Infrared Spectrophotometric Observations of the Adsorption of Fibrinogen from Solution at Optically Transparent Carbon Film Electrode Surfaces

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Using infrared transparent electrodes consisting of ~250 Å of carbon on germanium, and internal reflection spectroelectrochemistry, transitory co-adsorption of an unexpected form of fibrinogen has been observed along with adsorption of the native material at potentials more positive than the point of zero charge. The infrared spectra strongly suggest that the unexpected form has a secondary structure which is distinctly different from the native form.

Blood protein adsorption at solid–solution interfaces is known to be a factor in determining the thromboresistance of synthetic materials introduced into the cardiovascular system. It has been established (*1–4*) that the first event which takes place on such implantation is the adsorption of a layer of proteinaceous material at the solid–blood interface, probably due to van der Waals' forces. Thrombogenesis takes place subsequent to the formation of this initially adsorbed protein layer (*3, 4*). Some materials, such as isotropic pyrolytic carbon (*5*), have exhibited satisfactory thromboresistance, while other materials such as metals high in the electromotive series (i.e., gold or platinum) are generally unacceptable (*6*). The thromboresistance of a material is difficult to relate to its physical–chemical properties. Sawyer and co-workers (*6, 7*) have suggested that the formation of the protein film involves an electron-transfer reaction. Alternatively it has been suggested (*8*) that what is taking place is nothing more than electrostatic attraction to a charged surface. Baier and Dutton (*4*) have shown that there is instantaneous protein film formation on any surface regardless of charge, thus it is possible that a completely independent mechanism could be responsible for such spontaneously adsorbed films. It is not clear what the role of the electrochemical properties of the solid–solution interface is, but either electrostatic attraction or an electron-transfer reaction involving the surface and protein film is involved in the thromboresistance of synthetic biomaterials. The role of the adsorbed film will not be clear until we understand the way in which the structure and activity of the protein in the film is affected by surface electrodic processes.

There is, however, a phenomenon which we have referred to (*8*) as enhanced adsorption, also observed by Stromberg et al. (*9*), where the spontaneously adsorbed layer is covered with additional protein, with film thicknesses approaching 1000 Å or more. Enhanced adsorption takes place at a potential which is characteristic of the material and, according to Stromberg et al. (*9*), is dependent on the particular protein. In an earlier paper (*8*), enhanced adsorption of fibrinogen was observed at a germanium–solution interface at a potential of approximately −200 mV to a saturated calomel electrode (SCE). This *onset potential* (*9*) was several hundred millivolts positive to the point of zero charge (PZC) reported for germanium (*9, 10*). It is difficult to explain the need for such a strong driving force if the phenomenon is simply electrostatic, providing additional weight to Sawyer's theory that an electron-transfer reaction is involved (*6, 7*). In this paper, along with reporting the onset potential for fibrinogen adsorption at a carbon surface, it is noted that two different forms of fibrinogen appear to adsorb at potentials anodic to (more positive than) the onset potential, where only one form was expected. Furthermore, the unexpected form begins to disappear when the potential is adjusted more negative than the onset potential. This transitory nature may explain why it has not been observed previously.

EXPERIMENTAL

Infrared transparent electrodes used in this study were prepared by electron beam deposition from a glassy carbon source onto a hot germanium substrate (450–500 °C). The carbon films are made in two layers in order to avoid pinholes through to the germanium, and range from about 220 to 300 Å in total thickness. The initial carbon atoms probably form a carbide bond with the germanium. The films are more thoroughly described in a previous paper (*11*). The carbon films used in this study exhibited a resistivity of 3 to 4×10^{-3} Ω-cm. Germanium internal reflection elements were obtained from Harrick Scientific Corp. (P.O. Box 867, Ossining, N.Y. 10562) and the carbon films were prepared by a commercial coating laboratory (Lebow Co., 1407 Norman Firestone Rd., Goleta, Calif. 93107). Fibrinogen used in the instant study was 90% clottable, 99% human fibrinogen, obtained from Kabi (Stockholm, Sweden). The computer–spectrometer system employed in this and previous studies is described elsewhere (*12*), and the spectroelectrochemical cells are shown in Ref. 11.

RESULTS AND DISCUSSION

Figure 1 illustrates polarized internal reflection spectra of 1% fibrinogen in 0.17 M NaCl (0.34 M sodium citrate, 0.061 M sodium acetate, pH 7) in contact with a carbon surface at a controlled potential of −500 mV (to SCE). The open-circuit potential (rest potential) of a carbon OTE (optically transparent electrode) is about −180 mV, and the protein was added to the cell by forcing out a citrate buffer solution while holding the OTE potential at −500 mV. The initial potential of −500 mV was chosen in order to match as closely as possible the open-circuit potential of the germanium OTE's reported previously (*8*). The spectra shown in Figure 1 were obtained by subtracting the polarized internal reflection spectra of identical buffer solution from the spectra of the 1% fibrinogen solution. The maximum transmission of the carbon OTE's is about 1.5% T at 1800 cm^{-1}, and the spectra used to compute the difference spectra in Figure 1 were averages over 25 scans, smoothed once with a least-squares routine (*13*). The combination of averaging 25 spectra and using a 21-point smooth gives a signal-to-noise (S/N) enhancement of slightly more than 10.

