

Mixology of Protein Solutions and the Vroman Effect[†]

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Mixing rules stipulating both concentration and distribution of proteins adsorbed to the liquid–vapor (LV) interphase from multicomponent 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 $1/m$ 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 30 classical plasma proteins occupying the first 5 decades of physiological concentration. Mixing rules rationalize the experimental observations that (i) concentration-dependent liquid–vapor interfacial tension, γ_{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) γ_{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 γ_{lv} reflecting the linear combination of weight/volume 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.

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 state² 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 in vitro or in vivo biological response). Biomedical surface science³ 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, 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 (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, 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

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(1) Krishnan, A.; Sturgeon, J.; Siedlecki, C. A.; Vogler, E. A. *J. Biomed. Mater. Res.* **2004**, *68A*, 544.

(2) Horbett, T. A. Biological Activity of Adsorbed Proteins. In *Biopolymers at Interfaces*; Malmsten, M., Ed.; Surfactant science series 75; Marcel Dekker: New York, 1998; p 393.

(3) Castner, D. G.; Ratner, B. D. *Surf. Sci.* **2002**, *500*, 28.

(4) Vogler, E. A. How Water Wets Biomaterials. In *Water in Biomaterials Surface Science*; Morra, M., Ed.; John Wiley and Sons: New York, 2001; p 269.

(5) Vogler, E. A. *Interfacial Chemistry in Biomaterials Science*. In *Wettability*; Berg, J., Ed.; Marcel Dekker: New York, 1993; Vol. 49; p 184.

(6) *Biopolymers at Interfaces*; Malmsten, M., Ed.; Marcel Dekker: New York, 1998; p 656.

(7) *Protein Adsorption on Biomaterials*; Cooper, S. L., Peppas, N. A., Hoffman, A. S., Ratner, B. D., Eds.; American Chemical Society: Washington DC, 1982; Vol. 199, p 234.

(8) *Proteins at Interfaces: Physicochemical and Biochemical Studies*; Brash, J. L., Horbett, T. A., Eds.; American Chemical Society: Washington, DC, 1987.

(9) *Surface and Interfacial Aspects of Biomedical Polymers: Protein Adsorption*; Andrade, J. D., Ed.; Plenum Press: New York, 1985; Vol. 1–2, p 1.

(10) Vogler, E. A.; Martin, D. A.; Montgomery, D. B.; Graper, J. C.; Sugg, H. W. *Langmuir* **1993**, *9*, 497.

so-called "Vroman effect" (see refs 6, 7, and 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.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.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 paper 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.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.⁴ Using pendant-drop tensiometry, we find that concentration-dependent interfacial tension, γ_{lv} , of a broad array of purified human proteins spanning 3 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.

2. Methods and Materials

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 (citrate) 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. Reference 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 M Ω) water. Interfacial tension of PBS and water was checked periodically by Wilhelmy-balance tensiometry.

Liquid–Vapor Interfacial Tension Measurements. LV interfacial tensions γ_{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.¹ Briefly, the tensiometer employed a Tecan liquid-handling robot to aspirate between 10 and 12 μ L 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+% relative humidity) analysis chamber and dispense between 6 and 11 μ L 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 γ_{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 \pm 1 $^{\circ}$ C by means of a computer-controlled resistive heater. The upper-temperature limit was, however, not controlled but rather floated with laboratory temperature, which occasionally drifted as high as 29 $^{\circ}$ C during summer months. Thus, reported γ_{lv} values are probably not more accurate than about 1 mN/m on an intersample 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 γ_{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.^{5,32,33} Briefly, time-dependent γ_{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 seconds) as column values. It was generally observed that γ_{lv} data take on a sigmoidal shape when plotted on logarithmic–concentration axes,^{5,32} with a well-defined low-concentration asymptote γ_{lv}° and a high-concentration asymptote γ_{lv}' . Successive nonlinear least-squares fitting of a four-parameter logistic equation ($\gamma_{lv} = \{[(\gamma_{lv}^{\circ} - \gamma_{lv}')/(1 + (\ln C_B^{n/2}/\ln C_B)^M)] + \gamma_{lv}'\}$) to concentration-dependent γ_{lv} data for each time within the observation interval

(11) Brash, J.; Lyman, D. Adsorption of Proteins and Lipids to Nonbiological Surfaces. In *The Chemistry of Biosurfaces*; Brash, J. L., Ed.; Marcel Dekker: New York, 1971; p 177.

(12) Vroman, L. *Bull. N.Y. Acad. Med.* **1972**, *48*, 302.

