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

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Pendant-drop tensiometry of aqueous-buffer solutions of purified human proteins spanning nearly 3 orders of magnitude in molecular weight (MW) reveals that reduction in liquid–vapor (LV) interfacial tension γ lv followed a systematic progression in MW with the molar concentration required to reach a specified γ lv value decreasing with increasing MW in a manner reminiscent of the Traube rule for linear hydrocarbon surfactants. Furthermore, the concentration dependence of interfacial tension (∂γ lv/∂ ln C0, where C0 is bulk-solution concentration) is observed to be surprisingly invariant among this disparate group of proteins (i.e., approximately constant apparent Gibbs’ surface excess T = −RT ∂ ln γ lv/∂ ln C0).

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 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.

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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 and 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 and 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 discernible 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 γ
LV of purified human-protein solutions. A significant motivation of this work was to broaden the scope of previous investigations to include proteins with molecular weight (MW) spanning 3 orders of magnitude in search of a pattern missed in similar previous studies. As in these earlier studies, the LV interface was chosen as a molecularly smooth, model hydrophobic surface where interfacial energetics is directly accessible to tensiometric (surface thermodynamic) techniques and adsorbed-protein concentrations ι can be deduced by application of Gibbs’s isotherm. In a manner evocative of the brief history reviewed above, we observe that concentration-dependent γ
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 ι.

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 three-dimensional (3D) interphase with finite volume that accommodates larger protein molecules.

2. Methods and Materials

2.1. 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. Reference 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 (PBS) solution prepared from powder (Sigma Aldrich) in distilled-deionized (18 MΩ) water (the interfacial tension of PBS and water was checked periodically by Wilhelmy balance tensiometry).

2.2. Liquid–Vapor Interfacial Tension Measurements. LV interfacial tensions γ
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. Briefly, the tensiometer employed a Tecan liquid-handling robot to aspirate
between 10 and 12 mL 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 µL pendant drops (a smaller drop volume was 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. The precision of γlv was about 0.5 dyn/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. The 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 γlv values are probably not more accurate than about 1 dyn/cm 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 γlv, that is difficult to achieve on a routine basis. Instead, the veracity of arguments based herein depends 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.

3. Theory

3.1. 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 γlv among the diverse globular proteins studied herein spanning nearly 3 decades of molecular weight MW (see Tables 1 and 2) and (ii) the substantially constant value of the apparent Gibbs’ surface excess Γlv of 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 the 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.20 As a consequence, interphase concentrations Cn of larger proteins are lower than that of smaller proteins at constant P = Cn/Cb. 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.

3.2. Liquid–Vapor Interphase. The LV surface region is modeled as a layer with finite thickness bounded by the bulk-vapor phase on one side and by the 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 subsurface surface region (such as Ward and Tordai)21 but is not necessarily consistent with a strictly “2D” concept in which all adsorbate is constrained to a single interface layer (see refs 3 and 14 and citations therein for more discussion relevant to protein adsorption). More specifically, the interphase is modeled (following Schaff and Dejardin for example)22 as consisting of N slabs with thickness δ, the characteristic size of the protein molecule under study (in cm), such that the total interphase thickness (in cm) Ω = Nδ and volume Vc = Ω = ANδ (in cm³), where A is the interfacial area (cm²) and N is an integer number of slabs that may be fully or partially filled. The interphase solute concentration Cn (in mol/cm³) is related to these variables through eq 1:

\[
C_n = n/v_i = \left( \frac{n_A + n_B}{AN_d} \right) = (\Gamma_{lv}/N\delta) + C_b = \Gamma_{lv}/\delta + C_b/P = \Gamma_{lv}/\delta^{\left( \frac{p}{P - 1} \right)} \approx \Gamma_{lv}/(1).\\
\]

where n is the total number of solute moles (sum of adsorbate nA over and above nB contributed by the bulk phase) that reside within Vc, and Γlv is the Gibbs’ surface excess (mol/cm²). The approximation is specific to the case that adsorption is energetically favorable for which the (dimensionless) partition coefficient P = Cn/Cb >> 1, as generally anticipated for protein adsorption to a hydrophobic interface,20 so that the bulk-phase contribution to

(22) Schaff, P.; Dejardin, P. Colloids Surf. 1987, 24, 239.

