Characterization of Biomimetic Surfaces Formed from Cell Membranes

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ABSTRACT A method for fabricating biomimetic surfaces from intact cell membranes is described. A monolayer of alkanethiol on gold is covered by a second layer derived from the components of erythrocyte membranes either by self-assembly or by Langmuir-Blodgett methods. The resulting asymmetric hybrid layer was characterized by ellipsometry, surface plasmon resonance (SPR), contact angle, capacitance, voltammetry, and electron and atomic force microscopy. The erythrocyte membrane layer was measured to be ~30–40 Å in thickness. Using SPR, the presence of erythrocyte components on the surface was demonstrated by their selective removal by enzymatic action. The uniform deposition of membranous material on the substrate was shown by electron and atomic force microscopy. Demonstration of acetylcholinesterase (AChase) activity, a membrane-anchored enzyme, on the surface for at least 8 days, suggests that the outer leaflet of the erythrocyte membrane is present in its native form. Cyclic voltammetry demonstrates that enhanced electron transport from a solution redox species accompanies formation of the erythrocyte layer at the surface. This enhanced electron transport is blocked by 4,4′-diisothiocyanate stilbene-2,2′-disulfonic acid, a well known blocker of anion transport, suggesting that an erythrocyte anion transporter protein is incorporated into the surface layer in an active conformation.

INTRODUCTION

Many sensing and recognition molecules reside in cell membranes. Utilizing these molecules to form biomimetic surfaces for biosensors and biocompatible prosthetic devices hinges on retaining the functions of these molecules (Ahlers, et al., 1990). Many membrane components lose their specificity or activity with slight alterations of their lipid environment. Therefore, a method for fabricating biomimetic surfaces from intact cell membranes without isolation and reconstitution of membrane components is much desired. A variety of supported lipid membrane systems have been prepared by a number of different techniques, and on different solid supports, as has been reviewed recently (Sackmann, 1996). For a robust bilayer with longer temporal stability, the interactions of the molecular bilayer with the solid surface should be based on interactions stronger than the weak electrostatic interactions between phospholipid headgroups and the surface. Since the report of self-assembled monolayer (SAM) formation of alkanethiols on gold, considerable knowledge has been gained on these SAMs given the ease of preparations and their potential applications (Nuzzo et al., 1987; Ulman, 1991; Prime and Whitesides, 1991; Plant, 1993). Alkanethiol monolayers prepared from dilute alkanethiol solutions in ethanol can be pinhole-free, essentially homogeneous, and very stable. Addition of phospholipid vesicles to an alkanethiol monolayer results in the formation of a bilayer consisting of the alkanethiol monolayer covered with a single layer of phospholipid molecules such that the lipid polar headgroups are in contact with water and the alkane chains are sequestered from the water (Lang et al., 1992; Plant, 1993). Such hybrid bilayer membranes (HBMs) have been characterized by electrochemistry and impedance spectroscopy in our lab (Plant, 1993; Plant et al., 1994) and by FTIR (C. W. Meuse, G. Naiura, M. Lewis, and A. L. Plant, submitted for publication) and have been determined to be analogous to phospholipid bilayers in insulating properties and alkane structure.

The purpose of this study was to assess whether an intact cell membrane would associate with the hydrophobic alkanethiol surface in a manner similar to phospholipid vesicles. Recent kinetic analysis in our laboratory (unpublished observations) supports the hypothesis that the formation of a bilayer at a hydrophobic surface occurs not through the diffusion of individual lipid molecules to the surface, but by a kinetic process that accompanies vesicle diffusion. A fusion process, in which spreading of a continuous monolayer of a lipid at the surface, has been proposed (Kalb et al., 1992). Deposition of a layer of lipid and protein from a crude membrane preparation onto an alkanethiol SAM could be an efficient way of preparing bioactive surfaces as membranes from transformed cells could be enriched with specific proteins by molecular biological methods, and the tedium of purification and reconstitution of membrane proteins would be circumvented. In this paper we report the formation of supported erythrocyte membrane surfaces by the interaction of erythrocyte ghosts with alkanethiol SAMs. We have characterized the hybrid membrane surface by biochemical, electrochemical, optical, and imaging methods. Our goal was to determine whether the resulting surface is made of a single bilayer consisting of an alkanethiol monolayer plus a monolayer of membrane and whether

Received for publication 24 March 1997 and in final form 8 September 1997.

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0006-3495/97/12/3066/12 $2.00

certain activities of the erythrocyte membrane are retained in the resulting layer.

MATERIALS AND METHODS

Reagents

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine chloride, 4,4′-diisothiocyanate stilbene-2,2′-disulfonic acid (DIDS), chymotrypsin, and neuraminidase were purchased from Sigma Chemical Co. (St. Louis, MO). Concanavalin A was purchased from Boehringer Mannheim (Indianapolis, IN). Hexa-, deca-, and octadecanethiol were purchased from Aldrich (Milwaukee, WI). 1-Palmitoyl, 2-oleyl phosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Alabaska, AL). All other chemicals used were of analytical grade or better.

Preparation of erythrocyte ghosts and liposomes

Erythrocyte ghosts were prepared by osmotic lysis according to a published procedure (Steck and Kant, 1974). In brief, the procedure was as follows. Rabbit or human erythrocytes were washed three times in a buffer of 5 mM sodium phosphate, 150 mM sodium chloride (PBS), removing the buffy coat in the initial washes. Washed erythrocytes were lysed in precooled 5P8 buffer (5 mM phosphate buffer, pH 8.0) by mixing at a ratio of 1:20, and the lysate was centrifuged at 20,000 × g for 10 min. The translucent and whitish membrane pellets were pooled and washed three times in 5P8. Results reported here were independent of the source of blood. Unilamellar liposomes of POPC were made by extrusion through 0.2-μm filters, as described earlier (Sen et al., 1991). Protein concentration was estimated using bicinchonic acid (Pierce Chemical Co., Rockford, IL) and bovine serum albumin as a standard.