(13) Vroman, L.; Adams, A. L.; Klings, M.; Fischer, G. Fibrinogen, Globulins, albumins, and Plasma at Interfaces. In *Applied Chemistry at Protein Interfaces: A Symposium at the 166th Meeting of the American Chemical Society*; Advances in Chemistry Series 145; American Chemical Society: Washington, DC, 1975; p 255.

(14) Horbett, T. Protein Adsorption on Biomaterials. In *Biomaterials: Interfacial Phenomena and Applications*; Cooper, S. L., Peppas, N. A., Hoffman, A. S., Ratner, B. D., Eds.; American Chemical Society: Washington, DC, 1982; Vol. 199, p 234.

(15) Brash, J.; Hove, P. t. *Thromb. Haemostas.* **1984**, *51*, 326.

(16) Lensen, H. G. W.; Bargman, D.; Bergveld, P.; Smolders, C. A.; Feijen, J. *J. Colloid Interface Sci.* **1984**, *99*, 1.

(17) Vroman, L.; Adams, A. *J. Colloid Interface Sci.* **1986**, *111*, 391.

(18) Wojciechowski, P.; Hove, P. T.; Brash, J. L. *J. Colloid Interface Sci.* **1986**, *111*, 455.

(19) Elwing, H.; Askendal, A.; Lundstrom, I. *J. Biomed. Mater. Res.* **1987**, *21*, 1023.

(20) Shirahama, H.; Lyklema, J.; Norde, W. *J. Colloid Interface Sci.* **1990**, *139*, 177.

(21) Leonard, E. F.; Vroman, L. *J. Biomater. Sci., Polym. Ed.* **1991**, *3*, 95.

(22) Wahlgren, M.; Arnebrant, T. *Tibtech* **1991**, *9*, 201.

(23) Wojciechowski, P.; Brash, J. L. *J. Biomater. Sci., Polym. Ed.* **1991**, *2*, 203.

(24) Brash, J. L.; Hove, P. T. *J. Biomater. Sci., Polym. Ed.* **1993**, *4*, 591.

(25) Vroman, L. *J. Biomater. Sci., Polym. Ed.* **1994**, *6*, 223.

(26) Claesson, P. M.; Blomberg, E.; Froberg, J. C.; Nylander, T.; Arnebrant, T. *Adv. Colloid Interface Sci.* **1995**, *57*, 161.

(27) Lin, J. C.; Cooper, S. L. *J. Colloid Interface Sci.* **1996**, *182*, 315.

(28) Lee, J. H.; Lee, H. B. *J. Biomed. Mater. Res.* **1998**, *41*, 304.

(29) Jung, S.-Y.; Lim, S.-M.; Albertorio, F.; Kim, G.; Gurau, M. C.; Yang, R. D.; Holden, M. A.; Cremer, P. S. *J. Am. Chem. Soc.* **2003**, *125*, 12782.

(30) Derand, H.; Malmsten, M. Protein Interfacial Behavior in Microfabricated Analysis Systems and Microarrays. In *Biopolymers at Interfaces*; Malmsten, M., Ed.; Surfactant science series Vol. 75; Marcel Dekker: New York, 1998; p 393.

(31) Krishnan, A.; Siedlecki, C.; Vogler, E. A. *Langmuir* **2003**, *19*, 10342.

(32) Vogler, E. A. *Langmuir* **1992**, *8*, 2005.

(33) Vogler, E. A. *Langmuir* **1992**, *8*, 2013.

quantified γ_{lv}° and γ_{lv}' 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 $\Pi^{\max} \equiv \gamma_{lv}^\circ - \gamma_{lv}'$), as well as a parameter M that measured steepness of the sigmoidal curve. This empirical, multiparameter fitting to concentration-dependent γ_{lv} data was a purely pragmatic strategy that permitted quantification of best-fit protein and surfactant characteristics but is not a theory-based analysis.^{5,32,33} Three-dimensional (3D) representations of time-and-concentration-dependent γ_{lv} 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 matrixes at selected observation times.

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.^{1,31} This strategy was similar to that adopted by Ostuni et al.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 3 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 $r_v = 6.72 \times 10^{-8} \text{MW}^{1/3}$ (packing-volume radius in centimeters 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 mol/cm^3). Interestingly, it was found that P was essentially invariant among a diverse group of proteins studied ($P \sim 150$), 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^{\max} . C_i^{\max} can also be expressed in terms of fractional volume occupied by protein $\Phi_p^{\max} = C_i^{\max} V_p$ (dimensionless), where protein molar volume $V_p = 4/3\pi r_v^3 N_A$ (in cm^3/mol) if N_A is the Avogadro number. Elaboration of this model and calibration to experimental neutron-reflectivity data on albumin adsorption revealed that $\Phi_p^{\max} \sim 1/3$ 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