<table>
<thead>
<tr>
<th>name of protein (acronym)</th>
<th>γlv (dyn/cm)</th>
<th>γhv (dyn/cm)</th>
<th>In C[lv] (pM)</th>
<th>M (dimensionless)</th>
<th>P[imax] (dyn/cm)</th>
<th>apparent Γ0 (pmol/cm²)</th>
<th>ln C[max] (pM)</th>
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<tr>
<td>ubiquitin (Ub)</td>
<td>72</td>
<td>46</td>
<td>15</td>
<td>14</td>
<td>24</td>
<td>16</td>
<td>18</td>
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<tr>
<td>thrombin (FII)</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>
<td>17.41 ± 0.18</td>
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<td>human serum albumin</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>21.1 ± 1.2</td>
<td>160 ± 21</td>
<td>14.92 ± 0.28</td>
</tr>
<tr>
<td>fatty acid free (Fatty HSA)</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>
<td>16.26 ± 0.39</td>
</tr>
<tr>
<td>prothrombin (FII)</td>
<td>70.63 ± 0.30</td>
<td>53.3 ± 0.47</td>
<td>12.06 ± 0.16</td>
<td>−13.0 ± 2.0</td>
<td>18.33 ± 0.47</td>
<td>167.9 ± 9.4</td>
<td>14.15 ± 0.16</td>
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<tr>
<td>human IgG (IgG)</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>
<td>15.39 ± 0.27</td>
</tr>
<tr>
<td>human IgM (IgM)</td>
<td>70.81 ± 0.37</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>
<td>15.40 ± 0.26</td>
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<tr>
<td>complement component C1q(C1q)</td>
<td>71.13 ± 0.57</td>
<td>52.7 ± 1.6</td>
<td>14.65 ± 0.19</td>
<td>−20.1 ± 0.5</td>
<td>15.1 ± 0.92</td>
<td>177 ± 21</td>
<td>16.26 ± 0.17</td>
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<td>albumin macroglobulin (α mac)</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>17.6 ± 1.5</td>
<td>194 ± 33</td>
<td>13.08 ± 0.34</td>
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</tbody>
</table>

Table 2. Steady-State Protein Parameters

hence the FCC protein concentration
are consequently modeled as spheres with radius
Interphase.
Experimental measurements.
that differs from the apparent
symmetric, then
size that is approximately constant for all globular proteins
expressed in kDa; see ref 20 for a review of the literature
This strategy is similar to that adopted by Ostuni et al. 30
spherical dimensions and molecular packing of proteins).
concentrations are not at all likely either in bulk solution
or within the interphase. However, it seems reasonable
example protein with MW
will be discussed in greater detail below,
3.3. Proteins and Protein Packing within the LV Interphase.
Oblate-spheroid, globular-protein molecules are approximately spherical in aqueous solution 23,24
and are consequently modeled as spheres with radius
with the protein molar volume \( V_p = 4/3\pi r_v^3 N_A \) if \( N_A \) is the
Avogadro number. At interphase saturation, \( C_i = C_{i}^{\text{max}} \) so that
\( \Phi_i^{\text{max}} = 0.74 \rho \), revealing that \( \Phi_i^{\text{max}} \) is equivalent to
FCC packing at \( a = 1 \) (by model construction) and decreases linearly with (fractional) \( \epsilon \). \( \Phi_i^{\text{max}} \) is independent of MW
because the 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 \( \epsilon \) and that interphase capacity for protein is controlled by
the energetics of what amounts to be interphase dehydration. 34

3.4. A Traube-Like Rule for Protein Adsorption.
Equation 2 combines the notion of \( C_{i}^{\text{max}} \) with the MW-dependent
radius of globular proteins, where experimentally convenient dimensions of picomolarity (10^{-12} mol/L)
have been introduced:

\[
C_{i}^{\text{max}} = \epsilon(4 \text{ molecules}) (2\rho/\sqrt{2})^3 = \frac{0.177 \text{ molecules}}{(6.72 \times 10^{-9} \text{ MW}^{1/3} \text{ mol/L})} = \frac{9.68 \times 10^{11} \epsilon}{\text{MW}} \quad (2)
\]