Gold substrate preparation

Alkanethiol monolayers were allowed to self-assemble on gold films that were deposited on glass or silicon. This step simply involved placing freshly prepared or cleaned gold-coated substrates into a 1 mM solution of alkanethiol in ethanol for at least 12 h. The gold-coated substrates were prepared in-house for some experiments. The glass substrates (slides or coverslips) were cleaned in dichromic acid or NoChromix solution and then washed with distilled water and ethanol, dried under a stream of nitrogen, and placed immediately into a vacuum chamber where gold was deposited on them by thermal evaporation. For enzyme activity and imaging studies, 50 Å of Cr were deposited first for adhesion, followed by 50 Å of gold. For surface plasmon resonance (SPR) experiments, approximately 500 Å of Au was deposited directly on glass. Commercial, 2000-Å gold film electrodes, (Abtech, Yardley, PA) were used for electrochemistry experiments. These electrodes were cleaned by immersion in warm saturated potassium dichromate followed by immersion in 3% hydrofluoric acid before placement in alkanethiol solution. After formation of the alkanethiol monolayer, samples were removed from the ethanol solution, rinsed thoroughly with ethanol, dried under a stream of nitrogen, and used immediately for HBM formation.

Preparation of alkanethiol/erythrocyte hybrid membranes

Preparation of the hybrid membrane layers involved bringing together the membrane suspension and the hydrophobic alkanethiol surface. Variations in the method of preparation, as described below, were required for subsequent measurements.

Ellipsometry and contact angle measurements

For ellipsometry and contact angle measurements, alkanethiol-monolayer-coated substrates were placed in a petri dish, covered with the erythrocyte ghost suspension, and left overnight at 4°C. Samples were rinsed thoroughly with water by repeated dilution and removal of most of the fluid, while keeping them wet. After the final rinse, the water was removed from the dish, leaving only a thin film of liquid on the surface of the samples. At this point, the samples were allowed to air dry. Ellipsometric measurements were taken at an incident angle of 70° on dried HBM samples. Measurements were made at each stage of the treatment of the substrate for a reliable internal reference. The refractive index of organic monolayers was taken to be 1.5 (Ulman, 1991). Contact angle measurements were made on a custom-built instrument.

Electrochemical studies

Impedance measurements and cyclic voltammetry of the alkanethiol monolayers and erythrocyte membrane treated surfaces were performed by the methods described earlier (Plant, 1993). Briefly, the gold substrate served as the bottom of the electrochemical cell and as the working electrode with an exposed area of 0.32 cm^2. The reference electrode was Ag/AgCl, and a platinum wire served as an auxiliary electrode. Erythrocyte ghosts suspended in 20 mM Tris, 150 mM NaCl, 0.1% thimerosal, pH 7.4 (TBS), were added directly to the cell and association was allowed to occur in situ. Impedance measurements were made in the presence and absence of the ghost suspension. Removal of suspension of ghosts and rinsing of the cell was performed without disrupting the surface of the electrode. Electrochemical measurements were made with a Solartron electrochemical interface (model 1250, Schlumberger, Burlington, MA) and frequency generator (model 1286, Schlumberger). Impedance measurements were made at a DC potential of 0 V and a sinusoidal AC potential of ±10 mV. Capacitance values reported here were determined by impedance measurements in TBS. Cyclic voltammetry was performed with $K_3Fe(CN)_6$ or with an equimolar mixture of $K_3Fe(CN)_6/K_4Fe(CN)_6$ as the redox species in 1 M or 0.1 M KCl as the supporting electrolyte.

Microscopy

For environmental scanning electron microscopy (ESEM) measurements, octadecanethiol-coated substrates were floated face down on a suspension of erythrocyte ghosts in TBS overnight at 4°C. The samples were washed extensively by repeated removal of solution and addition of fresh water. After removing most of the final wash water, the samples were lifted out of the dish. At this point, the surface of the samples appeared wet, and they were allowed to air dry. No further sample preparation was required.

For preparation of replicas for transmission electron microscopy (TEM) observation, a thin layer of carbon was evaporated onto cleaved mica and was then coated with a thin layer of gold and treated with octadecanethiol. A drop of ghost suspension was placed in the center of alkanethiol-coated substrate, without completely covering the surface. After overnight incubation at room temperature, that area was carefully rinsed by repeated additions and removal of water at that spot. The spot was allowed to air dry and was shadowed with platinum at an angle of 29° from the surface plane for TEM, or gold sputtered for scanning electron microscopy (SEM). Replicas were floated on water and transferred onto grids for electron microscopy. TEM was performed on a Hitachi H-600 electron microscope. For SEM, the samples were examined in an ETEC Autoscan SEM.

For atomic force microscopy (AFM), erythrocyte membranes were transferred to the alkanethiol-coated supports by either self-assembly or by Langmuir-Blodgett (LB) transfer. The handling of the samples was similar to that described for hydrated phospholipid deposits (Hui et al., 1995). For LB transfer, ghost membranes were spread at the air-water interface by allowing the membrane suspension to drip down a rod onto the surface of a Langmuir trough. The surface pressure was maintained below 10 dyne/cm (He and Hui, 1985). Substrates covered with an alkanethiol monolayer were dipped vertically, either at 90° or at a smaller angle to the

surface through the interface from the air into the water. The cell membrane coated substrate was kept submerged under water for all subsequent operations. The sample was placed under the liquid scanner of a Topometrix Explorer Life Science atomic force microscope, such that a layer of solution was retained between the scanner and the sample. Images were obtained at room temperature using a silicon nitride tip on a cantilever with a spring constant of 0.032 N/m. The typical force applied to the sample was 10 nN, and forces up to 30 nN were applied.