The principal source of interference in these experiments is the intense 1633 cm^{-1} band of water, which has an intensity about 20 times that of the Amide I peak. Only by spectrum subtraction can the Amide I peak be observed in aqueous solution. In Figure 1, the solution fibrinogen exhibits the ex-

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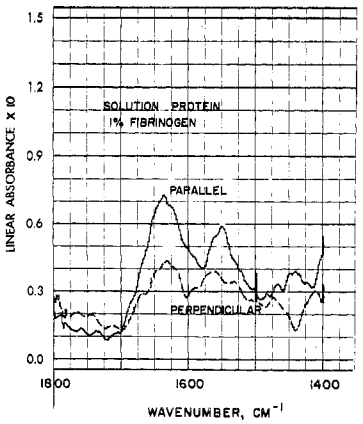

Figure 1. Parallel (TE) and perpendicular (TM) polarization difference spectra of fibrinogen in solution. Spectra obtained by subtracting spectra of citrate buffer solution

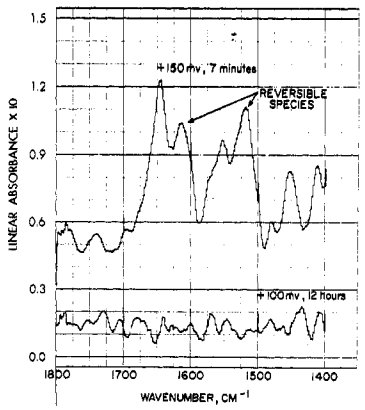

Figure 2. Bottom: TE polarization difference spectrum showing no enhanced adsorption after potentiostating for 105 min at +100 mV to SCE, then 10.5 h at 0 mV. Absence of water band is indicative of quality of subtraction procedure. Top: TE polarization difference spectrum, average of 10 spectra taken after potentiostating at +150 mV for 7 min, showing unexpected absorption bands at 1614 and 1520 cm⁻

pected Amide I and II bands at 1635 and 1550 cm^{-1}, respectively. In Figure 2, in the lower spectrum, the parallel (TE) polarization solution fibrinogen spectrum used for Figure 1 was subtracted from the TE polarization spectrum taken after the OTE surface had been potentiostated at +100 mV for 105 min, followed by potentiostating at 0 mV for 10.5 h. This spectrum shows that no adsorption took place after more than 12 h at potentials of 0 mV or greater. This is more than 200 mV anodic to (morepositive than) the onset potential for fibrinogen adsorption on germanium (*8*).

After the 12.25 h of controlled potential with no deposition, the potential was stepped to +150 mV for 2.5 min. Monitoring the Amide II band at 1553 cm^{-1} showed instantaneous deposition, with an increase of 0.04 absorbance unit in 2.5 min. Allowing the potential to drop back to the open-circuit value, the absorbance decreased slightly. We also noted that the open-circuit potential was −220 mV to SCE, as compared to −180 mV in citrate buffer. Averaging four spectra after the 2.5-min deposition yielded the surprising observation that an unexpected absorption band had shown up at around 1620 cm^{-1}. In an effort to examine this peak more carefully, ten TE-polarized spectra, at an average time of 36 min, were collected after the deposition had been stopped. After subtracting the baseline from this averaged spectrum, the 1620 cm^{-1} peak had disappeared.

It is possible to estimate the thicknesses of these aqueous protein films at the Ge–C–H_2O interface, using the following procedure. The thickness of a fibrinogen film prepared on germanium was measured ellipsometrically by R. E. Baier (Calspan Corp., Buffalo, N.Y.) as 980 Å (±10%, assuming n, the refractive index, = 1.5). By measuring the "reflectance absorbance" [$\equiv \log_{10}(R_0/R)$] of the same film in air with both parallel and perpendicularly polarized incident light, the equations of Hansen (*14*) can be used in nomograph fashion to compute the extinction coefficients for the amide bands. Figure 3 is such a nomograph, computed using Hansen's equations (*14*) and assuming $n_{protein} = 1.5$ and h, the film thickness, equals 980 Å. From the measured values of the reflectance absorbance of the Amide I band in both polarizations, an average value of 0.277 is obtained for $k_{protein}$. The two points are indicated by the crosses on Figure 3 ($R.A._{\parallel} = 0.0289$, $R.A._{\perp} = 0.0232$; $k_{2,\parallel} \cong 0.264$, $k_{2,\perp} \cong 0.290$).