Φ_p^{\max} can be further quantified in the more familiar units of weight/volume (w/v) concentration, W_i^{\max} , by expanding the definition above explicitly in terms of r_v

$$\begin{aligned} \Phi_p^{\max} &= C_i^{\max} V_p = C_i^{\max} \left[\frac{4}{3} \pi \text{MW} N_A (6.72 \times 10^{-8})^3 \right] = \\ &= (7.65 \times 10^2) W_i^{\max} \sim \frac{1}{3} \Rightarrow W_i^{\max} = \\ &= 4.36 \times 10^{-4} \text{ kg}/\text{cm}^3 = 436 \text{ mg}/\text{mL} \quad (1) \end{aligned}$$

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 Equation 1 also anticipates the experimental observation that γ_{lv} curves for diverse proteins spanning 3 decades of MW appear more similar than dissimilar when scaled on a w/v basis³¹ 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 $W_B^{\max} = W_i^{\max}/P$ 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 $C_i V_p$

$$\begin{aligned} \Phi_p^{\max} &= C_{i,i} V_{p,i} + C_{i,j} V_{p,j} = \\ &= \frac{4}{3} \pi N_A (6.72 \times 10^{-8})^3 [C_{i,i} \text{MW}_i + C_{i,j} \text{MW}_j] = \\ &= (7.65 \times 10^2) [W_{i,i} + W_{i,j}] \sim \frac{1}{3} \Rightarrow [W_{i,i} + W_{i,j}] = \\ &= 436 \text{ mg}/\text{mL} \quad (2) \end{aligned}$$

Equation 2 states that constant Φ_p^{\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 ~ 436 mg/mL. Given that P is approximately constant for all proteins and both i, j are at equal bulk concentration W_B^{\max} 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 is thus concluded that $W_{i,i} = W_{i,j} = 1/2 W_i^{\max} = 1/2 (P W_B^{\max})$. 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_B^{\max} and thus not capable of individually saturating the interphase, it cannot compete for space as effectively as in the preceding proposition, contributing at most $1/2 P W_{B,i}$. 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 $W_i^{\max} = (W_{i,i} + W_{i,j}) = 1/2 P (W_{B,i} + W_{B,j}^{\max})$. Thus, the j th protein dominates the saturated interphase

(34) Ostuni, E.; Grzybowski, B. A.; Mrksich, M.; Roberts, C. S.; Whitesides, G. M. *Langmuir* **2003**, *19*, 1861.

(35) Rao, C. S.; Damodaran, S. *Langmuir* **2000**, *16*, 9468.

(36) Vogler, E. A. *Adv. Colloid Interface Sci.* **1998**, *74*, 69.

(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^{\max} but $(W_{B,i} + W_{B,j}) \geq W_B^{\max}$, then the logic of the mixing model insists that each protein competes for space according to $W_I^{\max} = 1/2 P(W_{B,i} + W_{B,j})$.

A survey of many different purified blood-plasma proteins spanning 3 decades in MW shows that concentration dependence of γ_{lv} was very similar among this disparate group of molecules.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 γ_{lv}' (occurring at W_B^{\max}) that falls within the 20 mN/m band characteristic of all proteins studied.^{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 $\Pi_i^{\max} \equiv (\gamma_{lv}^\circ - \gamma_{lv}')_i$ and $\Pi_j^{\max} \equiv (\gamma_{lv}^\circ - \gamma_{lv}')_j$ are spreading pressures of pure i and j at W_B^{\max} for each (where $\gamma_{lv}^\circ = 71.97$ mN/m at 25 °C is the interfacial tension of phosphate-buffer-saline diluent), then the observed spreading pressure $\Pi_{\text{obs}}^{\max} \equiv (\gamma_{lv}^\circ - \gamma_{lv}')_{\text{obs}}$ of an i,j mixture should vary with the relative proportion of each component within the interphase. Expressing this proportion as a weight fraction $f_{i,j}^{\max} \equiv (W_{I,i}/W_{I,i} + W_{I,j})$ and recalling that $P_{ij} \equiv (W_{I,i}/W_{B,i,j})$, then it follows that $f_{i,j}^{\max} = (W_{B,i}/W_{B,i} + W_{B,j}) \equiv f_{B,i}^{\max}$ (where the superscript “max” is retained to emphasize the restriction that $(W_{I,i} + W_{I,j}) \geq W_I^{\max}$). It can be expected that Π_{obs}^{\max} should vary in some way with the bulk composition $f_{B,i}^{\max}$. We are unaware of any theoretical precedent for such a combining formula for interfacial tensions and so tender the linear combination of eq 3

$$\Pi_{\text{obs}}^{\max} = \Pi_i^{\max} (1 - f_{i,j}^{\max}) + \Pi_j^{\max} f_{i,j}^{\max} = \frac{\Pi_i^{\max} - f_{B,i}^{\max} (\Delta \Pi^{\max})}{\Pi_i^{\max} - f_{B,i}^{\max} (\Delta \Pi^{\max})} \quad (3)$$