Surface plasmon resonance measurements

Details of the design and calibration of the SPR setup used in our experiments were reported earlier (Silin et al., 1997). The device consists of an optical measuring unit, a flow cell (volume = 30 μl) into which the alkanethiol-coated substrate is mounted, and a peristaltic pump. All operations and data accumulation are controlled with a computer and custom software. A semiconductor laser provides *p*-polarized light at 750 nm. For these experiments, the delay time between measurements was 10 s and the sensitivity of the device was ± 0.6 Å for layers with a refractive index of 1.45. Calibration was performed with solutions of different concentrations of glycerol in water. Erythrocyte ghost suspensions, at a concentration of approximately 1 mg/ml total protein, was pumped through the cell at a rate of 50 μl/min. The change in the angle of the surface plasmon line minimum, indicative of an increase in thickness of the surface layer and corresponding to the association of membrane with the substrate, was monitored in real time. To minimize differences in the refractive index of various solutions, the buffer composition and temperature were kept constant throughout the experiment. For experiments involving neuraminidase and chymotrypsin, premixed solutions of enzyme were introduced into the flow cell as indicated, with thorough buffer washes between the changes of solutions.

Measurement of acetylcholinesterase activity

The alkanethiol substrates were carefully floated on diluted erythrocyte membrane or liposome suspensions in a petri dish to allow self-assembly of the bilayer to take place overnight at 4°C. The unadsorbed membrane material was removed and the surface was thoroughly rinsed with PBS. During rinsing, the surface was kept submerged in the buffer and was not allowed to pass through the air/water interface. For measuring the AChase activity, the PBS solution covering the substrates was replaced by a solution of acetylthiocholine chloride (12.5 mM) in PBS buffer. The formation of product was detected after 30 min by mixing an aliquot of the solution above the sample with Ellman's reagent (10 mM) and measuring the optical adsorbance at 410 nm (Steck and Kant, 1974).

RESULTS AND DISCUSSION

In this study on alkanethiol/erythrocyte membrane hybrids, we have applied a variety of techniques to address two types of questions: 1) the morphology of the resulting surface including thickness of the layer and 2) the orientation of the added cell membrane material and the reactivity of the layer components.

Morphological characterization

Contact angle measurements allowed assessment of the changes in wettability of the resulting surface. A freshly evaporated gold surface is hydrophilic (contact angle = 25° ± 5°; n = 5) but loses its wettability soon after being exposed to room air for several minutes. After a layer of alkanethiol is formed on freshly evaporated gold films, the surface becomes hydrophobic, with a contact angle of 90° ± 10° (n = 5). The surface has a lower contact angle of 40° ± 5° (n = 5) after association of the erythrocyte membrane. The addition of the membrane alters the hydrophobic surface, resulting in a surface with with a more polar character.

Two techniques were used to estimate the thickness of the supported membrane: ellipsometry and capacitance. Optical thickness measurements by ellipsometry on dry samples indicated a thickness of 12.5 Å for a decanethiol and 16 Å for an octadecanethiol monolayer, whereas the layer of erythrocyte membrane material contributes an additional apparent thickness of approximately 40 Å.

We also used the frequency-dependent impedance response as an in situ method for estimating the thickness of the added layer. The capacitance of the dielectric layer (alkanethiol plus cell membrane layer) that covers the electrode can be determined from the magnitude of the impedance response as a function of the frequency. Previous analysis of alkanethiol monolayers and phospholipid/alkanethiol bilayers (Plant et al., 1993) has shown that in the absence of electron transfer the impedance response for these systems reflects bulk resistance and capacitance properties and can be fit with a simple equivalent circuit model consisting of a resistor (the electrolyte solution) in series with a capacitor (the monolayer or bilayer). The alkanethiol/erythrocyte membrane hybrid demonstrated the impedance response shown in Fig. 1, which is similar to that observed for the alkanethiol/phospholipid hybrid, and can be fit with the same equivalent circuit model. The capacitance of the hybrid layer, C, is calculated by

$$Z = R + \frac{1}{j\omega C}$$

$$Z = \sqrt{R^2 + \left(\frac{1}{\omega C}\right)^2}$$

where Z is the impedance magnitude, $\omega = 2\pi f$, f = frequency of the applied AC potential, $j = \sqrt{-1}$, and R is the resistance. The capacitance is inversely proportional to the thickness, d, of the dielectric layer as shown by

$$C_m = \kappa\epsilon_0/d \qquad (1)$$

where C_m is the specific capacitance (normalized for electrode area), ϵ_0 is the permittivity of the free space (8.85×10^{-8} μF/cm), and κ is the dielectric constant of the layer.

The capacitance of the hybrid bilayer is the sum of the inverse of the capacitance of the individual layers. Thus, the capacitance for the POPC or the erythrocyte membrane layer (layer 2) is calculated from the capacitance of the complex (layer 1 plus layer 2) and the capacitance of the monolayer (layer 1) in the following way:

$$\frac{1}{C_{m(layer\,2)}} = \frac{1}{C_{m(complex)}} - \frac{1}{C_{m(monolayer)}} \qquad (2)$$

