^47-49\) Three-dimensional (3D) representations of time-and-concentration $\theta_a$ data were created in Sigma Plot (v8) from the data matrix discussed above and overlain onto fitted-mesh data computed from least-squares fitting. Two-dimensional (2D) representations were created from the same data matrices at selected observation times. Measured $\theta_a$ were converted to advancing adhesion tension $\tau_a = \gamma_{lv} \cos \theta_a$ for general interpretation;\(^49\) where $\gamma_{lv}$ is the interfacial tension of the contact-angle fluid. Adhesion tensions $\tau_a^o = \gamma_{lv} \cos \theta_a^o$ (pure saline) and $\tau_a' = \gamma_{lv} \cos \theta_a'$ (at the minimum contact angle observed $\theta_a'$) were computed with fitted parameters $\gamma_{lv}^o$ and $\gamma_{lv}'$ reported in ref.\(^34\) for the proteins under investigation. Smoothed adhesion-tension isotherms ($\tau_a$ vs. $\ln C_B$) were computed from
smoothed $\theta_a$ obtained from $\theta_a$ isotherms above, using smoothed $\gamma_h$ values computed from best-fit parameters reported in ref. 34. Likewise, smoothed spreading pressure isotherms ($\Pi_a$ vs. $\ln C_B$) were computed from smoothed $\tau_a$ curves where $\Pi_a \equiv (\tau_a - \tau_a^*)$.

8.3. Results

Contact-Angle Isotherms: The principal experimental observations of this work were time- and concentration-dependent solid-water (solid-liquid, SL) contact angles $\theta_a$ of human and animal (bovine, ovine, equine) blood plasma and serum. $\theta_a$ of serially-diluted, whole plasma or serum was measured using tilting-plate goniometry (TPG) as detailed in the Materials and Methods section, resulting in graphical constructions herein referred to as ‘$\theta_a$ curves’. Table 1 compiles quantitative results of this work. Contact angle parameters $\theta_a^\circ$, $\theta_a^\circ$, $\ln C_B^{\theta_a/2}$ and $M$ listed in cols 2-5 of Table 1 are the mean fitted values corresponding to final 25 $\theta_a$ curves recorded within the 60-minute time frame of the TPG experiment. Listed error is standard deviation of this mean. Corresponding adhesion tensions $\tau_a^*$ and $\tau_a^*$ (Columns 6, 7) were computed from $\theta_a^\circ$ and $\theta_a^\circ$ values, respectively, with uncertainty estimates computed by propagation of error in $\theta_a$ and $\gamma_h$ measurements (Methods and Materials). Maximum “spreading pressure” $\Pi_a^{\text{max}} \equiv (\tau_a^* - \tau_a^*)$ (Column 8) was computed directly from aforementioned $\tau_a$ values and associated uncertainty again estimated by propagation of error.

Figure 1 compiles representative TPG results for human (HS, panel A), bovine (BS, panel B), and equine blood serum (ES, panel C). Concentration-dependent $\theta_a$ was observed to follow a
sigmoidal-like function on logarithmic concentration axis; yielding parameters $\theta_0$, $\theta$, and $M$ when statistically-fit to a four-parameter logistic equation as described in Materials and Methods. In each case represented in Fig. 1, $\theta$ was observed to change with time to a final, steady state $\theta_0$ value. Dynamics were undoubtedly due to protein mass-transfer and adsorption-kinetic effects. We also note that the phenomenon of “SAM hydration” discussed previously, affects measurement of protein-solution contact angles because we observed that the whole contact angle isotherm ($\theta$ vs. concentration) slowly shifted lower with time (see Fig. 1B). Observation of time-dependence was important in this particular work only in so far as data demonstrate that $\theta$ dynamics dampen within the time frame of experimentation, achieving or approaching steady-state (equilibrium) within the 1 hr observation window. In fact, data collected in Table 1 refers only to steady-state measurements.

**Adhesion-Tension and Spreading-Pressure Isotherms:** Figure 2 traces sequential interpretation of steady-state (1 hr drop age), concentration-dependent $\theta$ data (Panel A) in terms of concentration-dependent $\tau$ (Panel B) and spreading pressure $\Pi$ (Panel C) for human serum (HS). Smooth curves through the data serve as guides to the eye. Steady-state (equilibrium) spreading pressure isotherms $\Pi$ were used as the basis of comparison of protein adsorption for the compounds listed in Table 1. Fig. 3 graphically compares steady-state concentration-dependent $\Pi$ data (SL interface, panel A) to $\gamma_{lv}$ (LV interface, panel B) between a purified protein, human immunoglobulin-G (IgG, MW=160kDa) to human plasma and serum on a single set of axis where the surrounding band(s) represents 99% confidence intervals around the best-fit human-plasma data listed in Table 1. It is clearly evident that concentration-dependent $\Pi$ or
\( \gamma_{lv} \) of IgG, plasma and serum cannot be confidently distinguished based on this data. Fig. 4 is similar in construct to Fig. 3 in that it graphically compares representative TPG steady-state \( \Pi_a \) curves (SL interface, panel A) to \( \gamma_{lv} \) (LV interface, panel B) for human, bovine, ovine and equine plasma on a single set of axis. Again, the surrounding band encloses data within 99% confidence intervals around the best-fit human-plasma data listed in Table 1. Figs. 1-4 collectively show that there is a striking similarity in interfacial energetics at two hydrophobic surfaces (\( \Pi_a \) or \( \gamma_{lv} \)) between whole-blood plasma, serum and purified-protein constituents. Serum, of course, is compositionally distinct from plasma by virtue of being depleted of fibrinogen in the process of coagulating plasma.