In order to find the apparent thickness of protein films at a solid–water interface, it is necessary to compute a change in reflectance absorbance (ΔR.A.) from that observed for the water band at 1633 cm^{-1}. At that frequency, water has a refractive index, n, of 1.34, and an extinction coefficient, k, of 0.10. In addition, carbon has a refractive index of about 3.2 at that frequency (*15*). From transmission infrared measure-

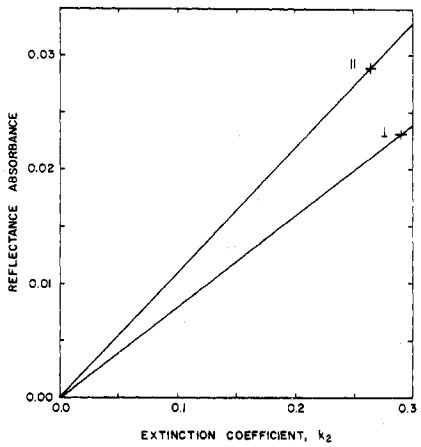

Figure 3. Plot of "reflectance absorbance" [$\log_{10}(R_0/R)$] vs. extinction coefficient, k, for a 980-Å-thick film of refractive index 1.5, on germanium, in contact with air, at 1640 cm^{-1}. Computed from exact equations of Hansen (*14*)

ments of carbon optically transparent electrodes on germanium, we find a value for $k_{carbon} \cong 1.4$ at 1640 cm^{-1}. Using a modified version of Hansen's equations in a program written by Randall and Mark (*16*), we can relate Amide I peak heights to an "apparent thickness" for the protein film for the 4-phase system Germanium–Carbon–Protein–Water. As it turns out, the presence of a carbon film does not change the *contrast* of the protein spectrum significantly over a film thickness range of 100 to 1600 Å (less than 1% difference in ΔR.A. with 270-Å carbon film present vs. bare germanium surface). Over the first 400 Å of protein thickness, the relationship between the apparent film thickness, h (in Å) and the *actual* Amide I peak height (not ΔR.A.) is approximately linear, as shown in Equation 1.

$$h \text{ (in Å)} \cong 5.67 \times 10^4 A_{\perp} \cong 3.22 \times 10^4 A_{\parallel} \quad (1)$$

The "apparent" film thickness, h, is computed based upon the assumption that the optical properties of fibrinogen measured in air ($n \equiv 1.5$, $k \cong 0.277$) are the same as those encountered at the solid–solution interface. If the wet film is swelled by included water molecules, this is obviously not the case. Such hydrated films could be mathematically modeled for n and k, but there is insufficient information contained in these

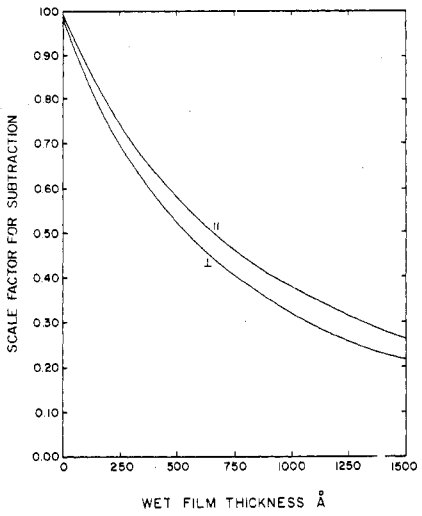

Figure 4. Fraction of water absorbance lost by imposition of a thin, nonabsorbing film ($k = 0$) of refractive index 1.5 between germanium and water, at 1640 cm^{-1}. Computed from exact equations of Hansen (*14*)

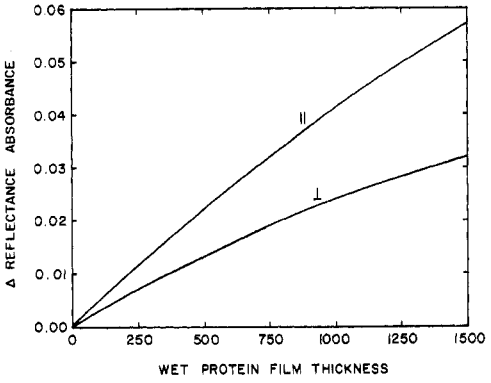

Figure 5. Resultant *change* in reflectance absorbance (ΔR.A.) which takes into account *both* an absorbing protein film ($n = 1.5$, $k = 0.277$) and the displacement of water ($n = 1.34$, $k = 0.10$) in a germanium–carbon–protein–water system (using Randall and Mark's (*16*) program). It is assumed, for the purpose of these plots, that there is no water included in the protein film