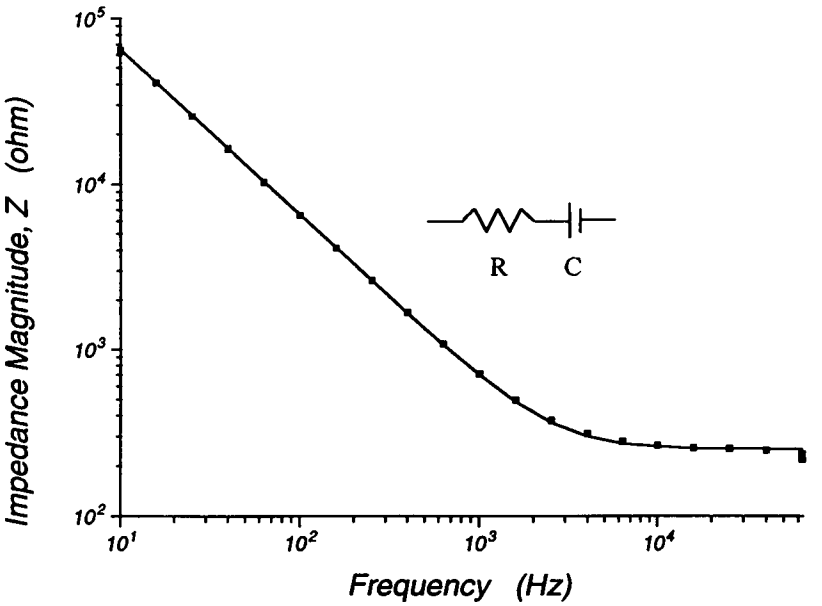

FIGURE 1 Impedance analysis of an octadecanethiol/erythrocyte membrane hybrid layer. Measurements were made in TBS. An AC potential of ±10 mV was applied with a DC potential of 0 V at frequencies from 64,000 to 10 Hz and back to 64,000 Hz. Points indicate the measured impedance at these frequencies (two data points per frequency); the line is the fit to an equivalent circuit model of a resistor and capacitor in series.

Table 1 shows the capacitance values measured for alkanethiol monolayers, alkanethiol/POPC hybrid bilayers, and for alkanethiol/erythrocyte membrane hybrids.

We assume a dielectric constant for alkanethiol of 2.25. Using Eq. 1 and the capacitance values of 1.01 and 1.47 $\mu F/cm^2$ that are reported in Table 1 for the octadecanethiol and decanethiol monolayers, respectively, we calculate that the thickness of the octadecanethiol layer is ~20 Å, and the thickness of the decanethiol layer is ~13.5 Å. The capacitance of the electrode decreases as a result of the formation of the HBM. The contribution of the POPC layer to the capacitance of the alkanethiol/POPC HBM is calculated using Eq. 2. The thickness of the insulating region (i.e., the acyl chain region) of the POPC layer is expected to be ~13 Å based on x-ray crystallography studies of similar lipids (Lewis and Engelman, 1983). Applying a dielectric constant of 2.7 (Plant et al., 1994) for the POPC layer, the capacitance values shown in Table 1 for the alkanethiol/POPC hybrids indicates a thickness of ~11 Å for this layer, regardless of the thickness of the alkanethiol layer. This calculation assumes that the alkanethiol monolayer is unaffected by the addition of the POPC layer, and so total capacitance reflects a sum of the inverse of the capacitance of layer 1 (alkanethiol) and layer 2 (POPC).

TABLE 1 Specific capacitance (C_m) measured and calculated for monolayer and hybrid systems

	Octadecanethiol C_m ($\mu F/cm^2$)	Decanethiol C_m ($\mu F/cm^2$)
Monolayer	1.01	1.47
Alkanethiol/POPC hybrid	0.68	0.96
POPC layer (calculated)	2.1	2.0
Alkanethiol/erythrocyte membrane hybrid	0.76	1.36
Erythrocyte membrane layer (calculated)	3.2	See text

If we examine the capacitance data for the erythrocyte membrane hybrids, this assumption does not appear to be unreasonable when the second layer is the erythrocyte membrane and the first layer is octadecanethiol. The capacitance of the octadecanethiol/erythrocyte ghost hybrid bilayer is ~10% larger than for the corresponding POPC-containing hybrid layer. However, the capacitance of the decanethiol/erythrocyte ghost hybrid is over 40% larger than that of the decanethiol/POPC hybrid bilayer and is nearly equivalent to the capacitance of the monolayer, even though other data indicate that a hybrid structure of significant thickness is formed. This discrepancy leads us to speculate that the assumption that the erythrocyte ghost membrane layer does not perturb the alkanethiol layer may not hold in the case of the decanethiol monolayer. Later in this report, additional data involving electron transport through this hybrid layer will be presented that also suggest perturbation of the decanethiol layer by the erythrocyte ghost membrane layer.

The contribution of the erythrocyte membrane layer to the capacitance of the octadecanethiol/erythrocyte membrane hybrid is similar to but slightly larger than that of a layer of POPC. Assuming a dielectric constant of 2.7 for the added layer, this corresponds to a thickness of the insulating (alkane) portion of the erythrocyte membrane of only ~7.5 Å. One interpretation of this apparent small thickness is that the erythrocyte ghosts form a thinner layer on the octadecanethiol surface than does POPC; however, the ellipsometry measurements indicate that a layer of substantial thickness is added to the surface. Alternatively, it is possible that the ghost membrane material only partially covers the surface or that the dielectric constant for erythrocyte membranes, which contain proteins and are obviously more

complex structures than pure lipid layers, is larger than that for pure lipid layers.

The extent of surface coverage by the ghost membrane was examined using ESEM. In Fig. 2, two samples are shown, an octadecanethiol monolayer sample (at right) and an octadecanethiol/erythrocyte membrane hybrid (at left). The membrane sample appears inhomogeneous in that there are areas of different contrast. The image in Fig. 2 *a* was acquired at 4°C and 5.5 torr. In Fig. 2 *b*, the pressure was increased to 5.6 torr, causing water to condense on the samples. Large, round droplets form on the hydrophobic monolayer, while flat areas of wetting, with shapes distinctly different from the shape of the water droplets on the alkanethiol-only sample, appear on the more hydrophilic membrane-containing sample. ESEM has been used to compare the wetting properties of hydrophobic and hydrophilic filters, where droplet shapes similar to what we have seen here were observed (de la Parra, 1993). As indicated by the contact angle measurements described earlier, the membrane-containing sample is clearly more polar. Importantly, the wetting of the membrane sample appears to be uniform over the entire area of the sample. These images thus indicate that the membrane covers the surface over a large area (hundreds of square microns).