8.4. Discussion

Concentration-Dependent \( \Pi_a \) of Plasma, Serum, and Protein Constituents Thereof:

Similarity in concentration-dependent \( \Pi_a \) of blood plasma and serum obtained from normal donors of diverse species is very intriguing in light of the substantial differences in protein composition. Observations collected in Figs. 1-4 corroborating general trends of experimental work dating back to the early 1900’s\textsuperscript{25,27,29,50,51} are all the more compelling in light of our recent findings that \( \frac{d\tau_a}{d\ln C_u} \) of a broad range of purified blood-protein constituents are likewise similar\textsuperscript{34}. Furthermore, we find that the saturating spreading pressures \( \Pi_{a,\text{max}} \) falls within a relatively narrow 5 mN/m band characteristic of all proteins and mixtures studied. Experiment thus suggests that \( \frac{d\tau_a}{d\ln C_u} \) of any combination of blood-proteins is very similar because \( \frac{d\tau_a}{d\ln C_u} \) of individual protein constituents are very similar\textsuperscript{34,44}. All taken together, we find this evidence
suggestive of a heretofore unresolved mechanistic commonality underlying protein adsorption from complex mixtures to a hydrophobic surface.

Our recently-developed theory of protein adsorption to two hydrophobic surfaces (liquid-vapor, LV and solid-liquid, SL surfaces)\(^4\) asserts that the mechanistic commonality mentioned above is the behavior of water at interfaces\(^5\). Theory suggests that interfacial capacity for protein is controlled by the extent to which interfacial water can be displaced by adsorbing protein molecules. This maximal interfacial capacity can be expressed either as a maximal volume fraction or, equivalently, a maximum weight/volume (not molar) concentration denoted \(W_i^{\text{max}}\) at which \(\tau_a\) occurs. Calibration of theory to experimental neutron-reflectivity or light scattering data has revealed that \(W_i^{\text{max}} \sim 436\) mg/mL, corroborating the conclusion drawn from diverse literature sources that adsorbed protein concentrations can be surprisingly large\(^5\). Importantly, \(W_i^{\text{max}}\) is found not to be dependent on protein identity or MW but rather is a generic, water-limited adsorption capacity for protein. Furthermore, \(W_i^{\text{max}}\) can be attained by any mixture of proteins, which is of relevance in the interpretation of concentration-dependent interfacial tensions (\(\gamma_v\)) or adhesion tensions (\(\tau_a\)) of multi-component protein mixtures such as blood plasma or serum, as further discussed below.

First, fixed \(W_i^{\text{max}}\) immediately explains the experimental observation that \(\Pi_a\) curves for diverse purified proteins spanning three decades of MW appear more similar than dissimilar when scaled on a w/v basis\(^4\). Serial dilution of a protein solution at surface-saturating concentration \(W_i^{\text{max}}\) exhibiting the characteristic adhesion tension \(\tau_a\) will trace similar concentration
dependence because \( \frac{d\tau_a}{d \ln C_a} \) of individual proteins are very similar. Second, fixed \( W_i^{\text{max}} \) rationalizes the experimental observation that \( \Pi_a \) curves for plasma and serum are substantially similar, regardless of species. At 60-85 mg/mL total protein (including fibrinogen)\(^\text{55}\), plasma and serum is well over the \( \sim 3 \text{ mg/mL} \) required to saturate a hydrophobic surface at \( W_i^{\text{max}} \), achieving minimum adhesion tension \( \tau'_a \) (see Fig. 1A). Depletion of coagulation proteins in the conversion of plasma to serum certainly changes the number and concentration of individual protein constituents in bulk solution, but does not alter \( W_i^{\text{max}} \) because the bulk-solution concentration is well above that required to saturate a hydrophobic surface. As a consequence, concentration-dependent \( \Pi_a \) curves of plasma and serum are nearly identical because other protein constituents compete for the interfacial vacancies effectively created by removal of proteins consumed in the coagulation process (e.g. conversion of fibrinogen to insoluble fibrin). Third, although variations in plasma proteome among species lead to a different weight-fraction distribution of proteins in the bulk phase and surface, these differences do not affect surface saturation (\( W_i^{\text{max}} \sim 436 \text{ mg/mL} \)). And, because \( \frac{d\tau_a}{d \ln C_a} \) of individual protein constituents are very similar, the net observed \( \Pi_a \) curves for plasma derived from different species are not significantly different.
8.5. Conclusions

