Contact activation of the plasma coagulation cascade. II. Protein adsorption to procoagulant surfaces

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A study of blood protein adsorption to procoagulant surfaces utilizing a coagulation time assay, contact angles, Wilhelmy balance tensiometry, and electron spectroscopy (ESCA) is presented. Using a new contact angle method of measuring protein adsorption termed “adsorption mapping” it was demonstrated that protein-adsorbed surfaces were inefficient activators of the intrinsic pathway of the plasma coagulation cascade whereas water-wettable, protein-repellent surfaces were efficient procoagulants. Repeated use of fully water-wettable (spreading) glass procoagulants in the coagulation time assay demonstrated that putative “activating sites” were not consumed in the coagulation of platelet-poor porcine plasma. Furthermore, these procoagulant surfaces retained water-wettable surface properties after incubation with blood proteins and saline rinse. The interpretation of these observations was that plasma and serum proteins were not adsorbed to water-wettable surfaces. However, ESCA of these same surfaces revealed the presence of a thin protein layer. Wilhelmy balance tensiometry resolved these seemingly divergent observations by demonstrating that protein was “associated” with a bound hydration layer, but not formally adsorbed through a surface dehydration or ionic interaction mechanism. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

Adsorption is one of the most important manifestations of surface and interfacial energetics. Protein adsorption has been the subject of intense research in biomaterials surface science for more than two decades because adsorbed protein, or lack thereof, effectively mediates the biological response to materials. Biomaterials may be broadly categorized as fully water wettable (contact angle = 0°, spreading) or not water wettable (contact angle > 0°) and, in this very general view, protein adsorption likewise may be categorized. Adsorption to poorly water-wettable materials from aqueous solution is reasonably well understood in terms of a surface dehydration phenomenon1-5 in which protein displaces water at an interface through a multiplicity of mechanisms. Reports of protein adsorption to nonwettable materials are quite uniform among and between different investigators with little controversy,6,7 except perhaps in the details associated with measurement protocols or data interpretation.7-12 Adsorption to water-wettable surfaces is apparently not so well understood however. In this case, water is so tightly surface bound that displacement by protein is energetically prohibitive.13,14 And yet different investigators have determined that wettable surfaces such as hydrogels support protein adsorption.15-20 Other investigators report that hydrogels are protein repellent.21-26 Scientific literature on the general subject of protein adsorption to ionic (nonhydrogel) surfaces also is somewhat controversial, again with some investigators reporting protein repellency27,28 and others confirming protein adsorption,29-31 although adsorption to water-wettable surfaces seems to be consistently lower than to poorly wettable surfaces.32-38

We have quantified the propensity of surfaces to contact activate the plasma coagulation cascade and related this quantity to procoagulant surface chemistry and energy. As described in the first of this two-part series on plasma coagulation, activation of the intrinsic pathway of blood plasma coagulation was shown to be a steep exponential-like function of procoagulant surface energy, with low activation observed for poorly wettable surfaces and very high activation for fully water-wettable surfaces.

Biochemical investigations of the intrinsic pathway of plasma coagulation implicate the interaction of four proteins involved in contact activation: the zy-
mogens Factor XII, prekallikrein, Factor XI, and the cofactor high-molecular-weight kininogen (see references 39 and citations therein). Surface contact of Factor XII leads to conversion to the active protease form XIIa. Kininogen is thought to transport Factor XI to surface-bound XIIa where Factor XIa is produced. Amplification of the surface-contact event occurs through prekallikrein conversion to kallikrein by XIIa, which itself rapidly converts XII to XIIa. This elegant, self-amplifying chain reaction is thus initiated by adsorption or binding of Factor XII onto a procoagulant surface, which, according to our method of quantifying procoagulant activity, is most efficient for fully water-wettable procoagulants. Therefore, toward a more complete understanding of the surface and interfacial chemistry aspects of contact activation of plasma coagulation, it is imperative to understand behavior of plasma and serum proteins at water-wettable surfaces.

We demonstrated herein that activating sites on clean, water-wettable glass procoagulant surfaces are not consumed or “poisoned” by irreversible or slowly reversible protein adsorption during plasma coagulation. We disclose strong evidence derived from newly developed contact angle methods that plasma and serum proteins are not formally adsorbed to these wettable glass surfaces through the previously mentioned surface dehydration mechanism, but instead are in some way “associated” with a bound interfacial hydration layer. The exact nature of this bound interfacial layer is not fully understood through this work and is an area for future research.