Several other imaging techniques were also used to provide a closer look at the morphology and uniformity of the membrane surface: SEM, TEM, and AFM.

By SEM, the octadecanethiol surface areas exposed to erythrocyte membranes appear rougher and thicker, although the exact thickness of the top layer cannot be determined due to the unknown thickness of sputter coating. There were no visible holes, or any evidence of individual membrane ghosts adhering to the substrate, indicating that the membrane ghosts are spread into extended sheets on the alkanethiol surface (results not shown). In TEM images of the replicas (Fig. 3), the membrane can be seen as darker areas less homogeneous in texture compared with the underlying alkanethiol layer. The area with adsorbed membrane is definitely rougher, and occasionally footprints from fused membrane ghosts and small, unfused membrane vesicles are seen (Fig. 3 *A*). Adjacent alkanethiol surfaces that were not exposed to the membranes were flat and featureless (Fig. 3 *B*). Fig. 3 *C* shows a boundary of erythrocyte membrane deposition area. The membrane material is present everywhere but the upper right corner where "bare" alkanethiol surface is seen. Like the SEM images, these observations indicate that areas exposed to membranes are rougher and thicker. Several vesicles appeared in exposed areas, although they were less than 1 μm in diameter, which is too small to be intact erythrocyte membrane ghosts, which measure approximately 8 μm in diameter. These vesicles may be the remnants of incompletely spread membrane ghosts.

AFM images of these samples observed under buffered solution show that the deposited membrane materials appear to be uniform with a roughness of 1.4 nm. Again, there is evidence for extensive coverage, over an area of 50 $\times$ 50 μm. Occasionally, small spherical vesicle structures of approximately 0.1 μm are observed (Fig. 4 *A*). These images are more consistent with the presence of molecular monolayers than of adsorbed intact membrane ghosts of 8 μm diameter on the surface. To ensure that the material seen on the deposited area is membrane material and is not the substrate, the force applied to the scanning tip was increased stepwise from 10 to 30 nN to perturb loosely adhering material (Fig. 4, *B* and *C*). The purpose of applying larger forces was, first, to find out the depth of removable material and, second, to determine the strength with which the deposited material adheres to the substrate. Fig. 4 *B* shows areas exposed to increasing tip scanning force, from the normal imaging force of 9 nN (lower right) to 16, 22, and 30 nN (lower left, upper right, and upper left, respectively). A close-up of an area scanned with an applied force of 30 nN is shown in Fig. 4 *C*. The height of the scanned area is approximately 5.7 nm below the deposited surface. This thickness is similar to the total thickness of approximately 4 nm measured by ellipsometry for the dry complex of alkanethiol and erythrocyte membrane. Some slight damage was seen from even the 9 nN force scan used for normal imaging. This is, however, the vertical force applied to the tip and should not be confused with the lateral force needed to remove materials from the scanned areas. The lateral force exerted by the torsion of the cantilever was not calibrated in our experiment.

Membrane chemistry and activity determinations

While the various microscopies provide morphological information, they tell us little about the identity of the components of the membrane layer. The data presented thus far indicate that material from the erythrocyte ghost preparations has associated with the surface and covered the surface in an apparently uniform manner. None of these measurements has addressed the question of whether individual proteins and cell membrane fragments randomly associate with the surface or whether the intact cell membrane reorganizes at the surface, depositing membrane components in a concerted manner. In the latter case, we would expect the composition of the hybrid surface to reflect the composition of the erythrocyte ghosts.

SPR was employed to provide information concerning the kinetics of ghost interactions with the surface, and what chemical groups are transferred to the surface. SPR, which is sensitive to the changes in refractive index at a metal surface, allows us to monitor the association of the erythrocyte membranes with the alkanethiol monolayer in real time. The shift of the angular position of the resonance minimum was calibrated for the changes in thickness of the surface layer (Silin et al., 1997). Fig. 5 shows that association of the erythrocyte membrane with an octadecanethiol monolayer-coated substrate reaches a steady state in less than 2 h under conditions of continuous flow. By this method, the apparent increase in the thickness due to the