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 \( C_{i}^{\text{max}} \) and must therefore
be equivalent to the bulk concentration \( C_{i}^{B} \) at which the
limiting interfacial tension \( \gamma_{lv} \) is achieved (i.e.,
the concentration at maximum spreading pressure \( \Pi_{lv}^{\text{max}} = \gamma_{lv}^{0} - \gamma_{lv} \)). \( C_{i}^{B} \) can be estimated from concentration-dependent
\( \gamma_{lv} \) curves (see the Appendix, section 6.1) and is related to \( C_{i}^{\text{max}} \) through the partition coefficient \( P = C_{i}^{B} / C_{i}^{\text{max}} \). Equation 3 states this relationship as a logarithmic
expression that is convenient to apply to (steady-state)
consideration of the concentration-dependent \( \gamma_{lv} \) data:

\[
\ln C_{i}^{B} = \ln(C_{i}^{\text{max}}/P) = \ln(9.68 \times 10^{11}) - \ln \text{MW} + \ln(\epsilon/P) = -\ln \text{MW} + [27.6 + \ln(\epsilon/P)] \quad (3)
\]

Assuming that \( \epsilon/P \) is constant for all proteins within this
study (as discussed above for \( \epsilon \) and below for \( P \), eq 3 predicts a linear relationship between \( \ln C_{i}^{B} \) and \( \ln \text{MW} \) with a slope of \(-1\). A value for the unknown ratio \( \epsilon/P \) can be
extracted from the intercept (see Results and Discussion).

Traube's rule for hydrocarbon surfactants stipulates that the concentration required to reduce \( \gamma_{lv} \) to an arbitrary
value \( \gamma_{lv}^{0} \) decreases in a regular progression with each
\(-\text{CH}_{2}-\) unit (i.e., MW) in a homologous series. 2,4,7 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 \( I \) is approximated by the slope of

\[ \frac{\text{d} \gamma_{lv}}{\text{d} \text{MW}} \approx -1\]

This conclusion is in general accord with a relatively new theory of protein adsorption introduced by Rao that concludes adsorption is
the linear-like region of experimental concentration-dependent \( \gamma_{IV} \) curves\(^{35,36} \) (i.e., the “surface excess region” falling between \( \gamma_{IV}^0 \) and \( \gamma_{IV} \) centered at \( \ln C_B^{1/2} \); see Figure 1A annotations):
\[
\delta \gamma_{IV} / \delta \ln C_B = \frac{-RT}{[\ln C_B^{\text{max}} - \ln C_B]} (4)
\]
\[
\gamma_{IV} = \gamma_{IV}^0 + RT[27.6 + \ln(e/P)] - \ln C_B^* - \ln MW
\]
(4a)

where \( C_B^* \) is the concentration required to achieve any arbitrary \( \gamma_{IV}^0 \) within the surface-excess region. Equation 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.\(^{20} \)

### 3.5. Protein Partition Constant

A central assumption of this work is that the partition coefficient \( C \) is approximately constant for all proteins. The Guggenheim interphase construction\(^{37,38} \) can be used to explore this notion more explicitly; for a two-component solution (surface-active solute \( 2 \) and solvent 1), the concentration dependence of the liquid–vapor interfacial tension is given by eq 5:
\[
\delta \gamma_{IV} / \delta \ln x_2 = \left[ n_1 - \frac{x_2}{n_1} \right] \approx \left[ n_2 - \frac{x_2}{n_2} \right] = \frac{-RT}{A} \left( n_1 - n_2 \right)
\]
(5)

where the superscript \( 1 \) differentiates the interphase region from the bulk phase (with no superscript), \( n \) is the number of moles of \( 1 \) or \( 2 \), and \( x \) is the mole fraction. The approximation applies to dilute-solute solutions where \( x_2 = n_2/n_1 \sim n_2/n_1 \) and \( x_1 \sim 1 \). Equation 5 can be written in terms of solvent and solute partition coefficients \( P_1 \equiv C_{1,2}/C_B, P_2 \equiv C_{1,2}/C_{B,2} = (n_2/n_1)/(V/V_1) \) and \( P_2 \equiv C_{1,2}/C_{B,2} = (n_2/n_1)/(V/V_1) \), respectively:
\[
\delta \gamma_{IV} / \delta \ln x_2 = -\frac{RT C_{B,2} V_1}{A} [P_2 - P_1] = -\frac{RT C_{B,2} \Omega P_2}{[P_2 - 1]} = \frac{RT C_{B,2} \Omega}{[P_2 - 1]} (6)
\]