MATERIALS AND METHODS

Preparation of platelet-poor porcine plasma (P4) and coagulation time assay

Whole citrated porcine blood was used as received under refrigeration (Environmental Diagnostics Inc., Burlington, NC). Preparation of platelet-poor plasma and use in coagulation time assays was described in detail in the first of this two-part series.4 EDTA was used to anticoagulate P4 used in adsorption map measurements and was prepared from citrated P4 by dilution with Dulbecco’s phosphate buffered saline (DPBS, Gibco) containing 2% EDTA. Additional anticoagulation was required to prevent clotting of P4 in contact with highly wettable surfaces.

Tensiometry and ESCA

Contact angle goniometry and preparation of glass coverslip test substrata was performed as described previously.14,27 Wilhelmy balance tensiometry techniques have been delineated elsewhere.41,42 Likewise, the adsorption mapping method of correlating protein adsorption with surface energetics previously has been described in detail.5,27 Plasma solutions in DPBS used for interfacial tension measurements (Fig. 2) and adsorption mapping (Fig. 3) were prepared approximately 1 h in advance of measurements. Wilhelmy balance measurements were carried out with 15–30 min solution aging. Contact angle measurements were made after a 15–30 min equilibration of a 10 µL droplet on test surfaces. Equilibration was carried out with test surfaces held in the horizontal mode and readings were made when angles were observed to be stable with time. The stage then slowly was tilted to 30–35° for advancing/receding angle (θ) readings. Although interfacial tension and contact angle measurements did not exhibit detectable time dependence after the above-stated equilibration times, further tests for equilibrium adsorption were not carried out. Small changes in γ or θ due to long-term adsorption effects would not substantially alter conclusions drawn from interfacial curves or the adsorption map. In any event, tensiometric measurements were made within a time frame relevant to the 2–20 min coagulation times observed for the P4 system in contact with various procoagulants. Electron Spectroscopy for Chemical Analysis (ESCA) was performed on a Surface Science SSX-100 spectrometer using standard operating conditions with electron gun charge neutralization and binding energies corrected to C(1s) = 285.0 eV.

RESULTS AND DISCUSSION

Results and discussion are combined into one section because some of the methods applied herein have been developed only recently and require brief introduction before data obtained can be fully appreciated. The work is organized in the following order. First, it will be shown by both indirect and direct methods that activating sites on clean, water-wettable glass disc procoagulant surfaces were not consumed in plasma coagulation. Second, application of a new contact angle method of monitoring protein adsorption to surfaces of varying water wettability (surface energy) will be discussed. This so-called “adsorption mapping” method demonstrates that plasma and serum proteins do not adsorb to modestly wettable surfaces under hydrated-state conditions in such a way that measurably affects solid–liquid energetics. The third part demonstrates, using conventional contact-angle goniometry, that fully water-wettable glass surfaces retain these wettable characteristics after incubation with plasma and serum. ESCA was applied to
reveal an internally inconsistent view of adsorption. Fourth, and finally, a robotic Wilhelmy balance that repetitively measures wetting forces extends the above contact angle goniometer observations and resolves the inconsistency by providing evidence that proteins are associated with a hydration layer bound to water-wettable surfaces. All of these results will be drawn together in the conclusion section.

The catalytic nature of coagulation activation

A rudimentary question that arises from the procoagulant surface perspective asks if activating sites increase in simple proportion to surface area and if these sites are consumed in the coagulation reaction. Herein the term “activating site” is used in a generic sense in reference to the localization of coagulation activity on a procoagulant surface. According to the biochemical description of contact activation, the activating site is surface-bound Factor XIIa. As alternative interpretations, activating sites might be thought of as the surface locations where XIIa binds or simply as points of chemical catalysis. The detailed distinction between these or other paradigms that might be offered for activating sites is very important in terms of a formal biochemical mechanism of coagulation activation, but is relatively unimportant in answering the above-stated rudimentary question.

The first article in this two-part series developed and applied a mathematical model of plasma coagulation that treated contact activation as simple chemical catalysis. This model was constructed on the premise that the number of activating sites on procoagulant surfaces increased in direct proportion to surface area and that these sites were neither permanently nor temporarily consumed during plasma coagulation. It was found that the closed-form mathematical expression derived from this model adequately fit experimental coagulation time data for procoagulants spanning a full range of surface energy (water wettability), including water-wettable glass surfaces. Maximum activation was observed for fully water-wettable surfaces and minimum activation for nonwettable surfaces. The indirect evidence derived from modeling is that activating sites, however interpreted mechanistically, are discrete surface locations that increase in surface density with procoagulant surface energy and are not consumed by contact activation of plasma coagulation.