The principal conclusion drawn from this and related work is that interfacial energetics of whole-blood plasma, serum and purified-protein constituents is strikingly similar across species at two hydrophobic surfaces studied herein (liquid-vapor, LV and solid-liquid, SL). Differences in concentration-dependent solid-water (solid-liquid, SL) interfacial energetics among plasma and serum from disparate species (human, bovine, ovine and equine) are not resolvable within 99% statistical confidence. Substantially conserved concentration-dependence

\[
\frac{d\gamma_{LV}}{d\ln C_g} \quad (LV \text{ interfacial tensions}) \quad \text{or} \quad \frac{d\tau_{SL}}{d\ln C_g} \quad (SL \text{ adhesion tensions})
\]

despite differences in plasma proteome among donor species is interpreted as a direct consequence of an invariant protein amphility (interaction energetics with water) with varying protein type (molecular weight or size)\(^{34}\).

Adsorption of proteins from heterogeneous aqueous solution can be accurately viewed as a competition for space in the hydrophobic surface region. Hydrophobic surfaces with concentrated protein solutions such as plasma or serum are always saturated with protein. Thus, enrichment or depletion of any particular protein in bulk solution does not have a significant effect on \(\frac{d\gamma_{LV}}{d\ln C_g}\) or \(\frac{d\tau_{SL}}{d\ln C_g}\) because substitution of one protein for another within the surface region does not affect surface saturation.
Citations


(28) Nouy, P. L. D. *Surface Equilibria of Biological and Organic Colloids*; The Chemical Catalog Co.: New York, 1926; Vol. 27.


### TABLE 1: STEADY-STATE PLASMA/SERUM ADSORPTION PARAMETERS

<table>
<thead>
<tr>
<th>TYPE (acronym)</th>
<th>$\theta_a$ (degrees)</th>
<th>$\theta'_a$ (degrees)</th>
<th>$\ln C_B^{\theta/2}$ PPT</th>
<th>$M$ (dimensionless)</th>
<th>$\tau_a$ (mN/m)</th>
<th>$\tau'_a$ (mN/m)</th>
<th>$\Pi_{\max}^a$ (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUMAN PLASMA (HP)</td>
<td>104.2±0.6</td>
<td>79.6±1.6</td>
<td>20.1±0.4</td>
<td>-9.2±1.6</td>
<td>-17.6±0.8</td>
<td>8.8±1.4</td>
<td>26.4±1.9</td>
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<tr>
<td>HUMAN SERUM (HS)</td>
<td>100.4±0.9</td>
<td>77.7±1.0</td>
<td>17.5±0.3</td>
<td>-13.6±3.2</td>
<td>-12.7±1.1</td>
<td>10.1±0.8</td>
<td>22.9±1.4</td>
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<tr>
<td>BOVINE PLASMA (BP)</td>
<td>100.5±0.6</td>
<td>76.6±1.8</td>
<td>20.3±0.5</td>
<td>-9.8±1.8</td>
<td>-13.2±0.8</td>
<td>8.9±1.5</td>
<td>22.1±1.7</td>
</tr>
<tr>
<td>BOVINE SERUM (BS)</td>
<td>101.6±0.6</td>
<td>86.4±0.8</td>
<td>17.7±0.4</td>
<td>-12.9±3.1</td>
<td>-14.3±0.7</td>
<td>2.6±0.6</td>
<td>16.9±0.9</td>
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<tr>
<td>EQUINE PLASMA (EP)</td>
<td>101.4±0.5</td>
<td>80.2±0.5</td>
<td>18.1±0.2</td>
<td>-36.1±7.3</td>
<td>-13.6±0.6</td>
<td>8.1±0.4</td>
<td>21.6±1.1</td>
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<tr>
<td>EQUINE SERUM (ES)</td>
<td>100.6±0.5</td>
<td>79.1±0.9</td>
<td>18.9±0.3</td>
<td>-9.4±1.2</td>
<td>-12.9±0.4</td>
<td>8.4±0.6</td>
<td>20.7±0.7</td>
</tr>
<tr>
<td>OVINE PLASMA (OP)</td>
<td>100.9±0.5</td>
<td>86.1±1.1</td>
<td>21.1±0.4</td>
<td>-20.8±6.6</td>
<td>-13.6±0.6</td>
<td>3.0±0.9</td>
<td>16.6±1.1</td>
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<tr>
<td>OVINE SERUM (OS)</td>
<td>101.7±0.5</td>
<td>85.5±0.9</td>
<td>19.9±0.3</td>
<td>-14.9±3.3</td>
<td>-14.5±0.6</td>
<td>3.5±0.7</td>
<td>17.9±0.9</td>
</tr>
</tbody>
</table>