where \( V_1 \) is the interphase volume used as above, \( V \) is the bulk-solution volume, and the identity \( V_1 = A \Omega \) has been applied. The approximation is valid for a dilute-solute solution for which the change in solvent concentration within the interphase due to adsorption is negligible. Equation 6 is valid for any two proteins \( i \) and \( j \) that might have quite different MWs (MW \(_i \approx \) MW \(_j \)) but which we observe experimentally to exhibit similar \( \delta \gamma_{IV} / \delta \ln x_2 \) (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 \( P_1 \) and \( P_j \) in terms of interphase thickness and bulk concentrations:
\[
\begin{align*}
\frac{[P_j - 1]}{[P_i - 1]} &= \frac{[\Omega^{\text{max}} + \gamma_{IV}^{\text{max}} \Omega^{\text{max}}]}{[\Omega^{\text{max}} + \gamma_{IV}^{\text{max}} \Omega^{\text{max}}]} = \frac{[\Gamma / C_{i,1}^{\text{max}} + \gamma_{IV}^{\text{max}} \Gamma / C_{j,1}^{\text{max}}]}{[\Gamma / C_{i,1}^{\text{max}} + \gamma_{IV}^{\text{max}} \Gamma / C_{j,1}^{\text{max}}]} = P_j / P_i
\end{align*}
\]
(7)

where eq 7 makes use of the approximation \( C_i = n_i / V_i \approx \Gamma / \Omega \) that is commensurate with \( P \approx 1 \). Thus eq 7 leads to the conclusion that \( P(P_j - 1) = P(P_i - 1) \), a condition that can be met only if \( P_i = P_j \). Evidently then, a similar \( \delta \gamma_{IV} / \delta \ln C_B \) constant for proteins with widely varying MW, strongly suggesting that any activity-related discrepancy between \( \Gamma \) and \( \gamma_{IV} \) is roughly constant among these proteins. The ratio \( \Gamma / \gamma_{IV} \) measures this discrepancy and can be written in terms of an activity coefficient \( \sigma = \sigma(dC/dx) \) by noting that \( \gamma_{IV} = -aRT \ln(CV/dx) \) and \( \Gamma = -\dot{x}_2 \dot{RT} (dC/dV/dx) \):
\[
\Gamma / \gamma_{IV} = \frac{(x_2 \delta x_2 / dV_2 / dx_2) (dx_2 / dV_2 / dx_2)}{1 / (\sigma(dC/dV_2 / dx_2))} \approx C
\]
(8)

Thus, \( \Gamma / \gamma_{IV} \) = \( c \Gamma \) under the circumstance that the (generally unknown) activity coefficient \( \sigma \) is not a strongly nonlinear function of protein concentration.

### 3.7. Interphase Thickness

The maximal interphase thickness \( \Omega^{\text{max}} \) that occurs when the interphase is saturated with adsorbent can be computed from eq 1 in combination with eq 2, assuming that \( \Gamma \) = \( \gamma_{IV}^{\text{max}} \).
\[
\Omega^{\text{max}} = c \Gamma / C_i^{\text{max}} = \frac{c \Gamma MW}{9.68 \times 10^8 e}
\]
(9)

where units of picomoles and centimeters have been used.

Equation 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 \( N^{\text{max}} = \Omega^{\text{max}} / \Omega^{\text{max}} = 2 / [\gamma_{IV}^{\text{max}}] \).

### 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 LV interfacial tension \( \gamma_{IV} \) of

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Figure 1. Interfacial tension profiles in 3D (γlv as a function of analysis time (drop age) and logarithmic (natural) solution concentration \(C_B\)) and 2D (γlv as a function of logarithmic solution concentration \(C_B\) at selected times) formats comparing Aerosol-OT (panel A), human serum albumin (panel B), and human immunoglobulin M (panel C, preparation 3, Table 1). In each case, solute concentration \(C_B\) is expressed in pmol/L (pM). The symbols in the 2D panels represent time slices through the 3D representations (filled circles = 0.25 s, open circles = 900 s, filled triangles = 1800 s, and open triangles = 3594 s; annotations in panel A indicate maximum and half-maximum spreading pressure). Notice that adsorption kinetics dominates IgM adsorption, requiring 1 h to reach steady state, whereas kinetics dampens within about 2000 s for HSA. Dynamic effects dampen within 900 s for AOT, the small-molecule reference surfactant.
aqueous-buffer solutions of purified human proteins with MW spanning nearly 3 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.