Figure 1 collects coagulation times of porcine platelet poor plasma (P4) observed with varying surface areas of clean, water-wettable glass disc procoagulants. Filled symbols correspond to two different trials with glass discs used immediately after cleaning. The smooth curve through the data is the best fit of the above-described coagulation model to this first-use data. Open symbols correspond to data obtained for two corresponding trials in which first-use discs were recovered from coagulated plasma, rinsed in saline, and used in a second coagulation trial. Surface area was calculated from glass discs' dimensions (5 x 10^-3 cm diameter) and expressed as a ratio to the constant volume of P4 (0.5 mL) used in each coagulation time measurement. The smooth curve drawn through the data was obtained by least-squares fit of a coagulation time model to the first-use data (see text).

Observations at hand are an insufficient basis on

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Figure 1. Coagulation time curve for platelet poor porcine plasma (P4) with water-wettable glass-disc procoagulants. Closed symbols correspond to two separate trials with glass discs used immediately after cleaning. Open circles correspond to data obtained for corresponding trials in which these first-use discs were recovered from coagulated plasma, rinsed in saline, and used in a second coagulation trial. Surface area was calculated from glass discs' dimensions (5 x 10^-3 cm diameter) and expressed as a ratio to the constant volume of P4 (0.5 mL) used in each coagulation time measurement. The smooth curve drawn through the data was obtained by least-squares fit of a coagulation time model to the first-use data (see text).

Figure 2. Liquid–vapor interfacial tension \( \gamma \) curve for EDTA-stabilized porcine plasma as a function of dilution in saline. Note that plasma exhibits the biosurfactant property of adsorbing to the hydrophobic liquid–vapor interface, reducing \( \gamma \) from 73 dyne/cm characteristic of pure saline to a lower limit near 60 dyne/cm.
which to advance a detailed molecular description of the physicochemical events that transpire at a water-wettible procoagulant surface during contact activation of the plasma coagulation cascade. Surface thermodynamics seldom can be interpreted in molecular terms without ancillary information. However, it is of interest to speculate how the descriptive biochemistry of plasma coagulation links to the above surface chemistry findings. One possibility is that adsorption and generation of Factor Xlla is instantaneous relative to the time frame of plasma coagulation so that the number of surface-bound Xlla molecules is proportional to the number of adsorption sites on a procoagulant surface, which, in turn, is proportional to procoagulant surface area. According to this scenario, adsorbed Xlla must rapidly convert XI to Xla in a manner that does not temporarily or permanently consume protease activity, so that the number of surface-bound Xlla molecules appears constant over the time course of coagulation. Furthermore, immediate reuse of this surface-bound Xlla in a second coagula-

tion reaction must not alter overall observed coagulation activation. Another possibility is that Factor XII association with the surface and production of Xlla is transient in nature and surface sites where XII → Xlla are quickly recycled and thereby conserved in the over all coagulation process. However constructed, the biophysical events at the solid–liquid interface must accommodate constant procoagulant surface activity throughout the time course of coagulation in a manner that increases in proportion to procoagulant surface area.

Adsorption mapping of blood plasma

Biomaterial surface scientists have applied the traditional tools of surface physical chemistry to probe biocompatibility. Among these surface-science tools are those falling under the general umbrella of tensiometry (see reference 5 and citations therein), the most popular and well known of which probably is contact angle goniometry. Contact angle techniques offer a number of distinct advantages to biomaterials research that are unique to tensiometry. First, contact angles are sensitive to only the outermost atomic layers responsible for the surface energetics that drive adsorption. Second, contact angle measurements can be interpreted directly in terms of the surface energetics of the adsorbed layer that mediates many important secondary biological outcomes, such as cell/tissue adhesion or the triggering of different biochemical cascades controlling blood clotting and immune responses. Third, contact angles provide access to equilibrium or steady-state adsorption since angle measurements do not perturb adsorption dynamics. This is of particular value to biomaterial scientists studying protein adsorption because biological macromolecules are easily denatured and the hydrated, adsorbed state is most relevant to the biological environment encountered in end-use applications. A related factor is that radioisotope or chromophore tagging is not required, thus eliminating a potential source of artifacts.

One difficulty encountered in practical application of contact angles in the measurement of adsorption, however, lies in the complexity of interpretation of results. Another particularly relevant problem to biomaterial investigators dealing with chemically undefined biological milieu, such as blood, plasma or serum, is that quantifying adsorption through contact angles using conventional surface thermodynamic theories is not generally possible because there are no extensive concentration factors, such as mole fraction or molarity, readily available to scale interfacial activity.

A new method of correlating surface energetics
with protein adsorption, known as adsorption mapping, recently has been proposed that takes advantage of the above-stated positive attributes of contact angles and circumvents many, if not all, of the interpretive difficulties, particularly for complex protein mixtures of unknown composition. Adsorption map theory has been discussed in detail elsewhere, and only a few salient features need to be reiterated here so that the reader readily can interpret the adsorption map of porcine plasma and appreciate the revealed relationship among protein adsorption, surface energy, and plasma coagulation.