Notes: Listed data result from statistical fit (see Materials and Methods)
**Figure 1:** Advancing contact angle isotherms in 3D ($\theta_a$ as a function of analysis time (drop age) and logarithmic (natural) solution concentration $C_a$) and 2D ($\theta_a$ as a function of logarithmic solution concentration $C_a$ at selected times) formats comparing human (HS, panel A, Table 1), ovine (OS, panel B), and equine (ES, panel C) serum. In each case, solute concentration $C_a$ is expressed in v/v units of parts-per-trillion (PPT, mL/$10^{12}$ mL solution). Symbols in 2D panels represent time slices through 3D representations (filled circle = 0.25 sec, open circle = 900 sec, filled triangles = 1800 sec, and open triangles = 3594 sec; annotations in Panel A indicate maximum and half-maximum contact angle reduction, $\theta_a^{\max}$ and $\frac{1}{2} \theta_a^{\max}$ respectively. Note also decrease in $\theta_a$ with time, attributed to slow hydration of the SAM surface (Panel B, arrow annotation; see Results section for more discussion).
Figure 2: Sequential interpretation of a steady-state (3600 sec drop age) contact angle adsorption isotherm for human serum (HS, Table 1); Panel A, advancing contact angles, $\theta_a$; Panel B, advancing adhesion tension, $\tau_a$; Panel C, advancing spreading pressure $\Pi_a$. Smoothed curves through the data serve as guides to the eye. Annotations identify low- and high- concentration asymptotes for contact angles ($\theta_a^0$, $\theta_a^\prime$), adhesion tensions ($\tau_a^0$, $\tau_a^\prime$) and maximum spreading pressure $\Pi_a^{\text{max}}$ that are used to characterize isotherms (Table 1).
Figure 3: Comparison of steady-state, concentration-dependent $\Pi_u$ (panel A) as well as $\gamma_\nu$ (panel B) data for (human) plasma (HP, Table 1), serum (HS), and IgG (hIgG) on a single concentration axis, showing that protein mixtures cannot be confidently distinguished from a purified protein at two hydrophobic surfaces. Band represents 99% confidence intervals around best-fit-human plasma data. (closed circle = HP, open square = HS, open triangle = hIgG). Notice that maximum spreading pressure $\Pi_u^{\text{max}}$ is conserved at about 25mN/m at the two hydrophobic surfaces for a purified protein as well as complex protein mixtures, plasma and serum.
\( \Pi_a (\text{mN/m}) \)

\( \ln C_B \) (\(C_B\) in PPT)

\( \Pi_{\text{max}} \approx 25 \text{ mN/m} \)

\( \gamma_L, \gamma_V \) (\( \text{mN/m} \))
Figure 4: Comparison of steady-state, concentration-dependent $\Pi_a$ (panel A) as well as $\gamma_v$ data for human (HP, filled circle, Table 1), ovine (OP, open triangle), equine (EP, open square) and bovine (BP, open circle) plasma on a single concentration axis. Band represents 99% confidence intervals around best-fit-human plasma data, suggesting that species cannot be confidently distinguished from this data. In each case, solute concentration $C_a$ is expressed in v/v units of parts-per-trillion (PPT, mL/10^{12} mL solution). Notice that maximum spreading pressure $\Pi_a^{\text{max}}$ is conserved at about 25mN/m at the two hydrophobic surfaces regardless of species differences.
\( \Pi (\text{mN/m}) \)

\( \gamma (\text{mN/m}) \)

\( \Pi_{\text{max}} \approx 25 \text{ mN/m} \)

\( \Pi_{\text{max}} \approx 25 \text{ mN/m} \)

\( \ln C_B (C_B \text{ in PPT}) \)
Abstract

Mass of adsorbed protein derived from a simple sphere-packing model based on energetics of protein adsorption closely matches experimental results from high-precision quartz crystal microbalance (QCM) technique. Results from four purified proteins, human serum albumin (FV HSA), Fibrinogen (Fb) and Immunoglobulins (IgG; IgM) have been used to compare theory to experiment by computing total mass of adsorbed protein as well as water molecules within the interphase. Outcomes from the comparison of these two complementary methods (biophysical theory to a modern surface-analytical tool) not only reinforce the basic principles of the protein adsorption model but also constitute first steps towards achieving a complete mass and energy inventory necessary for a comprehensive understanding of the protein adsorption process.

9.1. Introduction

A critical need in the development of successful biomaterials is firm structure-property relationships that link the propensity of a material to promote protein adsorption to measurable surface characteristics such as surface chemistry and energy (water wettability). Our recent work on interfacial energetics has revealed a pattern in protein adsorption understandable in terms of known physical principles of amphilicity (interaction energetics with water) and surfactancy (propensity to adsorb to surfaces). Surprisingly perhaps, we find that protein adsorption has more to do with the properties of water, the universal biological solvent, than the molecular properties of proteins per se. This suggests that surface aspects of the protein-adsorption problem have, in fact, a general
solution that can be articulated in a set of tangible biophysical rules or laws. Indeed, we find that a relatively straightforward theory of protein adsorption applies to complex proteinaceous mixtures such as blood plasma or serum, not just single-protein solutions, suggesting that these rules will have practical medical utility that transcends the physical chemistry that underlies theory.