where $\Delta \Pi^{\max} \equiv \Pi_i^{\max} - \Pi_j^{\max}$. Equation 3 stipulates that Π_{obs}^{\max} varies between the two boundaries Π_i^{\max} and Π_j^{\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—Multicomponent 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 multicomponent protein solutions

$$\frac{1}{m} \sum_i^m W_{Ii} = \frac{P}{m} \sum_i^m W_{Bi} = W_I^{\max} \Rightarrow \sum_i^m W_{Bi} = \frac{m}{P} W_I^{\max} \sim \frac{4.36 \times 10^{-4}}{1.5 \times 10^2} m = (3 \times 10^{-6}) m \text{ (kg/cm}^3 \text{ for MW in kDa)} = 3m \text{ (mg/mL)} \quad (4)$$

Equation 4 states that the weight/volume (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 $P_{\text{eff}} \equiv P/m$ 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.

4. Results

Plasma, Serum, and Purified Proteins. Figure 1 collects time-and-concentration-dependent “ γ_{lv} 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, γ_{lv} as a function of time and concentration) and two-dimensional (2D, γ_{lv} as a function concentration at specified times) representations. Note that the logarithmic-solute-dilution ordinate $\ln C_B$ in Figure 1 is expressed in either w/v or v/v units of parts-per-trillion (PPT, grams of solute/10¹² grams of solution for purified proteins and mL/10¹² mL of 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 γ_{lv} as a function of bulk solution concentration C_B . Adsorption results in sigmoidally shaped, concentration-dependent γ_{lv} curves on a $\ln C_B$ axis, with a well-defined low-concentration asymptote γ_{lv}° and a high-concentration asymptote γ_{lv}' .⁵

We observed that most proteins within the $10 \leq \text{MW} \leq 1000$ kDa achieve a limiting γ_{lv}' within the 1 h observation period employed in this work.¹ Results shown in Figure 1A for hIgG are somewhat exceptional in this regard and arguably did not reach equilibrium. Either the bulk solution concentration was insufficient to fully saturate the surface (despite 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 γ_{lv}' possibly due to slow change in adsorbate configuration at the LV surface. In any event, γ_{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 Methods and Materials. Characteristic parameters listed in Table 1 for albumin, plasma, and serum are more certain in this regard because a secure limiting γ_{lv}' was achieved (see Figure 1). Figure 2 graphically compares equilibrium, concentration-dependent γ_{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 these data that concentration-dependent γ_{lv} of hIgG, plasma, and serum cannot be confidently distinguished.

Binary Protein Mixtures: Figure 3 compares time-dependent γ_{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). Figure 3A,B corresponds to experiments performed at a total protein concentration of 2.1 mg/mL whereas Figure 3C,D corresponds to 27 $\mu\text{g/mL}$ 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), γ_{lv} was observed to asymptotically approach a characteristic steady-state value γ_{lv}' . Comparison of parts A and C of Figure 3 shows that whereas steady state was arguably reached at 2.1 mg/mL total protein, γ_{lv} drifted continuously lower with time at 27 $\mu\text{g/mL}$ total protein. For the 2.1 $\mu\text{g/mL}$ total protein case, steady-state γ_{lv}' was estimated by simply