4.1 Experimental Results. Concentration-Dependent LV Interfacial Tension. The principal experimental observations of this work were time- and concentration-dependent LV interfacial tension $\gamma_{lv}$ of aqueous-buffer solutions of purified human proteins with MW spanning 3 orders of magnitude. Interfacial tension measurements were made using a pendant-drop tensiometer as described in Methods and Materials. Figure 1 compares selected results for the anionic surfactant Aerosol-OT (Figure 1A, AOT, MW = 444 Da), human serum albumin (Figure 1B, fatty-acid-free FAF HSA, MW = 663.6 kDa), and immunoglobulin M (Figure 1C, IgM, 1000 kDa) in both three-dimensional ($\gamma_{lv}$, as a function of time and concentration) and two-dimensional ($\gamma_{lv}$ as a function of concentration at specified times) representations, termed "$\gamma_{lv}$ curves" herein. Note that the logarithmic-solute-concentration ordinate $\ln C_{lv}$ in Figure 1 is expressed in picomolarity (pM, 10^{-12} mol solute/L solution). It was observed that these $\gamma_{lv}$ curves were generally sigmoidal in shape, with well-defined low-concentration limits $\gamma_{lv}^0$ and high-concentration asymptotes $\gamma_{lv}^\infty$. Smooth curves through the data of Figure 1 result from least-squares fitting of a four-parameter logistic equation $\gamma_{lv} = (\ln C_{lv}^0 - \gamma_{lv}^0)/(1 + (\ln C_{lv}^0/\ln C_{lv}^* + \gamma_{lv})^2)$ to concentration-dependent $\gamma_{lv}$ data for each time within the observation interval, as described elsewhere.15,35,36 Empirical application of a logistic equation is a purely pragmatic approach aimed at quantifying variable parameters with a measure of statistical confidence. In this way, data fitting recovered $\gamma_{lv}^0$, $\gamma_{lv}^\infty$ and a parameter measuring concentration at half-maximal change in interfacial activity, $\ln C_{lv}^{1/2}$ (where $1/2 = 1/2\mu_{lv}^\infty$ and $\mu_{lv}^\infty = \gamma_{lv}^\infty - \gamma_{lv}^0$), as well as a parameter M that measured the steepness of the sigmoidal curve. Results for HSA and IgM (Figure 1B,C, respectively) were similar to those for AOT in that sigmoidal-shaped $\gamma_{lv}$ curves connected low- and high-concentration asymptotes. Significantly more pronounced time dependence in $\gamma_{lv}$ was observed for proteins, however, especially for intermediate concentrations.

The dynamics was 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 was observed. Observation of time dependence was important in this particular work only insofar as data demonstrate that $\gamma_{lv}$ kinetics dampened within the time frame of experimentation and achieved steady state within the 3600 s observation window. Data collected in Table 2 refer only to steady-state measurements. The bulk-solution concentration at which the limiting interfacial tension $\gamma_{lv}$ occurs ($C_{lv}^{\infty}$) is of theoretical interest in this work and was estimated from fitted parameters compiled in Table 2 as described in the Appendix, section 6.1. $C_{lv}^{\infty}$ 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 $\gamma_{lv}$.

Figure 2 compiles smoothed, steady-state $\gamma_{lv}$ curves for proteins selected from Table 2 spanning 3 decades in MW (data are not shown in Figure 2 for the sake of clarity; see Figure 1 for similar plots including authentic data and Table 2 for statistics of fit). The Traube-rule-like progression in Figure 2 was not at all obvious on a w/v basis (not shown) because data are compressed into a single band.15 However scaled, the similarity of $\gamma_{lv}$ curves for such a diverse group of proteins is rather striking ($\mu_{lv}^\infty < 30$ dyn/cm with $\gamma_{lv}$ lying within ±10 dyn/cm) and corroborates results of previous studies of proteins covering a narrower range of MW.19 Bearing in mind that the $\mu_{lv}^\infty$ range for synthetic surfactants can exceed 60 dyn/cm, with 25 < $\mu_{lv}^\infty$ < 50 dyn/cm typical of ordinary hydrocarbon soaps and surfactants,42,43 it is apparent from the data of Table 2 and Figures 1 and 2 that aqueous-protein solutions are relatively weak surfactant systems with $\mu_{lv}^\infty$ only slightly overlapping with the weakest of surfactant systems. In view of the substantial structural diversity of the human plasma proteome sampled by the proteins of Table 1,31 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 values for the 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 $\gamma_{lv}$ curve (i.e., the surface excess region between $\gamma_{lv}^0$ and $\gamma_{lv}^\infty$) centered at $\ln C_{lv}^{1/2}$; see Figure 1) corresponding to steady state, as described previously.35,36 The term “apparent” alerts the reader to the fact that casual application of the adsorption isotherm $\Gamma = (-1/RT)\ln C_{lv}^*/\ln C_{lv}$ treats solutes (proteins and surfactants) as isomerically pure, nonionized polyelectrolytes at infinite dilution with unit