It is our contention that a comprehensive understanding of the protein adsorption process will require both a complete mass and energy inventory. A number of modern methods are available to measure the mass of protein adsorbed (see, for example, ref. \(^2\) and citations therein) including ellipsometry, IR spectroscopy, surface plasmon resonance, and reflectometry. This work focuses on the application of high precision quartz crystal microbalance (QCM) techniques, including QCM arrays, as described in the following sections. This is a very well established method that has minimal complexities in the experiments and interpretation compared to other methods, thus serving to make the systematics of our approach optimally efficient. There are but two basic methods to measure interfacial energetics of protein adsorption: tensiometry (contact angle and wetting techniques) and microcalorimetry. We advocate use of tensiometry herein because it is particularly well-suited for micro-scale measurements of adsorption of proteins available only in small quantities on well-defined surfaces with low surface area prepared using modern methods of surface engineering.\(^3,4\) Also, experience from recent work on energetics has refined interpretation of tensiometric results in terms of protein adsorption.\(^5-9\) Hence, a novelty of this work is the concerted application of methods that will provide both the needed mass and energy inventory.

There are many different ways to measure adsorption and these different techniques have assorted advantages/drawbacks (esp. surface sensitivity).\(^4\) Results are not typically intercomparable, and this
has no doubt contributed to the general lack of consensus within the protein-adsorption literature (see review ref. 10 and citations therein). Adsorption mechanisms can be broadly classified into chemisorption and physisorption categories, although protein adsorption need not occur exclusively through one mechanism or another. Adsorption through the former process leads to more strongly-bound adsorbate than through the latter. The need to account for weakly-bound, physisorbed protein emphasizes the use of in situ techniques that do not perturb/destroy structure of the interfacial layer into which protein becomes entrained.10 This work focuses on gravimetry and tensiometry, two complementary in situ techniques measuring adsorbate mass and adsorption energetics, respectively.

Quartz-crystal microbalance (QCM) techniques have emerged as highly sensitive means of detecting and monitoring adsorption to solid surfaces11-15 and will be used in our studies to measure adsorbed protein mass. A useful experimental attribute of QCM is that it can be successfully married with surface engineering methods (e.g. self-assembled monolayer or SAM technology). The basic QCM measurement consists of applying an AC voltage, generally in the 5-10 MHz frequency region but often higher, to an AT-cut thin quartz crystal16 with metal electrodes, typically gold, and tracking the resonant frequency of the crystal using a simple oscillator circuit. Observed frequency shift is proportional to change in mass, such as by adsorption of molecules.17

Tensiometry or goniometry involves measurement of time-and-concentration-dependent interfacial tensions (LV) or contact angles (SL) respectively, of protein-solutions. A substantial amount of work on energetics of protein adsorption to the two hydrophobic surfaces (LV and SL) have already been outlined in Chapters 1-8. This chapter focuses mostly on testing and interpreting our theory of protein adsorption in terms of adsorbate mass measurements from QCM. QCM and tensiometry are
highly complementary techniques because results can be co-interpreted in terms of a complete mass-energy inventory that is essential to secure identification of adsorption mechanisms, thickness of the adsorbate layer(s), and protein organization within adsorbed layer(s). The same techniques can be applied equally, without modification, to purified-protein solutions and multi-component protein solutions such as plasma/serum that are especially biomedically relevant.

9.2. Materials and Methods

Quartz Crystal Microbalance: Details of the quartz crystal microbalance (QCM) technique have been described elsewhere\textsuperscript{15,18}. The basic QCM measurement consists of applying an AC voltage, generally in the 5-10 MHz frequency region, to an AT-cut thin quartz crystal (\(\sim 1\text{mm}\)) with metal electrodes on both sides, typically gold, and tracking the resonant frequency of the crystal using a simple oscillator circuit. When the mass of the electrodes change, such as by adsorption of protein molecules, the frequency shifts proportionally.\textsuperscript{17,19} Shift in the resonance frequency of the quartz crystal can be used to calculate the adsorbed mass using the classical Sauerbrey relationship\textsuperscript{17,19}

\[
\Delta f = \frac{-f_0^2 \Delta m}{N \rho_0}; \quad \text{where } f_0 \text{ is the resonant frequency of the oscillator, and } N \text{ is a frequency constant of the quartz crystal. The mass sensitivity constant } C = \frac{-N \rho_0}{f_0^2} \text{ is equal to 17.7 ng/(cm}^2\text{.Hz) at } f_0 = 5\text{MHz. Thus, total mass of adsorbed protein can be computed as } \Delta m = -\Delta f \times 17.7\text{ng/cm}^2. \]

QCM response as a function of time was monitored for each of 24-30 serial protein dilutions covering a dynamic range between \(10^{-10}\) to 1 % (w/v), constituting a complete protein adsorption isotherm. About 500 \(\mu\text{L}\) of protein solution was pipetted into the closed sample cell at the start of data collection. At the end of an hour-long measurement period (equilibrium), data collection is
discontinued and solution replaced with the next higher concentration with intermediate buffer rinses (3X).