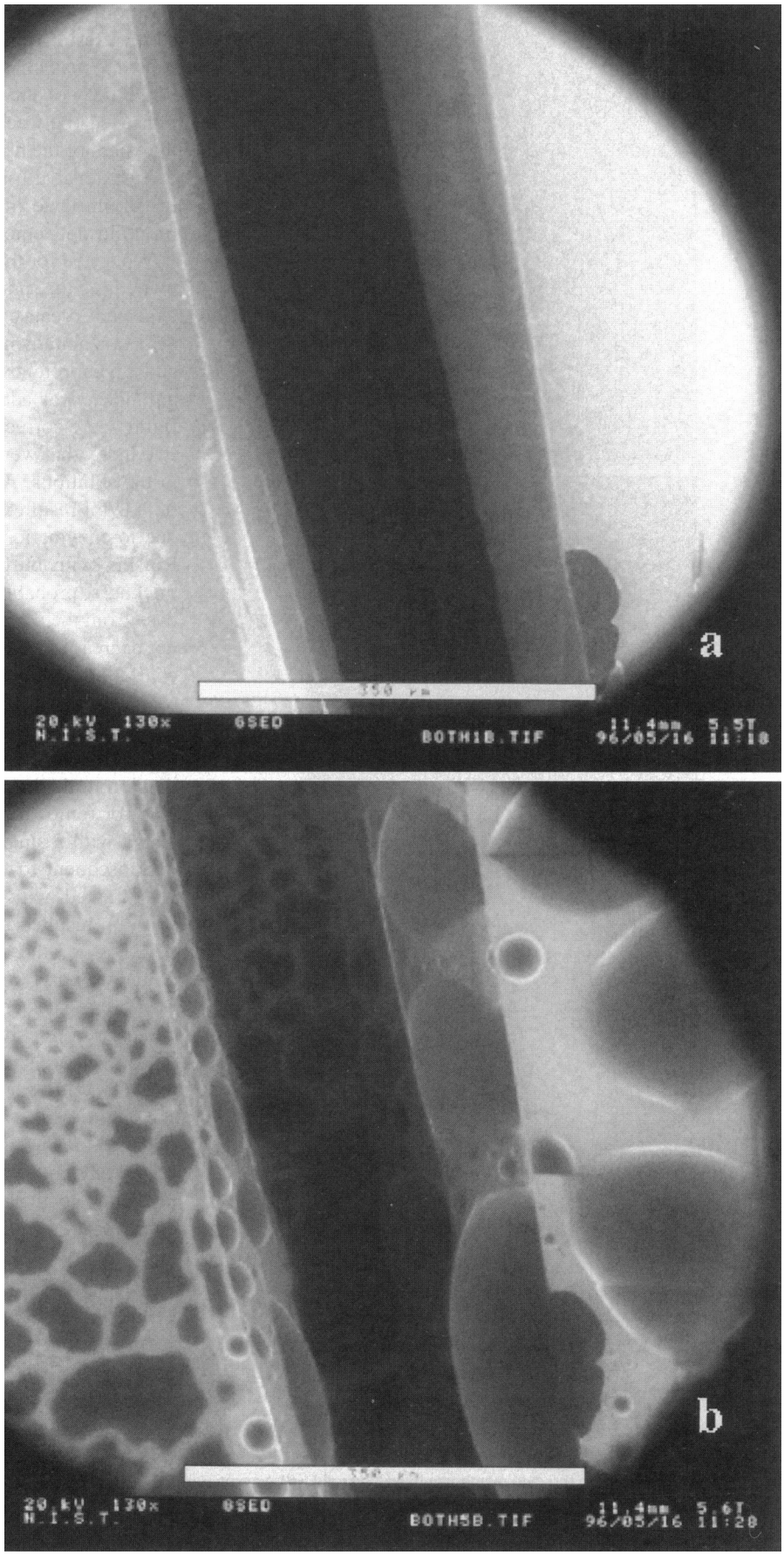

FIGURE 2 Environmental scanning electron micrographs of the erythrocyte membrane hybrid (*left*) and the octadecanethiol monolayer (*right*). (*a*) Before water condensation. (*b*) Shortly after the start of water condensation. Condensation was initiated by increasing the pressure in the chamber slightly.

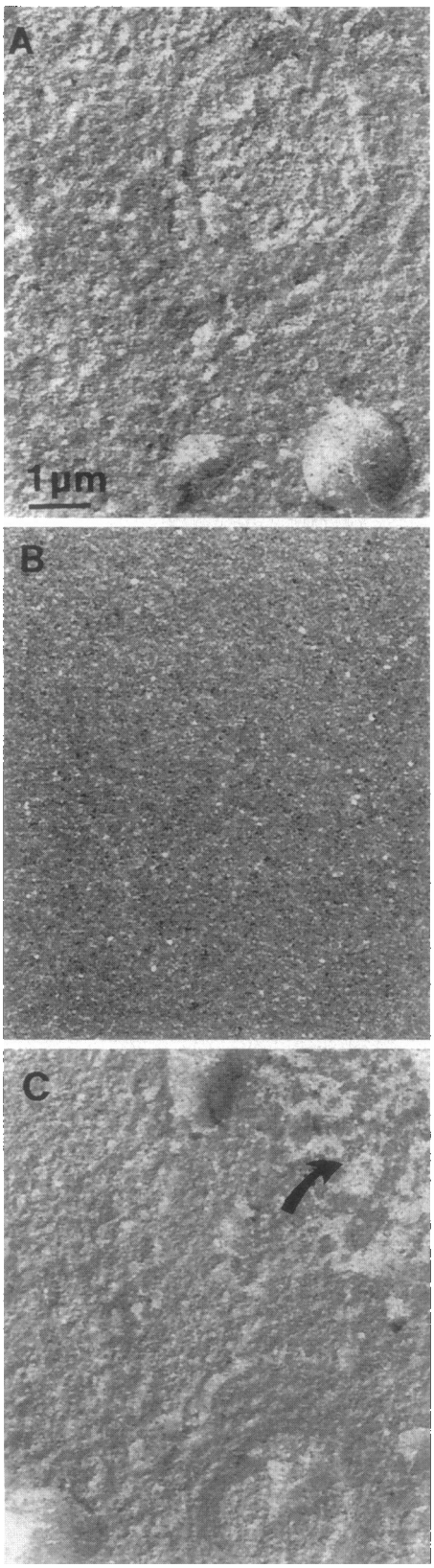

FIGURE 3 Transmission electron micrographs of shadowed replicas. (*A*) Area of octadecanethiol/erythrocyte membrane hybrid on gold. (*B*) Area of octadecanethiol monolayer. (*C*) Edge of an erythrocyte membrane hybrid area and an area of uncovered octadecanethiol (*upper right corner*). Magnification, ×80,000.

addition of membrane material is approximately 30 Å. A similar result of a 27 Å increase was observed when the substrate was coated with a decanethiol monolayer instead. Regardless of the concentration of the membrane preparation used (between 0.2 and 1 mg of total protein/ml), the thickness of the hybrid layer for all measurements was 30 ± 3 Å. Extensive (overnight) flowing of buffer solution over the erythrocyte membrane-covered surface (Fig. 5, dotted line) did not remove the adsorbed material.