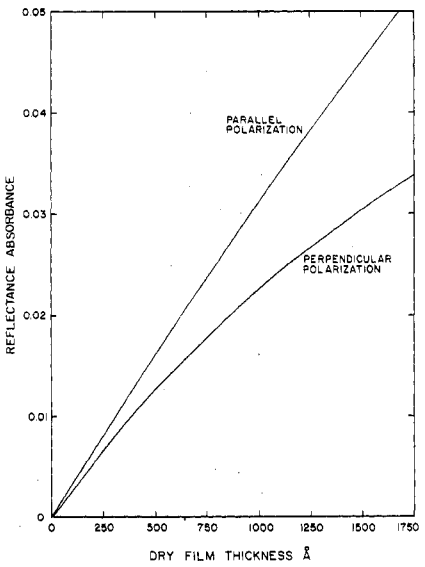

Figure 6. The thickness of a protein film of refractive index 1.5, extinction coefficient 0.277, can be related to the measured reflectance absorbance (based on *one* reflection) at the germanium–air boundary, at 1640 cm^{-1}

experiments to specify the molar composition of a hydrated film.

The linear relationships above are valid when one measures the Amide I band intensity above the water band; i.e., by the familiar tangent-line baseline approach. If, as in most of these experiments, one is simply monitoring a band maximum frequency, then in the case of the Amide I band maximum at 1640 cm^{-1}, there is an additional complication introduced by the water, with its band maximum at 1633 cm^{-1}.

For single-frequency monitoring, as the Amide I band increases in intensity because of film formation, so does the water band *decrease* in intensity as the solvent is literally being "pushed away" from the interface. By mathematically interposing a nonabsorbing film between the solid surface and the water, we can compute (*14, 16*) the fraction of the water band absorbance that must necessarily be subtracted from the original water band absorbance as the film grows. This is shown in Figure 4, where it can be seen that a 750-Å thick, nonabsorbing film would decrease the water band height by 50–60%. The result of this correction is that any observed change in absorbance (ΔR.A.) at 1640 cm^{-1} is due partly to an increase in the Amide I band and partly to a decrease in the intensity of the water band. In Figure 5, these effects are combined to yield a plot of ΔR.A. vs. the apparent wet film thickness. (The values of ΔR.A. are based on a single reflection, multiple reflection ΔR.A. values must be divided by N, where N is the number of reflections where solution contact exists.) As a side benefit to these calculations, the observed value of $k = 0.277$ for fibrinogen at 1640 cm^{-1} makes it possible to create a plot of *dry* film thickness vs. R.A. for fibrinogen adsorbed to germanium which, for the benefit of the several investigators using this procedure, is given in Figure 6. (The curves given in Figure 6 are based on Hansen's (*14*) exact equations and the optical constants $n_{Ge} = 4.0$, $k_{Ge} = 0$, $n_{fibrinogen} = 1.5$, and $k_{fibrinogen} = 0.277$.)

From Figure 5, we can compute an apparent thickness for the film which appeared briefly, and was discussed above. An increase of 0.04 absorbance unit, for a 2-mm thick, 52.5-mm long prism with 11 reflections at the solid–solution interface, corresponds to a ΔR.A. of ca. 3.6×10^{-3}. This was measured for parallel polarized light, and from the near-linear section of Figure 5 from 0- to 250-Å film thickness, we can use the relationship $h \cong 2.2 \times 10^4 \cdot \Delta R.A._{\parallel}$, or in this instance the initially formed film exhibited a thickness of 80 Å, assuming that the value of the k for the unexpected species absorbing at 1620 cm^{-1} is similar to that of normal fibrinogen at 1640 cm^{-1}.

The top spectrum in Figure 2 was obtained by depositing at +150 mV for 7 min, and then averaging 10 spectra with the current switched off, at 1.75 min per spectrum. In this case, the unusual peaks shown in the top spectrum of Figure 2 were observed after subtracting the fibrinogen solution baseline. This spectrum clearly shows *two* Amide I peaks (at 1644 and 1614 cm^{-1}) and two Amide II peaks (at 1553 and 1520 cm^{-1}). In addition to the new set of peaks, the relative intensities are reversed, with the Amide II peak > the Amide I peak for the unexpected species, and vice-versa for the usual species. Continued computer-averaging at the open-circuit potential (now dropped to −320 mV) showed that it required about 150 min for the unexpected species to completely disappear.

As the water background has been subtracted for the top spectrum in Figure 2, the relationships given in Equation 1 are applicable. The 1640 cm^{-1} Amide I band corresponds to an apparent film thickness of 360 Å, ignoring the overlap from the adjacent (1614 cm^{-1}) unexpected peak. If that overlap