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activity coefficients. In particular, this treatment does not explicitly take into account counterions which, for a 1:1 ionic surfactant such as AOT dissolved in pure water, means that the actual surface excess \( \Gamma_{\text{sw}} = 1/2 \Gamma_{\text{LV}} \). Thus, \( \Gamma = 460 \pm 31 \, \text{pmol/cm}^2 \) derived from data of Figure 1A was found to be in good agreement with results from drop-volume tensiometry (\( \Gamma_{\text{LV}} = 221 \, \text{pmol/cm}^2 \)) and predicts an adsorbed footprint \( \Gamma_{\text{LV}}/\Gamma_{\text{sw}} = 70 \, \text{Å}^2/\text{mol} \) in reasonable agreement with neutron reflectometry (NR) measurements. No counterion 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 \( \Gamma_{\text{LV}} \), as discussed in the theory section, bearing in mind, of course, the aforementioned assumptions of purity and applicability of the infinite-dilution approximation.

Figure 3 plots apparent \( \Gamma \) values collected in Table 2 against protein MW, with error bars representing intraexperiment uncertainty calculated by propagation of fitted-parameter errors, as described previously. Interexperiment error suggested by replicate protein preparations (sometimes different lots obtained from different vendors, see Table 1) appeared not to be significantly larger than intraexperiment uncertainty. Taken as a whole, these data support the contention that the concentration dependence of interfacial tension \( \gamma_{\text{LV}} \) is relatively constant among proteins with MW spanning nearly 3 orders of magnitude (10–1000 kDa). Again, in view of the substantial structural diversity of the human plasma proteome sampled by the proteins of Table 1, it seems reasonable to conclude that variability in protein structure does not confer widely varying LV interfacial activity.

4.2. Theoretical Interpretation of Results. Secure interpretation of concentration-dependent interfacial ten-

sions in terms of adsorbed concentrations and interphase thicknesses is critically dependent on the 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 Refs 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 and thus comparing proteins derived from different tissues (e.g., blood, ocular, mammary) and sometimes from different species (e.g., bovine vs 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 NR of FAF HSA at the LV interface reported by Lu et al., 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 Interface. 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. This finding is somewhat consistent with a protein-sphere radius \( r_{\text{v}} = 2.7 \, \text{nm} \) calculated from molecular-packing density calculations discussed in the theory section or, as described in the cited NR literature, a \( 14 \, \text{nm} \) ellipsoidal molecule adsorbed with the major axis oriented parallel to the interface. The adsorbate mass/unit area deduced from NR was \( 2.1 \pm 0.3 \, \text{mg/m}^2 \) or 3.2 pmol/cm\(^2\) and was construed to be equivalent to the Gibbs' surface excess \( \Gamma_{\text{LV}} \). Interpreted in terms of eq 9 of the sphere-packing model, this implies that \( \Omega_{\text{max}} = 4.8 \times 10^{-7} \, \text{cm} \) and that \( C_{\text{LV}}^{\text{max}} = 7 \times 10^{-9} \, \text{mol/cm}^2 \) or \( \Gamma_{\text{LV}}^{\text{max}} = 22.6 \, \text{mN/m} \) for comparison with the scaling of Figures 1, 2, and 5. With \( \Gamma_{\text{LV}}^{\text{max}} = 1.5 \times 10^{-10} \, \text{mN/m} \) for HSA (in \( \Gamma_{\text{CC}} = 23.4 \)), this implies that \( \chi = 0.45 \) or that the saturation interfacial concentration is approximately half of the hypothetical face-centered-cubic arrangement of hard spheres with radius \( r_{\text{v}} \), or \( \Phi_{\text{v}}^{\text{max}} = 0.74 _{\text{e}} \sim 1/3 \). The effective-packing radius \( R \) is thus 30% larger than \( r_{\text{v}} \) with \( \chi = 1.3 \) (as calculated from \( \delta = 2R = 2r_{\text{v}}^{\text{x}} = 1.3 \, \text{nm} \)), which is consistent with a 3.6 nm hydrodynamic radius obtained by dynamic light scattering of albumin solutions. This inferred value of \( \chi \) might also be compared to the so-called "jamming limit" of 0.55 at which adsorbing disks saturate a surface without overlap and to reports of adsorbed-protein densities exceeding this limit. It is of further interest that \( C_{\text{LV}}^{\text{max}} \) exceeds the estimated solubility limit for HSA