**Proteins:** Purified proteins, human serum albumin (FV HSA), Fibrinogen, IgG and IgM were used as-received from Sigma-Aldrich and were the highest purity available (>96% as assessed by SDS PAGE). Mass, concentration, and molecular weights supplied with the proteins were accepted without further confirmation.

**Surfaces:** Methyl-terminated self-assembled monolayer surfaces were prepared on the quartz-crystal sensor element of the QCM apparatus using standard surface engineering techniques outlined elsewhere\textsuperscript{20-24}. Briefly, the sensor element was pre-cleaned in hot 1:4 H\textsubscript{2}O\textsubscript{2} (30%)/H\textsubscript{2}SO\textsubscript{4} solution. The cleaned crystal surface was rinsed with absolute ethanol and immediately immersed in 1mM solutions of 1-hexadecanethiol (CH\textsubscript{3}(CH\textsubscript{2})\textsubscript{15}SH) in ethanol for 3 days. After SAM formation, the samples were rinsed with ethanol and dried with nitrogen.

**QCM Gravimetry:** An important attribute of this part of the work on QCM gravimetry is that it involves collaborative efforts with Liu and Allara of the Molecular Surface Science Laboratory. This collaboration is ongoing from our recent work on self-assembled monolayers as model substrates for protein adsorption studies at the SL interface. Methyl-terminated hexadecane thiol SAM surfaces will be prepared on QCM crystals using standard techniques described in previous sections. Initial studies measuring protein adsorption by QCM will deploy a micro liquid cell and computer-controlled QCM setup. QCM response as a function of time will be monitored for each of 24-30 serial dilutions covering a dynamic range between 10\textsuperscript{-10} to 1 % (w/v), constituting an
adsorption isotherm for each test surface. Analysis will proceed from most dilute (control blank) to most concentrated (~1%). Briefly outlining the experimental protocol, ~500 µL of protein solution will be pipetted into the closed sample cell at the time data collection is started. After equilibrium is achieved (≤ 1 hour based on preliminary data), data collection will be discontinued and the solution replaced with the next higher in the series with 3X serial buffer rinse between serially-increasing solution concentrations but avoiding desiccation of the sample cell.

Co-interpretation of QCM and Tensiometric Data: This part of the work, will, for the first time, permit sensible co-interpretation of QCM (mass) and goniometric (energy) data because the theory of adsorption that will be applied to both is written in tangible terms of adsorbate surface concentration (in moles/volume), and not in the more abstract notion of adsorbate surface density (in moles/area) typically used in the interpretation of small-molecule adsorption. Importantly, tensiometry and QCM are independent and highly complementary techniques from which a comprehensive energy-mass inventory can be assembled that tests adsorption mechanisms.

9.3. Theory of Protein Adsorption

Previous work probing energetics of protein adsorption to the water-air (liquid-vapor, LV) as well as solid-water (solid-liquid, SL) interphases using tensiometric techniques has revealed important biophysical relationships among diverse proteins spanning three decades in molecular weight (MW). Significant results from this work have led to a model of protein adsorption based on close-packing of oblate-spheroid, globular protein molecules (approximately spherical in aqueous solution). Consequently, globular proteins are modeled as spheres with radius \( r_v = 6.72 \times 10^{-8} MW^{1/3} \) (packing-volume radius in cm for MW in kDa). At surface-saturating protein concentrations, this
model suggests that proteins with hydration shells pack within the interphase to nearly face-centered-cubic (FCC) concentrations. Thus, the model envisions close-packing of hydrated protein molecules of characteristic radius $R = \chi r_v$ where $\chi$ represents excluded volume surrounding each molecule. Maximal interphase concentrations of hydrated proteins (radius, R) was reasoned to be proportional to the maximum conceivable concentrations resulting from face-centered cubic packing of core proteins (radius $r_v$ ) through a packing-efficiency parameter. This packing-efficiency factor $\varepsilon$ was estimated to be 0.45 from independent calibration to two sources from neutron-reflectometry$^{27}$ and quasi-electric light scattering$^{28,29}$ respectively. All features of the protein adsorption model are clearly detailed in refs. $^7,^8,^{25,26}$. Select aspects of the model relevant to this work are reproduced herein.

**Interphase Protein-Water Mass Proportions:** Equation 1 computes the maximal interphase concentration $C_i^{\text{max}}$ of hydrated proteins, per unit volume of the interphase corresponding to the core protein thickness ($2r_v$). $C_i^{\text{max}}$ follows an inverse function of protein MW and is related by the packing efficiency parameter $\varepsilon$ -

$$C_i^{\text{max}} = \varepsilon(4 \text{ molecules}) = \frac{0.17\varepsilon \text{ molecules}}{(2r_v\sqrt{2})^3} = \frac{9.68\times10^8\varepsilon}{MW}\text{ (pmol/cm}^3\text{)} \quad \text{.........Eq. 1}$$

The theory also derives volume fractions of adsorbed protein and the proportions of water molecules within the interphase. At interphase saturation ($C_i^{\text{max}}$), the maximum volume fraction of protein $\Phi_p^{\text{max}}$ is 0.74$\varepsilon$ or $\Phi_p^{\text{max}} = 0.33$ when $\varepsilon = 0.45$. Thus, maximum volume fraction of adsorbed protein is constant irrespective of protein MW. Knowledge of protein volume fraction can be extended to compute volume fractions of bound- as well as free-water within the interphase, thus generating an
Table 1 shows the proportions of protein and water within the interphase in terms of volumes, moles and mass as determined from respective volume fractions. Computations from the packing model can be used to predict mass proportions of protein and water derived experimentally from QCM techniques.