An adsorption map is a graphical construction formed by plotting the domain of an adsorption index \( [\tau' - \tau^*] \) against the range of surface wettability in pure water (or buffered saline as appropriate herein) \( \tau^* \). Here, wettability is expressed as adhesion tension \( \tau = \gamma \cos \theta \), where \( \gamma \) is the liquid–vapor interfacial tension and \( \theta \) is the contact angle of that liquid on the surface under study. The adsorption index \( [\tau' - \tau^*] \) compares the adhesion tension \( \tau' \) measured with a protein mixture (having characteristic interfacial tension \( \gamma' \)) to the adhesion tension \( \tau^* \) obtained with pure saline (having characteristic interfacial tension \( \gamma^* = 72.8 \) dyne/cm). The adsorption index is sensitive to adsorption because adsorbed protein causes measurable changes in \( \theta \), \( \gamma \), and consequently \( [\tau' - \tau^*] \). According to theory, surfaces that support adsorption have \( [\tau' - \tau^*] > 0 \) whereas surfaces that do not support adsorption are characterized by \( [\tau' - \tau^*] \leq 0 \). Boundaries of the map are derived from surface thermodynamics (the Young equation) and enclose all observable data for a particular protein system in what is termed an adsorption triangle (see Fig. 3(A) for example). Area of an adsorption triangle falling on the map where \( [\tau' - \tau^*] > 0 \) corresponds to surfaces supporting adsorption whereas area of the adsorption triangle falling below \( [\tau' - \tau^*] = 0 \) correspond to protein repellent surfaces. Thus the adsorption map is a new tool in biomaterials surface science that allows straightforward characterization of the wetting behavior of heterogeneous biological fluids.

Figure 2 plots observed liquid–vapor interfacial tension \( \gamma \) of porcine plasma at different dilutions in saline. As observed with other protein and peptides, porcine plasma exhibits the biosurfactant property \( \gamma' \) of reducing interfacial tension from \( \gamma^* = 73 \) dyne/cm characteristic of pure saline to an asymptotic lower limit. In the porcine plasma case, the asymptotic limit \( \gamma' = 59.7 \) dyne/cm is typical of serum albumin solutions (see annotations on Fig. 2 and Table I). Interfacial tension decreases with concentration because protein adsorbs to the hydrophobic vapor interface and the lower limit results from surface saturation. Boundaries of the porcine plasma adsorption map discussed below are derived from this data.

Adsorption occurs to solid surfaces as well. In this case, the adsorption map is a convenient means of relating adsorption to surface energy. Figure 3(A) is an adsorption map for EDTA-anticoagulated P\(^4\) (see Materials and Methods) constructed from the data summarized in Table I, which includes identity of test substratum. Note that \( [\tau' - \tau^*] \) data corresponding to advancing and receding contact angles fall along a monotonic trend line. This monotonic behavior is found to be characteristic of all surfactants and proteins studied, including human and porcine serum. Consequently, only a few measurements on surfaces with different \( \tau^* \) are required to completely characterize the biosurfactant system. Interpolation of the trend line to \( [\tau' - \tau^*] = 0 \) leads to the conclusion that plasma proteins do not absorb to surfaces more wettable than \( \tau^* = 37 \) dyne/cm \( (\theta_{adv} = 59^\circ) \). Contact angle measurements employed in adsorption mapping

### Table I

<table>
<thead>
<tr>
<th>Sample Description*</th>
<th>Advancing Mode</th>
<th>Receding Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \theta^\circ ) (degrees)</td>
<td>( \tau^* ) (dyne/cm)</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>0</td>
<td>73.0</td>
</tr>
<tr>
<td>O(_2) plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polystyrene</td>
<td>40</td>
<td>55.9</td>
</tr>
<tr>
<td>( \gamma )-irradiated</td>
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<td></td>
</tr>
<tr>
<td>in air</td>
<td>67.5</td>
<td>27.9</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>90</td>
<td>0.0</td>
</tr>
<tr>
<td>as received</td>
<td>90</td>
<td>0.0</td>
</tr>
<tr>
<td>OTS treated</td>
<td>103</td>
<td>-16.4</td>
</tr>
</tbody>
</table>

*All contact angles are single observations and corresponding adhesion tensions are nominal values calculated from \( \tau = \gamma \cos \theta \) where \( \gamma = 73.0 \pm 0.2 \) and \( \gamma' = 59.7 \pm 1.0 \) dyne/cm (see Fig. 2). Variation in contact angles on the same specimen is observed to be about \( \pm 2^\circ \).