(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 (\( \Gamma = 337.3 \pm 28.2 \, \text{pmol/cm}^2 \) at steady state taken near \( 1 \, \text{h} \) drop age).
(49) Note that the 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.
Figure 4. Relationship between the surface-saturating bulk-solution concentration $C_0^{\text{max}}$ and protein MW (open circles = preparation 1, filled circles = preparation 2, filled triangles = preparation 3). Linear regression through the data yielded a slope of $-1.4 \pm 0.2$ consistent with the expectation of unit slope and an intercept of $21.8 \pm 1.3$ ($R^2 = 71.6\%$). Notice that low-MW proteins require greater bulk-phase concentrations to saturate the interphase than higher-MW proteins.

A Traube-rule-like dependence of concentration-dependent $\gamma_i$ for proteins selected from Table 1 spanning 3 decades in MW (reading from left to right on the 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 the Appendix, section 6.3, for details). The Traube rule effect is especially evident as viewed along the $\ln C_0$ axis where monotonically increasing concentrations of lower-MW proteins are required to reach a universal limiting interfacial tension $\gamma_i^{\text{lv}}$.

Higher-MW proteins. Given that the $C_0^{\text{max}}$ values plotted in Figure 4 approach $10\%$ w/v, it is reasonable to anticipate that extrapolated $C_0^{\text{max}}$ values for yet-lower-MW proteins must equal or exceed protein-solubility limits. As a consequence, surface saturation and the related limiting interfacial tension $\gamma_i^{\text{lv}}$ are not expected for low-MW proteins at fixed P. In this regard, it is noteworthy that $\gamma_i$ 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 Figure 4 and further below).

Surface Excess and Interphase Thickness. Again using NR of HSA as a single-point calibration by taking $\Gamma_{\text{nr}} = 3.2$ pmol/$\text{cm}^2$, the factor relating actual and apparent surface excesses $c = \Gamma / \Gamma_{\text{nr}} = 179/3.2 = 56$. Equation 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 Figure 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.

5. Conclusions

The two principal experimental observations of this work were that (i) reduction in 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 \(\delta\gamma_{lv}/\delta \ln C\) was relatively constant among proteins with MW spanning nearly 3 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 protein size (MW). When calibrated to previously reported neutron reflectometry 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 to the hydrophobic LV interface, simultaneously reducing interfacial energetics and solution concentration of a relatively hydrophobic solute for which water is an ambivalent solvent. Amphilicity is the word coined by Hartley in 1936 to express this ambivalent solvency from the perspective of the solute molecules (originally amphiaphily). Apparently in the case of proteins, molar variability in \(\gamma_{lv}\) is achieved by aggregating greater mass of similar amphiaphilic character (blocks of amino acids), as opposed to accumulating greater amphilicity with MW. The extent to which this amphillicity 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 \(\phi_p^{\text{max}} = 1/3\). 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 seem 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, bearing in mind that hydrophobic hydration is a, perhaps the, key phenomenon controlling adsorption.