**9.4. Results and Discussion**

Figure 1 plots shift in resonance frequency ($\Delta f$) for two purified proteins measured using QCM techniques described in Materials and Methods. Mass of adsorbed protein can be computed from the frequency shift using the Sauerbrey equation$^{17}$ (see Materials and Methods). Accordingly, maximum adsorbed-protein mass was computed using the high-concentration parameter from a four-parameter sigmoidal fit to $\Delta f$ data for each of the four proteins. Results were found to be consistent with recent literature$^{18}$ as well. Table 2 compares mass proportions of protein and water calculated from the adsorption model to total mass from QCM measurements. Moles (columns 5-7) and mass (columns 8-10) proportions of protein, bound water and free water were computed per unit area of the interphase (see Table 1 and Eq. 2 for respective formulations). The sum total mass of core protein, bound water and free water (column 12) serves for direct comparison to QCM results (column 13). Remarkable statistical agreement was seen for FVHSA (MW=66.3kDa), IgG (MW=160kDa) and Fb (MW=340kDa) in comparing mass computed from model to that from experiment. However, adsorbate-mass measured for IgM (MW=1000 kDa) falls short of the computed theoretical value by over 60mg/m$^2$ (row 4 Table 2). This substantial mismatch is speculated to be an outcome of the exceptionally large size of IgM$^{30}$ and the expectation from theory that it occupies five whole layers at the interphase$^{31}$. Further limitations from the QCM experimental techniques, such as viscosity or extent of dissipation$^{32,33}$ is also speculated and
warrants further investigation. Figure 2 compares adsorption isotherms for FV HSA from
goniometry (\( \Pi_a \) vs. \( \ln C_B \)) to those from QCM (\( \Delta f \) vs. \( \ln C_B \)) with concentration scaled in w/v
units. Similarity in adsorption trends from two different techniques is evident in Fig. 2 and this is
particularly useful in co-interpretation of data derived from energetics and mass.

9.5. Conclusions

Close agreement was seen between theory and experiment in calculations of mass of protein and
water within a hydrophobic interphase. A relatively-simple model of protein adsorption derived
from tensiometric data computes mass proportions of protein and water within a hydrophobic
interphase. Calculations from the model demonstrated significant statistical match with
experimental mass measurements from quartz-crystal microbalance technique for three out of four
purified proteins studied (FVHSA, Fb and IgG). Further investigations refining both theory and
QCM experimental techniques is expected to resolve any discrepancies between the theoretical and
experimental outcomes for purified proteins as well as multi-protein mixtures, plasma and serum.
Citations


<table>
<thead>
<tr>
<th>Type</th>
<th>Volume fraction (cm³)</th>
<th>Moles within Interphase (moles)</th>
<th>Mass within Interphase (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein</strong></td>
<td>( \Phi_p V^\text{max}_I ) = 0.33( V^\text{max}_I )</td>
<td>( \frac{0.33V^\text{max}_I \rho_p}{MW_p} )</td>
<td>0.33( V^\text{max}_I \rho_p )</td>
</tr>
<tr>
<td><strong>Bound water</strong></td>
<td>( \Phi_x V^\text{max}_I ) = 0.41( V^\text{max}_I )</td>
<td>( \frac{0.41V^\text{max}_I \rho_w}{MW_w} )</td>
<td>0.41( V^\text{max}_I \rho_w )</td>
</tr>
<tr>
<td><strong>Interfacial free water</strong></td>
<td>( \Phi_{Iw} V^\text{max}_I ) = 0.26( V^\text{max}_I )</td>
<td>( \frac{0.26V^\text{max}_I \rho_w}{MW_w} )</td>
<td>0.26( V^\text{max}_I \rho_w )</td>
</tr>
</tbody>
</table>

Notes: Interphase volume \( V^\text{max}_I \) = thickness of hydrated protein layer (cm) X area (A) of interphase (cm²)

\( \rho, MW \) represent density and molecular weight of protein or water as denoted by subscripts \( p \) or \( w \) respectively.
Table 2: Mass Proportions of Protein and Water within the Interphase – Theory and Experiment