\*\text{\( \gamma \)}-Irradiation for different time intervals results in varying levels of surface oxidation. OTS = octadecyltrichlorosilane.
of P4 positively detect plasma protein adsorption on surfaces less wettable than this ($\tau^* < 37$ dyne/cm). It is important to reiterate here that contact angle measurements represented in the adsorption map are biomedically relevant because of the aforementioned favorable characteristics of tensiometry. As such, the adsorption map is a unique view of protein adsorption from chemically undefined biological milieu.

Figure 3(B) reproduces from the first paper of this series an observed relationship between the propensity of a procoagulant surface to contact activate plasma coagulation (K value in m$^{-2}$, data corresponds to a system of oxidized polystyrene procoagulants) and saline wettability expressed as $\tau^*$. Note the correspondence between the [$$] = 0$$ point on Figure 3(A) and where K just begins a transition from a relatively flat response to surface energy to an exponential-like increase with surface energy in Figure 3(B). The interpretation is that protein-adsorbent surfaces do not efficiently activate the plasma coagulation cascade whereas protein repellent surfaces readily activate the plasma coagulation cascade. To our knowledge, Figure 3 is the first quantitative illustration of a relationship among surface energy, protein adsorption, and the response of a fully potenti-ated biological system.

Procoagulant wettability

Contact angle goniometry and electron spectroscopy (ESCA)

Figure 4 diagrams an experiment in which clean, water-wettable glass plates ($\tau^* = 72.8$ dyne/cm, $\theta^* = 0^\circ$) first were incubated for 15 min at room temperature in polystyrene culture dishes containing (a) saline, (b) citrated P4, (c) porcine serum, and (d) recalci-fied P4. Following incubation, plates were removed from the culture dishes, rinsed by 3x sequential immersion in fresh saline, edge blotted to remove excess saline rinse, and mounted in the humidified chamber of a contact angle goniometer. In all cases, including the recalified P4 which coagulated into a gel, glass slides could be recovered from the protein mixtures with no visually apparent protein film. By contrast, a protein film was clearly evident on a glass plate bearing a hydrophobic ($\theta_{adv} = 106^\circ$, $\theta_{rec} = 90^\circ$) octade-cyltrichlorosilane surface chemistry when removed from coagulated P4. This adherent film did not rinse away with saline rinses (not shown in Fig. 4). Figure 4 annotations show that there was no observable contact angle ($\theta^* = 0^\circ$) due to complete spreading of saline on all surfaces both before and after incubation in saline and proteinaceous fluids. These results corroborate findings from the adsorption map by demon-strating that water-wettable glass surfaces do not adsorb proteins from serum or plasma in such a way that observed surface wettability is changed.

At this juncture it is of interest to comment on the sensitivity of contact angles to surface adsorption. It is a common laboratory observation that water-wettable surfaces are very easily contaminated due to adsorption and that the fully water-wettable condition is a transient state under normal, ambient labor-atory conditions. Indeed, rather rigorous cleaning procedures and application of flames or oxygen plasmas generally are required to render a surface completely wettable. Thus it is the expectation that protein adsorption from aqueous solution to a water-wettable surface, however slight, would yield an observable contact angle because surface-bound protein (or any other organic molecule) is poorly water-wettable relative to a highly oxidized glass or polymer surface. Adsorption of Factor XII has been ascribed to ionic interactions between a negatively charged surface and positively charged moieties on the protein, presumably through a histidine-rich region.44,45 Divalent cations or phospholipids do not appear to play a role in Factor XII adsorption.44 A similar mechanism is involved in the adsorption of cationic surfactants onto clean glass. As an example, it has been shown that adsorption of cetyldimethylammonium bromide (cetyl bromide) from water can be detected by contact angle goniometry from as little as $1 \times 10^{-7}%$ W/V solutions.44 Cetyl bromide adsorption
causes contact angles to increase (dewetting or autophobic effect) because the surfactant adsorbs in a head-down configuration due to charge interactions between the surfactant and surface. This adsorbed conformation extends hydrophobic residues into solution causing the surface to become less wettable (see reference 27 for cetyl bromide autophobic behavior in an adsorption map). We see no evidence of this kind of behavior of blood proteins at water-wettable surfaces, neither through the experiments outlined in Figure 4 nor in the adsorption map. Thus, all taken together, tensiometric measurements provide no evidence for adsorption of plasma proteins from water solution to water-wettable surfaces. This is consistent with the theoretical expectations that water is too tightly bound to \( \theta = 0^\circ \) surfaces \([W_{\text{adhesion}} = 2\gamma = 2(72.8) = 145.6 \text{ dyne/cm}]\) to be displayed by adsorbate, except perhaps through highly energetic ionic interactions, such as that discussed above for cetyl bromide.