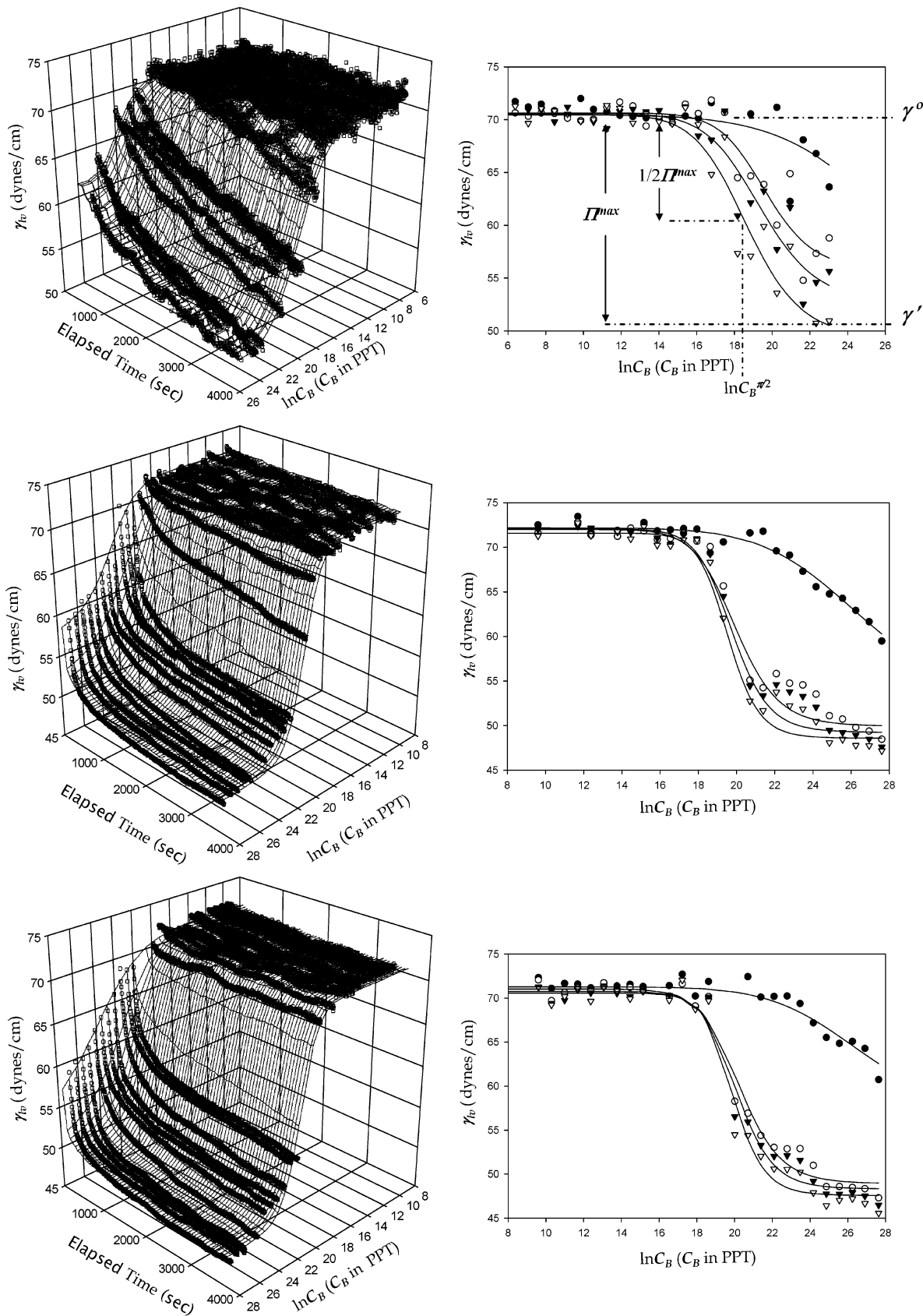


Figure 1. Interfacial tension profiles in 3D (γ_{iv} as a function of analysis time (drop age) and logarithmic (natural) solution concentration C_B) and 2D (γ_{iv} as a function of logarithmic solution concentration C_B at selected times) formats comparing (human) immunoglobulin-G (hIgG, panel A, preparation 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_B is expressed in either w/v or v/v units of parts-per-trillion (PPT, grams of solute/ 10^{12} grams of 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 s; open circle, 900 s; filled triangles, 1800 s; open triangles, 3594 s; 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.

Table 1. Protein Parameters

name of protein (acronym)		γ_{lv}° (mN/m)	γ_{lv}' (mN/m)	$\ln C_{\beta}^{\Pi/2}$ (PPT)	M (dimensionless)	Π^{\max} (mN/m)
human serum albumin fraction V (FV HSA)	prep 1	72.3 ± 1.2	50.3 ± 1.2	16.32 ± 0.28	-9.9 ± 2.5	21.4 ± 1.2
	prep 2	70.8 ± 1.1	46.2 ± 2.5	16.61 ± 0.51	-7.3 ± 2.2	25.5 ± 2.5
human IgG (hIgG)	prep 1	70.48 ± 0.57	48.7 ± 3.2	17.99 ± 0.71	-8.6 ± 2.1	23.1 ± 3.2
	prep 2	71.13 ± 0.57	51.6 ± 1.9	18.60 ± 0.39	-10.4 ± 2.7	20.1 ± 1.9
	prep 3	71.09 ± 0.42	56.48 ± 0.92	19.72 ± 0.19	-20.1 ± 5.1	15.21 ± 0.92
human IgM (hIgM)	prep 1	70.98 ± 0.39	51.4 ± 1.2	16.82 ± 0.19	-13.2 ± 3.5	20.3 ± 1.2
	prep 2	71.65 ± 0.55	50.2 ± 3.1	18.52 ± 0.35	-14.2 ± 4.2	21.5 ± 3.1
	prep 3	70.51 ± 0.59	55.4 ± 1.3	17.59 ± 0.12	-11.7 ± 3.3	16.3 ± 1.3
human plasma (HP)	prep 1	71.70 ± 0.62	48.55 ± 0.71	19.56 ± 0.19	-23.3 ± 3.6	23.15 ± 0.71
	prep 2	71.38 ± 0.74	45.5 ± 1.1	19.19 ± 0.26	-10.9 ± 1.6	26.5 ± 1.1
human serum (HS)	prep 1	70.54 ± 0.46	47.61 ± 0.62	19.91 ± 0.17	-23.4 ± 3.7	24.08 ± 0.62
	prep 2	72.64 ± 0.72	45.64 ± 0.69	18.47 ± 0.18	-13.4 ± 1.6	26.33 ± 0.69