<table>
<thead>
<tr>
<th>Protein</th>
<th>$MW_p$ (kDa)</th>
<th>$V_r^{max}$ (cm³) $[2r_i \times \text{Area}]$</th>
<th>$V_i^{max}$ (cm³) $[2R \times \text{Area}]$</th>
<th>Per Unit Area of Interphase</th>
<th>QCM mass (mg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Moles (pmol/cm²)</td>
<td>Mass (mg/m²)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Protein</td>
<td>Bound water</td>
</tr>
<tr>
<td>FVHSA</td>
<td>66.3</td>
<td>$5.4 \times 10^{-7}$ A</td>
<td>$7.2 \times 10^{-7}$ A</td>
<td>3.6</td>
<td>1.6×10⁴</td>
</tr>
<tr>
<td>IgG</td>
<td>160</td>
<td>$7.2 \times 10^{-7}$ A</td>
<td>$9.3 \times 10^{-7}$ A</td>
<td>1.9</td>
<td>2.1×10⁴</td>
</tr>
<tr>
<td>Fb</td>
<td>340</td>
<td>$9.4 \times 10^{-7}$ A</td>
<td>$12.2 \times 10^{-7}$ A</td>
<td>1.2</td>
<td>2.8×10⁴</td>
</tr>
<tr>
<td>IgM</td>
<td>1000</td>
<td>$13.4 \times 10^{-7}$ A</td>
<td>$17.4 \times 10^{-7}$ A</td>
<td>0.6</td>
<td>4.0×10⁴</td>
</tr>
</tbody>
</table>

Notes: $V_r^{max}$ = thickness of core protein layer (cm) × area (A) of interphase (cm²)

$V_i^{max}$ = thickness of hydrated protein layer (cm) × area (A) of interphase (cm²)

* Total mass (mg/m²) = 1 layer mass (Protein + Bound water + Free water) × Number of layers
Figure 1: Comparison of resonance frequency shift ($\Delta f$) for two purified proteins FV HSA (MW=66.3kDa, closed triangles) and IgG (MW= 160kDa, closed circles) measured from quartz crystal microbalance. Smooth curves result from sigmoidal fit to the data. Steady-state, limiting frequency measurements can be used to deduce adsorbed protein mass from Sauerbrey equation (see Materials and Methods)
Graph showing the relationship between $\ln C_B$ (in PPT) and $\Delta f$ (Hz). The x-axis represents $\ln C_B$, ranging from 5 to 25, and the y-axis represents $\Delta f$, ranging from -20 to 120 Hz. The graph includes two curves and data points represented by circles and triangles.
Figure 2: Comparison of adsorption isotherms from QCM (resonance frequency shift, $\Delta f$, open circles) with goniometry (spreading pressure, $\Pi_s$, closed circles) for albumin (FV HSA). Smooth curves result from sigmoidal fit to the data.
Anandi Krishnan was born at Tirunelveli in Southern India on the 19th of June 1978. She is the elder of three children, and spent her early years in Trivandrum, Kerala – the so-called God’s Own Country. She later moved with her family to Kuwait, where she was pampered through high-school at the Indian School, Kuwait. She managed to study a bit and landed an admission to the Govt. College of Engineering, Trivandrum – where she spent four joyous years under her grandmother’s care. Graduating with a Bachelor of Technology degree in Electrical and Electronics Engineering in 2000, she spent a short stint in software engineering at Bangalore, India. Later in the Fall of 2001, she and her husband moved to the United States of America to pursue graduate studies. She has since been pursuing her Ph. D in Bioengineering at Penn State under the able guidance of Dr. Erwin Vogler of the Department of Materials Science and Engineering. Anandi’s research interests include biomaterials, medical devices and their clinical applications in cardiovascular health and disease. In addition to simple books, such as those of Italo Calvino or R.K. Narayan, Anandi enjoys carnatic music.

Some Publications and Presentations -

**PUBLICATIONS**

1. Krishnan A, Siedlecki CA, Vogler EA

2. Krishnan A, Sturgeon J, Siedlecki CA, Vogler EA

3. Krishnan A, Siedlecki CA, Vogler EA


**PRESENTATIONS**

1. Controlling Role of Water in Protein Adsorption

2. Mixology of Protein Solutions and the Vroman Effect

3. Traube-Rule for Protein Adsorption

**Some Publications and Presentations**

1. **Traube-Rule Interpretation of Protein Adsorption to the Liquid-Vapor Interface.** Langmuir; 19:10342-10352, 2003


3. **Mixology of Protein Solutions and the Vroman Effect.** Langmuir; 20: 5071-5078, 2004

4. **Liquid-Vapor Interfacial Tensions of Blood Plasma, Serum and Purified Protein Constituents Thereof;** Biomaterials; 26; 3445-3453, 2004


7. **An Evaluation of Goniooinetric Methods**

   Colloids and Surfaces. In Press, May 2005

**PRESENTATIONS**

1. Oral Presentation, **AVS 51st International Symposium**

   Anaheim, CA; November 2004

2. Poster Presentation, **Gordon Research Conference on Chemistry at Interfaces;** Meriden NH; August 2004

3. Poster Presentation, **Gordon Research Conference on Biomaterials**

   Plymouth, NH; July 2003