We applied ESCA as a check of contact angle measurements. Surprisingly, ESCA detected nitrogen on all glass slides of Figure 4, with the exception of the saline control. Relevant ESCA data is summarized in Table II. Note that for glass slides immersed in plasma or serum, more than 5 atom % nitrogen attributable to protein \(^{33,46}\) was detected. Roughly twice that amount was observed on the previously mentioned silane-treated plate to which a visible protein film was bound. Comparison of data gathered at a 20° take-off angle (higher surface sensitivity) to that at 35° (lower surface sensitivity) suggests that the protein layer adsorbed was quite thin (less than an infinite thickness to ESCA) because the Si signal arising from the glass substrate was always observable and significantly lower at 20° than at 35°.

Rows 1 to 5 of Table III summarize observations made to this point regarding blood proteins at water-wettable surfaces. It is apparent that ESCA measurements are inconsistent with the interpretation of plasma coagulation and tensiometric observations. A proposition that reconciles these divergent views asserts that protein is "associated" with the clean glass slides in such a way that the surface retains complete water wettableness under humidified conditions but yields measurable protein nitrogen under the dehydrated, high vacuum environment of an ESCA spectrometer. Wilhelmy balance measurements discussed in the next section provide evidence for such an associated protein layer.

Wilhelmy balance tensiometry

Low contact angles (<10°) are rather difficult to measure with a contact angle goniometer. Consequently, we applied the Wilhelmy balance method of measuring adhesion tension of pure saline to the glass coverslips of Figure 4 as a check of goniometer measurements discussed in the previous section. In the Wilhelmy balance method, glass slides are suspended from a continuously reading force transducer (a microbalance with milligram sensitivity) by a torsion wire.\(^{31}\) Adhesion tension \( \tau \) is determined from weight measurements as the slide is immersed (advancing) or withdrawn (receding) from a test saline solution. Typically, data is graphically interpreted from so-called hysteresis curves that plot weight (wetting forces) against immersion depth. We prefer to display force data in a continuous data-point format because dynamic events transpiring at the solid-liquid three-phase line due to adsorption can be easily observed and individually analyzed.\(^{41}\) Figure 5(A) is an example set of Wilhelmy balance hysteresis curves in the point-number format.

Another difference in our implementation of the

<table>
<thead>
<tr>
<th>Substratum</th>
<th>Incubation Fluid</th>
<th>Take-Off Angle</th>
<th>C</th>
<th>N</th>
<th>O</th>
<th>Si</th>
<th>Cl</th>
<th>All Other</th>
</tr>
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<tbody>
<tr>
<td>Clean glass plate</td>
<td>Saline</td>
<td>20°</td>
<td>22.2</td>
<td>—</td>
<td>50.0</td>
<td>21.1</td>
<td>—</td>
<td>6.7</td>
</tr>
<tr>
<td>( \theta_{\text{adv}} = \theta_{\text{rec}} = 0^\circ ) (saline)</td>
<td>Citrated P(^4)</td>
<td>20°</td>
<td>64.7</td>
<td>8.2</td>
<td>19.6</td>
<td>3.0</td>
<td>1</td>
<td>2.6</td>
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<tr>
<td></td>
<td>Porcine serum</td>
<td>35°</td>
<td>48.8</td>
<td>6.2</td>
<td>29.2</td>
<td>10.2</td>
<td>1.9</td>
<td>3.7</td>
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<td>Recalified P(^4)</td>
<td>20°</td>
<td>40.7</td>
<td>6.0</td>
<td>37.5</td>
<td>13.7</td>
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<td>2.1</td>
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<td>Recalified P(^4)</td>
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<td>32.6</td>
<td>4.6</td>
<td>39.8</td>
<td>15.9</td>
<td>—</td>
<td>7.1</td>
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<tr>
<td>OTS treated glass plate (( \theta_{\text{adv}} = 106^\circ ))</td>
<td>—</td>
<td>—</td>
<td>74.4</td>
<td>5.3</td>
<td>16.0</td>
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<td>Recalified P(^4)</td>
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<td>Recalified P(^4)</td>
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<td>66.9</td>
<td>11.1</td>
<td>15.4</td>
<td>1.4</td>
<td>—</td>
<td>5.2</td>
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</tbody>
</table>

ESCA = electron spectroscopy for chemical analysis. P\(^4\) = porcine platelet poor plasma. OTS = octadecytrichlorosilane.