We tested for the presence of carbohydrates at the surface by treating the sample, in situ, with neuraminidase. This was expected to cause selective removal of *N*-acetylneuraminic acid, an abundant carbohydrate in membrane glycolipids and glycoproteins. In Fig. 5 (at the second arrow), neuraminidase was added into the flow stream. The data in this figure were corrected for the changes in the solution refractive index that were due to the high concentration of protein in the solutions. Addition of the enzyme at a concentration of 1 U/ml resulted in an apparent decrease in the thickness due to enzymatic cleavage of surface carbohydrate groups. Rinsing with buffer resulted in an additional decrease in thickness, probably by removing weakly bound neuraminidase from the surface. The result was a small but significant net decrease in the thickness of the layer of ~2 Å. Polysaccharides account for approximately 8% of the total erythrocyte ghost composition by dry weight, and acetylneuraminic acid constitutes ~20% of the total saccharides (Rosenberg and Guidotti, 1969). This observation of a reduction in thickness of approximately 2 Å is not unreasonable for removal of the sugar residues that would be associated with a single monolayer of ghost membrane.

Subsequent to the neuraminidase treatment, the surface was treated with 1 mg/ml chymotrypsin. Chymotrypsin addition resulted in an initial dramatic increase in apparent thickness of the surface layer, as the enzyme apparently bound rapidly (and probably with high affinity) to the membrane surface. This increase was then followed by a decrease in thickness over time. The initial and more rapid phase in the decrease in thickness is probably due to proteolytic activity, whereas the slow decrease during washing (beginning at the arrow marked w) is probably the result of both chymotrypsin activity and slow desorption of tightly bound chymotrypsin. The net change in the thickness of the layer due to the activity of chymotrypsin was ~6 Å and confirms the presence of a substantial amount of protein associated with the surface. Treatment with 10 μg/ml chymotrypsin showed a similar result, although the kinetics of, and total increase in, thickness due to chymotrypsin binding was less. The kinetics of decrease in thickness due to proteolytic activity was also slower, although approximately the same amount of material was removed from the surface.

To be useful, a supported membrane formed by this method should exhibit specific activity associated with the parent membrane. The activity of AChase, an erythrocyte marker enzyme that is associated with the outer leaflet of the membrane, was measured after exposing erythrocyte

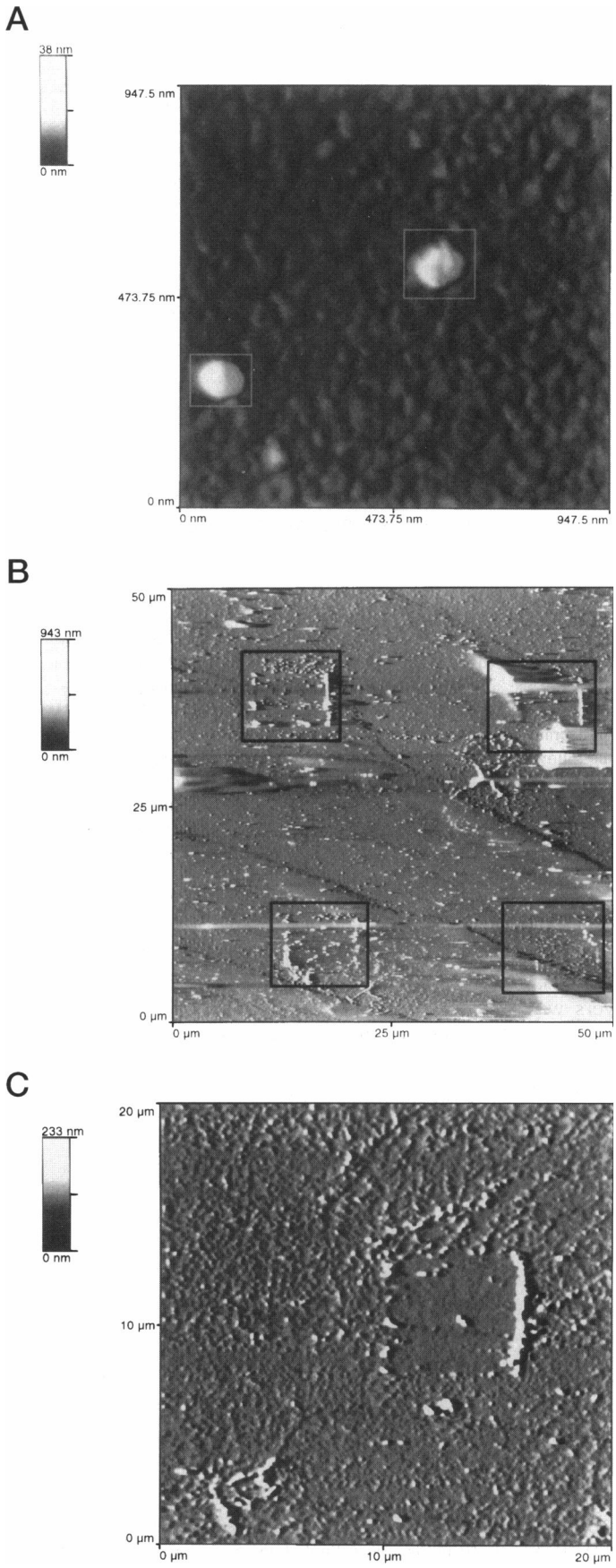

FIGURE 4 Atomic force micrograph of an octadecanethiol/erythrocyte membrane hybrid. (*A*) A 1-μm scan showing small vesicles (~100 nm in diameter) on a rough but evenly covered surface, imaged at the constant force mode with a tip force of 9 nN under liquid. (*B*) A 50-μm scan area of four 10-μm areas (*upper left*, *right*, *lower left*, and *right*, respectively) that were previously and repeatedly scanned with 30, 22, 16, and 9 nN of tip force under liquid. (*C*) Area that was previously and repeatedly scanned with 30 nN of tip force under liquid.