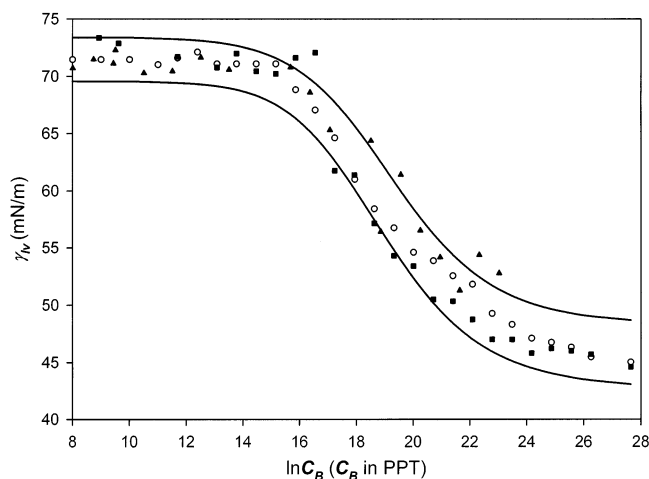


Figure 2. Comparison of steady-state, concentration-dependent γ_{lv} data for (human) plasma (HP, preparation 2, Table 1), serum (HS, preparation 2, Table 1), and IgG (hIgG, preparation 3, Table 1) on a single concentration axis, showing that protein mixtures cannot be confidently distinguished from a purified protein. Band represents 99% confidence intervals around best-fit-human plasma data (open circle, HP; filled square, HS; filled triangle, hIgG).

Table 2. Time-Dependent γ_{lv} of FV-HSA in hIgM Solutions

%	2.1 mg/mL total protein steady-state parameters		27 μ g/mL total protein kinetic parameters	
	γ_{lv}' (mN/m)	Π_{obs} (mN/m)	slope of $t^{1/2} > 30$	R^2 (%)
FV HSA in hIgM				
0	56.21 ± 0.22	15.76 ± 0.22	-0.14 ± 0.01	95
25	54.59 ± 0.17	17.39 ± 0.17	-0.15 ± 0.01	94
50	53.92 ± 0.11	18.17 ± 0.11	-0.11 ± 0.01	86
75	52.15 ± 0.12	19.82 ± 0.12	-0.15 ± 0.01	92
100	51.42 ± 0.17	20.55 ± 0.17	-0.16 ± 0.01	87

averaging the final 25 γ_{lv} observations. Results were expressed in terms of steady-state spreading pressure $\Pi_{obs} = (\gamma_{lv}^{\circ} - \gamma_{lv}')$ listed in columns 3 and 4 of Table 2, where $\gamma_{lv}^{\circ} \equiv 71.9$ mN/m, consistent with the interfacial tension of water at 25 °C. Figure 3B plots Π_{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 γ_{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 Π_{obs} could not be reliably estimated in the 27 μ g/mL total protein case because γ_{lv} did not achieve steady state. However, it was noted that data corresponding to $t > 900$ s was quite linear on $t^{1/2}$ coordinate (Figure 3D, see annotations) following a lag phase that was

especially noticeable in the 100% IgM case (columns 4 and 5 of Table 2 compile linear-least-squares parameters 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 γ_{lv} 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.

5. Discussion

Plasma and Serum. An extensive survey of concentration-dependent γ_{lv} 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 γ_{lv} for plasma and serum are effectively the same (see Figures 1 and 2), even though serum is compositionally distinct from plasma by virtue of being depleted of fibrinogen—one of the 12 most abundant proteins (2.5–4.5 mg/mL).³⁷ Taken together, experimental results show that γ_{lv} of blood plasma and serum are very similar to that of purified constituents. These findings supplement the long-known, but heretofore unexplained, observation that γ_{lv} of plasma derived from different (normal) mammalian species (bovine, ovine, canine, human) fall within a narrow 5 mN/m window,^{38–45} despite 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 γ_{lv} .¹ 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 γ_{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 amphiphilicity among different proteins manifests

(37) Putnam, F. W. Alpha, Beta, Gamma, Omega—The Roster of the Plasma Proteins. In *The Plasma Proteins: Structure, Function, and Genetic Control*; Putnam, F. W., Ed.; Academic Press: New York, 1975; Vol. 1, p 58.