*Estimated accuracy is ±0.1%. All other includes (in decreasing levels) Na > P > K > T, > Al ~ Ca.
### TABLE III
Summary of Experimental Observations

<table>
<thead>
<tr>
<th>Probe</th>
<th>Observation</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mathematical model of P₄ coagulation</td>
<td>Single parameter fit to CT data obtained with procoagulant surfaces spanning full range of water wettability.</td>
<td>Activating sites increase in proportion to procoagulant surface area and are not consumed in coagulation of P₄. Functional relationship between procoagulant activity and surface energy revealed.</td>
</tr>
<tr>
<td>Consecutive use of procoagulants in separate coagulation assays</td>
<td>Re-use of a procoagulant in second assay does not reduce propensity to activate coagulation.</td>
<td>Activating sites on procoagulant surface are not consumed in coagulation.</td>
</tr>
<tr>
<td>Adsorption mapping</td>
<td>Adsorption map of EDTA-anticoagulated P₄ correlates with propensity to activate coagulation.</td>
<td>Protein adsorbent surfaces do not significantly activate P₄ coagulation. Protein repellent surfaces efficiently activate P₄ coagulation. Blood proteins do not adsorb to water wettable surfaces (hydrated state).</td>
</tr>
<tr>
<td>Contact angle goniometry</td>
<td>Water-wettability retained after incubation in blood proteins.</td>
<td>Blood proteins do not adsorb to water wettable surfaces (hydrated state).</td>
</tr>
<tr>
<td>Robotic Wilhelmy balance tensiometry</td>
<td>Erratic hysteresis curves attributable to dynamic events at solid-liquid–vapor three phase line.</td>
<td>Protein associated with a bound hydration layer that collapses onto the glass slide upon dehydration.</td>
</tr>
</tbody>
</table>

Wilhelmy balance from conventional balance tensiometry was used of a robotic Wilhelmy balance that automated data acquisition from a multiplicity of test liquids. The design employed a commercial XYZ translation stage to locate a force transducer over any one of an XY array of liquid-sample positions. A glass slide was attached to the force transducer using a torsion wire, and Z motion performed the immersion/withdrawal cycles. As mentioned above, samples analyzed in this work were the glass coverslips illustrated in Figure 4 examined directly after incubation in protein solutions and rinsing. Test solutions were five identical saline solutions.

Figure 5(A) shows the hysteresis curves obtained with the control coverslip incubated in saline. Directly after saline rinse, this slide was attached to the robotic balance transducer and carried through 4 advancing-receding cycles. Upon completion of the test in the first pure saline solution, the balance was programmed to move to the four subsequent saline sample positions and repeat wetting measurements. Four immersions in a test saline solution required about 1 min, and the plate was suspended in air for a period of 38 s between saline samples, which included a 10-s delay to allow oscillation of the torsion wire to dampen. Thus, there was opportunity for the test plate to air dry, in whole or in part, between test solutions. Mean advancing and receding $\tau^\circ$ values corresponding to the four immersion-withdrawal cycles for each of the five separate saline solutions are listed directly on Figure 5(A). Note that in each case results were statistically identical and not different from the expected $\tau^\circ = 73$ dyne/cm for saline at ambient laboratory temperature. It is concluded that this glass slide exhibited $\theta_{\text{adv}} = \theta_{\text{rec}} = 0^\circ$ and that, consequently, $\tau^\circ = \gamma^\circ \cos \theta = \gamma^\circ$. Final mean $\gamma^\circ$ values for the five test solutions are given at the foot of Figure 5(A).

Figure 5(B) is the same style of data presentation as in Figure 5(A), but the data are obtained in this case with a glass coverslip used to coagulate recalcified P₄ (see Fig. 4). Test saline solutions were those used in the experiments of Figure 5(A). The first hysteresis curve obtained directly after removal from the clotted P₄ and rinsing was very similar to that obtained with the clean-glass control coverslip, yielding advancing/receding $\tau^\circ$ consistent with a fully wettable surface exhibiting $\theta_{\text{adv}} = \theta_{\text{rec}} = 0^\circ$. This result corroborates contact angle goniometer observations reported in the previous section. Interestingly, hysteresis curves of the subsequent saline solutions exhibited increasingly erratic behavior, attributable to dynamic events at the solid–liquid–vapor three-phase line that control wetting forces. This is particularly evident on first immersion-withdrawal cycles after dehydration in air.