### Glossary of Symbols

- \(a\): protein activity
- \(A\): area of interphase (cm²)
- \(c\): proportionality constant, \(c \equiv \Gamma \gamma_{lv}\)
- \(C_B\): bulk-solution concentration (moles/volume)
- \(C_B^{\max}\): bulk-solution concentration at limiting interfacial tension (moles/volume)
- \(C_B^{1/2}\): bulk-solution concentration at half-maximal change in interfacial activity (moles/volume)
- \(C_B^f\): bulk-solution concentration at arbitrary interfacial tension \(\gamma_{lv}^f\) (moles/volume)
- \(C_{FCC}\): face-centered-cubic close-packing concentration (moles/volume)
- \(C_i\): interphase concentration (moles/volume)
- \(C_i^{\max}\): maximal interphase concentration (moles/volume)
- \(C_{SL}\): solubility limit concentration (moles/volume)
- \(\chi\): proportionality constant, \(\chi \equiv R\gamma_{lv}\)
- \(\delta\): interphase thickness element (cm)
- \(\epsilon\): packing efficiency
- \(\phi_p\): volume fraction of protein in the interphase
- \(\phi_p^{\max}\): maximum volume fraction of protein in the interphase
- \(\gamma_{lv}^0\): liquid–vapor interfacial tension (dyn/cm)
- \(\gamma_{lv}^l\): low-concentration asymptote of a concentration-dependent \(\gamma_{lv}\) curve (dyn/cm)
- \(\gamma_{lv}^{\text{high}}\): high-concentration asymptote of a concentration-dependent \(\gamma_{lv}\) curve (dyn/cm)
- \(\Gamma^v\): arbitrary interfacial tension (dyn/cm)
- \(\Gamma^v\): apparent Gibbs' surface excess (moles/area), \(\Gamma = \gamma_{lv}^{\text{high}}\)
- \(M\): parameter fitted to concentration-dependent \(\gamma_{lv}\) curve

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where the identity \( \Pi^{\text{max}} \equiv \gamma_{lv}^0 - \gamma_{lv}^0 \) has been used. All of the parameters on the right-hand side of eq A1 are derived from nonlinear, least-squares fitting of concentration-dependent \( \gamma_{lv} \) to a four-parameter logistic equation as described in Methods and Materials. Confidence in the \( C_B^{\text{max}} \) values listed in Table 2 and plotted in Figure 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%.

\[
\sigma_{C_B^{\text{max}}}^2 = \frac{1}{4} \left( \frac{\sigma_{\gamma_{lv}^0}}{\gamma_{lv}^0} - \sigma_{\gamma_{lv}}^2 \right) \left( \frac{\sigma_{\gamma_{lv}^0}}{\gamma_{lv}^0} - \sigma_{\gamma_{lv}}^2 \Gamma^2 \right) \sigma_\Gamma^2 \quad (A2)
\]

where the \( \sigma \)'s represent standard errors in \( \ln C_B^{\text{max}} \) and the best-fit parameters \( \ln C_B^{\text{lv}}, \gamma_{lv}^0, \gamma_{lv}^0, \) and \( \Gamma \).

6.2. Estimation of Parameters for Ubiquitin. The parameters for ubiquitin (Ub) listed in Table 1 and shown in Figures 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 \( \gamma_{lv}^0 \) is not achieved at physically realizable concentrations. However, the highest-concentration \( \gamma_{lv} \sim 46 \text{ dyn/cm} \) 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 \( \gamma_{lv} = 46 \text{ dyn/cm} \) is not too far from the hypothetical \( \gamma_{lv}^0 \) that would be achieved if Ub was sufficiently soluble to fill the interphase. Thus, we estimate that \( \Pi^{\text{max}} \sim 13 \text{ dyn/cm} \) would occur at \( C_B^{\text{lv}} = 15 \text{ pM} \). The low-concentration asymptote is clearly defined at \( \gamma_{lv}^0 = 72 \text{ dyn/cm}, \) 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 section 6.1.

6.3. Steady-State \( \gamma_{lv} \) as a Function of MW and Concentration. The smooth mesh through the summary graphic Figure 5 was computed from eq 4a of the theory section assuming that the nominal values \( \gamma_{lv}^0 = 72, \gamma_{lv}^0 = 45, \) and \( \Gamma = 179 \) approximate all proteins of this study. Equation 4a permits calculation on any interfacial tension within the linear-like Gibbs’ excess region (i.e., the surface excess region between \( \gamma_{lv}^0 \) and \( \gamma_{lv}^0 \) centered at \( C_B^{\text{lv}} \); see Figures 1A and 3), above and below which calculated \( \gamma_{lv} \)'s are truncated to \( \gamma_{lv}^0 \) and \( \gamma_{lv}^0 \) respectively.

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