(38) Morgan, J. L. R.; Woodward, H. E. *J. Am. Chem. Soc.* **1913**, *35*, 1249.

(39) DuNouy, P. L. *J. Exp. Med.* **1925**, *41*, 779.

(40) Harkins, H. N.; Harkins, W. D. *J. Clin. Invest.* **1929**, *7*, 263.

(41) Harkins, W. D.; Brown, F. E. *J. Am. Chem. Soc.* **1916**, *38*, 228.

(42) Harkins, W. D.; Brown, F. E. *J. Am. Chem. Soc.* **1916**, *38*, 246.

(43) Zozaya, J. *J. Biol. Chem.* **1935**, *110*, 599.

(44) Zozaya, J. *J. Phys. Chem.* **1938**, *42*, 191.

(45) Zozaya, J. *J. Phys. Chem.* **1938**, *42*, 657.

(46) Swenson, M. J. Physiological Properties and Cellular and Chemical Constituents of Blood. In *Duke's Physiology of Domestic Animals*; Swenson, M. J., Ed.; Cornell University Press: London, 1977.

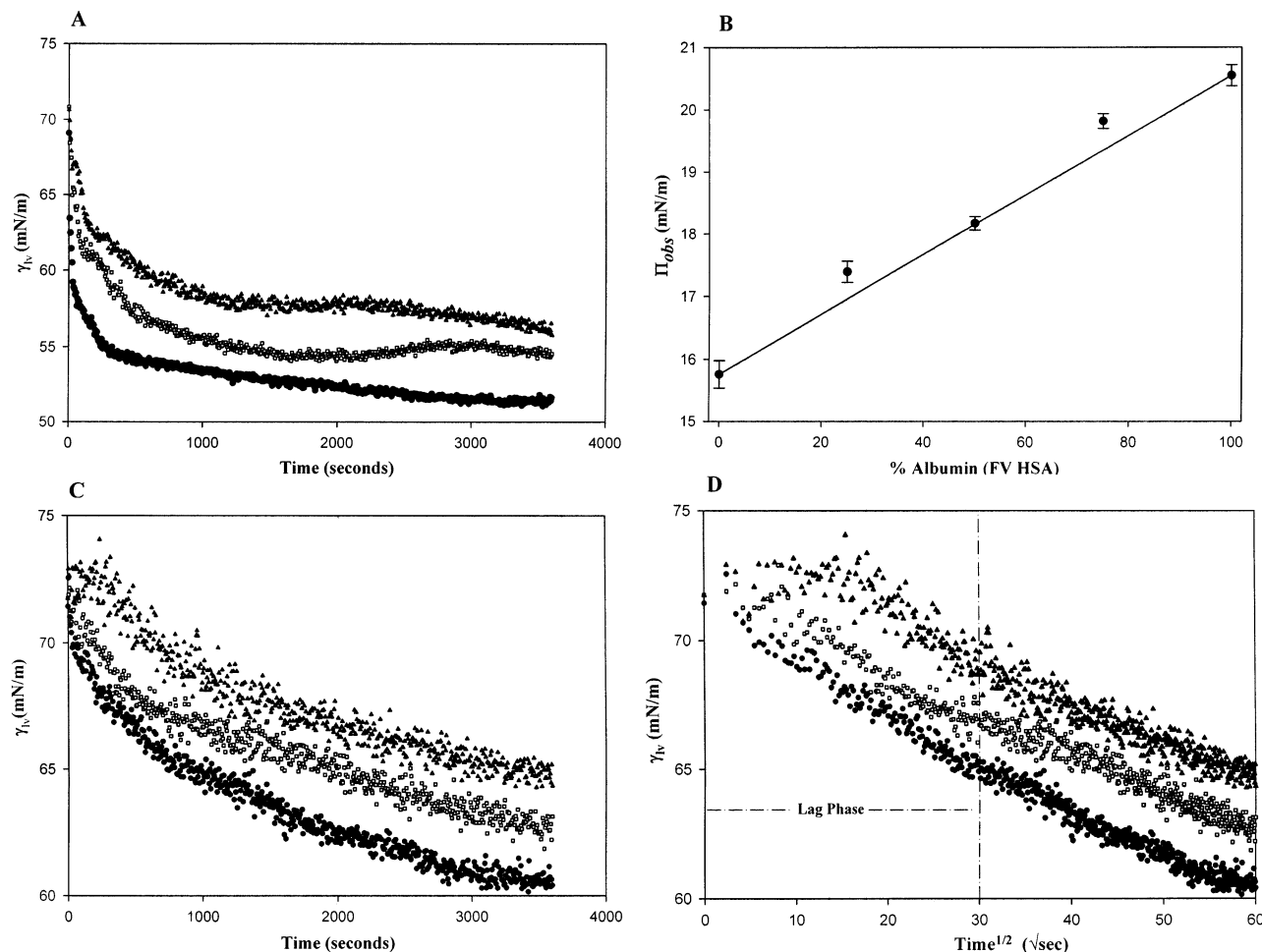


Figure 3. Interfacial tension γ_{iv} of (human) FV HSA and hIgM mixtures in different proportions (A, B at 2.1 mg/mL; C, D at 27 $\mu\text{g/mL}$ total protein concentration). Symbols represent different proportions of FV HSA in hIgM (filled circle, 100%; open square, 25%; filled triangle, 0% FV HSA in hIgM). Equilibrium (steady state) is reached within the 3600 s drop age of the PDT mixture experiment at 2 mg/mL (panel A), while γ_{iv} drifted continuously lower with time at 27 $\mu\text{g/mL}$ (panel C). Panel B plots the observed spreading pressure (Π_{obs}) as a function of proportion of FV HSA in hIgM, with error bars corresponding to the standard deviation of the mean γ_{iv} . Line representing eq 3 (see theory) demonstrates close correlation with experimental results. Panel D plots γ_{iv} as a function of $t^{1/2}$, revealing a linear trend (following a lag phase of $t < 900$ s) 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 $t > 900$ s.

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 γ_{iv}' will not vary significantly with bulk phase composition.

At 60–85 mg/mL total protein (including fibrinogen),⁴⁷ plasma and serum are well over the ~ 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 γ_{iv} . So it happens that concentration-dependent γ_{iv} 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 γ_{iv} 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 hydration⁴⁸ of blood proteins is approximately constant across a broad range of MW.^{1,31}

Mixing rules articulated in Theory suggest that protein adsorption to the LV surface from multicomponent solutions can be accurately viewed as a competition for space within the interphase region. Competition is 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 γ_{iv} . For example, we have reported that blood factor XII is only weakly surface active at physiological concentration of 4 mg/100 mL.³¹ Bearing