In light of the ESCA observations of the previous section, a reasonable interpretation of the Wilhelmy balance hysteresis curves of Figure 5(B) attributes erratic wetting behavior to hydration–dehydration–rehydration of a protein layer bound to the glass coverslip as it is sequentially immersed in saline, dehydrated in air, and immersed again in saline through the course of obtaining hysteresis curves in five consecutive saline solutions. Protein (or contaminant) adsorption to the glass plate from saline solutions can...
Figure 5. Wilhelmy balance hysteresis curves in sequential point number format for four immersion (advancing) and withdrawal (receding) cycles repetitively measured in five separate saline solutions. (A) corresponds to a clean glass plate. Note that there is no statistically significant difference between advancing and receding cycles among or between the five test solutions, leading to the expected $\gamma' = 73$ dyne/cm for saline at ambient temperature. (B) corresponds to the same test saline solutions as in (A) and tested with a glass slide used to coagulated porcine plasma (see Fig. 4). In this case, only the first hysteresis curve exhibited behavior consistent with a fully wettable glass slide (compare to A). Increasingly erratic wetting behavior observed for each successive test solution was traced to rehydration-dehydration of a protein film bound to the glass slide.

be ruled out because these solutions were shown to be free of adsorbate in the previous analysis with the control coverslip (Figure 5(A)). Significant desorption of protein from the coverslip into the sequential saline test solutions seems unlikely because $\gamma'$ was not statistically different before and after use in the experiment of Figure 5(B). Furthermore, since hysteresis was most erratic on first immersions and became more regular with each successive immersion-withdrawal cycle, it is evident that erratic wetting behavior diminishes with saline immersion time. This is consistent with a rehydration event and inconsistent with a desorption event, which would be expected to become more pronounced with multiple immersions.

Similar hysteresis curves were observed with citrated P₄ (not shown) and porcine serum (Figure 6(A)). It is of interest to compare wetting phenomena of proteins at hydrophilic and hydrophobic surfaces. Figure 6(B) displays hysteresis curves obtained for a hydrophobic, silane-treated glass slide immersed in porcine serum and rinsed in saline. Note that, in contrast to the behavior of proteins associated with wettable surfaces (Figures 5(B) and 6(A)), the protein layer adsorbed to the hydrophobic surface was stable to hydration/dehydration as indicated by regular and repeating hysteresis curves both among and between test saline solutions.

CONCLUSIONS

Table III collects all of the observations regarding protein adsorption events involved in contact activation of the intrinsic pathway of plasma coagulation from this work. We conclude from this summary that protein adsorbent surfaces do not significantly activate P₄ coagulation, whereas protein repellent surfaces efficiently activate coagulation. Furthermore, we conclude that blood proteins do not adsorb directly to water-wettable glass surfaces in such a way that alters solid-liquid interfacial tension. Rather, protein appears to be "associated" with a hydration layer tenaciously bound to water-wettable surfaces. This hydrated layer collapsed onto the surface upon de-hydration, simultaneously accounting for ESCA observations of bound protein under dry-state vacuum conditions and fully water-wettable behavior under the hydrated conditions probed by tensiometry.
Figure 6. Wilhelmy balance hysteresis curves in sequential point number format for four immersion (advancing) and withdrawal (receding) cycles repetitively measured in five separate saline solutions. (A) corresponds to a clean glass plate exposed to porcine serum (see Fig. 4). Only the first hysteresis curve exhibited behavior consistent with a fully wettable glass slide (compare to Figure 5B). Increasingly erratic wetting behavior observed for each successive test solution was traced to rehydration–dehydration of a protein film bound to the glass slide. (B) corresponds to hysteresis curves observed for a silanized glass plate exposed to porcine serum. Note that, in contrast to protein bound to the water wettable glass slide of (A), the adsorbed protein layer exhibited uniform wetting behavior for all five test saline solutions.

The exact nature of this "associated" protein is not clear from this work, and we are aware of only a single report describing nonadsorbed protein held adjacent to surfaces. However, it is very clear that the behavior of water at surfaces strongly affects protein adsorption and, more generally, biology at interfaces. Perhaps associated protein is physically entrapped in a surface-bound water layer or connected to the surface through hydrated-ion bridges, similar to the co-adsorbed electrolytes reported for protein adsorbed to negatively charged lattices.

These results have importance beyond the biophysical implications for blood coagulation. Possibly, some of the controversy in the protein adsorption literature mentioned in the introduction can be reconciled by recognition of protein associated with bound water at wettable surfaces. This hydrated, bound protein layer is transparent to conventional tensiometry but observable by spectroscopic and biochemical methods, especially by those employing protocols that include a dehydration step. The mechanistic distinction between protein adsorbed to a surface (either through surface dehydration or ionic interactions) and protein associated or entrapped in a bound hydration layer is profound. In the former means of adsorption, protein can be concentrated within an interfacial region separating bulk liquid and bulk solid phases in excess of that anticipated from bulk liquid composition. Furthermore, adsorption from a proteinaceous milieu can be selective (e.g., Vroman effect) causing surface composition to be different from bulk composition. By contrast, a physically entrapped protein layer seems more likely to reflect both bulk liquid concentration and composition.

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