(47) Adkins, J. N.; Varnum, S. M.; Auberry, K. J.; Moore, R. J.; Angell, N. H.; Smith, R. D.; Springer, D. L.; Pounds, J. G. *Mol. Cell. Proteomics* **2002**, *1*, 947.

(48) Yaminsky, V. V.; Vogler, E. A. *Curr. Opin. Colloid Interface Sci.* **2001**, *6*, 342.

(49) Anderson, N. L.; Anderson, N. G. *Mol. Cell. Proteomics* **2002**, *1*, 845.

this in mind, eq 4 interprets total plasma/serum protein as

$$60 < \sum_i^m W_{B_i} < 85 \text{ mg/mL}$$

or $20 < m < 28$, which is roughly consistent with the 30 classical plasma proteins³⁷ that occupy the first five decades of physiological concentration.⁴⁹ In other words, the effective partition coefficient $P_{\text{eff}} \equiv (P/m) \sim 150/30 = 5$ 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 $\sum W_B \gg W_B^{\text{max}}$. As a consequence concentration-dependent γ_{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 γ_{lv} 's 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. Figure 3 summarizes results of such an approach at two total protein concentrations. Figure 3A shows that limiting γ_{lv} is achieved at 2.1 mg/mL for 100% hIgM or HSA. Figure 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 γ_{lv} observation (1 h). 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 $\mu\text{g/mL}$ total protein solutions regardless of HSA/hIgM relative composition.

It is noteworthy that the lag phase in γ_{lv} dynamics observed in the 100% hIgM case (see annotations in Figure 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 $t < 900$ s. 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. However, the 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, and 51 and citations therein).

It is of further interest that post-lag-phase kinetic data were quite linear on $t^{1/2}$ coordinates for all protein compositions listed in Table 2 (see Figure 3D), suggesting that approach to steady state was dominated by diffusion and followed $(Dt/\pi)^{1/2}$ 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.

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 $P_{\text{eff}} \equiv P/m$, where $P = C_i/C_B$ 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 30 classical plasma proteins occupying the first 5 decades of physiological concentration. Liquid–vapor interfacial tension, γ_{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 γ_{lv} among these specimens cannot be clearly distinguished when scaled by w/v concentration. Furthermore, equilibrium (steady-state) γ_{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.

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(50) Malmsten, M. J. *Colloid Interface Sci.* **1998**, *207*, 186.

(51) Lassen, B.; Malmsten, M. J. *Colloid Interface Sci.* **1996**, *180*, 339.