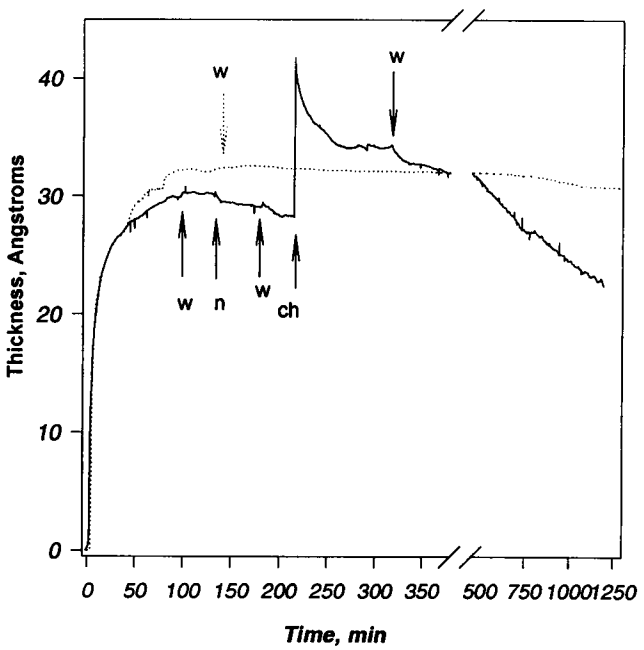

FIGURE 5 Surface plasmon resonance response for two experiments as erythrocyte membranes (0.4 mg/ml protein) were added into the flow stream (time = 0) and allowed to associate with the octadecanethiol surface. For the solid line, the first arrow labeled w indicates the replacement of ghost suspension with buffer solution. At the arrow labeled n, 1 U/ml neuraminidase was injected into the flow stream, followed by buffer at the following w. At the arrow labeled ch, buffer containing chymotrypsin was introduced, followed by buffer only at the following w. Data have been corrected to eliminate the effect of differences in solution refractive index. For the dotted line, a ghost suspension was added at time 0, replaced by buffer solution at the w, and the erythrocyte membrane layer was allowed to remain under flowing buffer overnight.

membranes for 12 h to either octadecanethiol or decanethiol monolayers. AChase activity associated with the hybrid layers was assayed over a period of several days. Assays were done at room temperature, and the samples were stored at 4°C between assays. Fig. 6 shows the activity of AChase associated with the hybrid layers over a period of several days and after extensive washing and handling. Each experimental point was from a surface area of 2.6 cm^2. The activity profile of AChase that is shown here is representative of at least five experiments. The activity was high initially but decreased rapidly with time. The decline in activity is comparatively slower after 2 days.

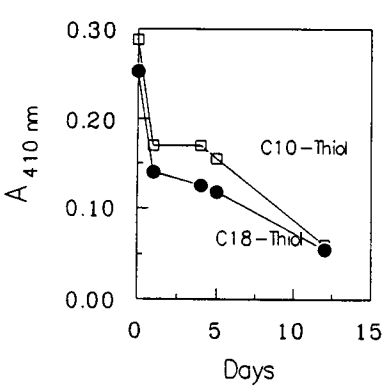

FIGURE 6 The activity of AChase of erythrocyte membranes deposited on decanethiol (C10) or octadecanethiol (C18) SAMs as a function of storage time. These data are representative of at least five replicate measurements.

Cyclic voltammetry also indicated the presence of possible protein-associated ion transport activity (Fig. 7). The potential-dependent current response associated with electron transfer between a bilayer-modified gold electrode and a soluble redox species, potassium ferricyanide, is indicative of the insulating properties of the alkanethiol monolayers and the alkanethiol/phospholipid hybrid layers (Fig. 7 *A*, *inset*). Compared with a bare gold electrode, an electrode covered with an alkanethiol monolayer exhibits significantly reduced current and requires the application of increasingly negative potentials to produce sufficient driving force to reduce ferricyanide. The addition of a phospholipid layer to the alkanethiol layer increases this insulating effect (Plant, 1993; Plant et al., 1994). At a highly insulating electrode, such as a phospholipid/alkanethiol hybrid bilayer, electron transfer does not appear to be diffusion dependent, exhibiting no or little effect of concentration of redox species or scan rate on current or reduction potential. Unlike what we have observed when forming bilayer membranes from phospholipid and alkanethiol, the decanethiol/erythrocyte membrane hybrid surface showed an increase in electron transfer compared with the monolayer and the appearance of electron transfer at a potential closer to its formal potential (Fig. 7 *A*). This redox response was also diffusion dependent (Fig. 7 *B*, *inset*) as determined by altering the rate of the potential scan (Bone and Zaba, 1992). This response suggests that the erythrocyte membrane layer at the surface enables the transfer of electrons between ferricyanide and the gold electrode by allowing the diffusion of ferricyanide closer to the electrode surface. Fig. 7 *B* also shows the result of treatment of the hybrid membrane with 2 mM DIDS, a potent inhibitor of anion transport by Band 3 protein in erythrocyte membranes. After removing the DIDS solution and adding back ferricyanide, it is observed that electron transfer is significantly blocked again. The potential required for reduction is more negative, and the reaction shows less diffusion dependence compared with before DIDS addition. Thus, at least part of the electron transfer at a supported erythrocyte hybrid membrane is blocked by a compound that is known to block protein-dependent anion transporter activity. Although we are unaware of other observations of ferricyanide transport by Band 3, this protein is known to be an anion transporter with broad specificity (Knauf, 1979). In control experiments, DIDS was ineffective at blocking electron transfer at an electrode covered with a discontinuous alkanethiol monolayer.

Supported erythrocyte hybrid membranes formed from octadecanethiol showed much less dramatic increases in electron transfer than did the hybrid membranes formed at decanethiol monolayers. The capacitance data for decanethiol/erythrocyte hybrid membranes (see Table 1) suggest

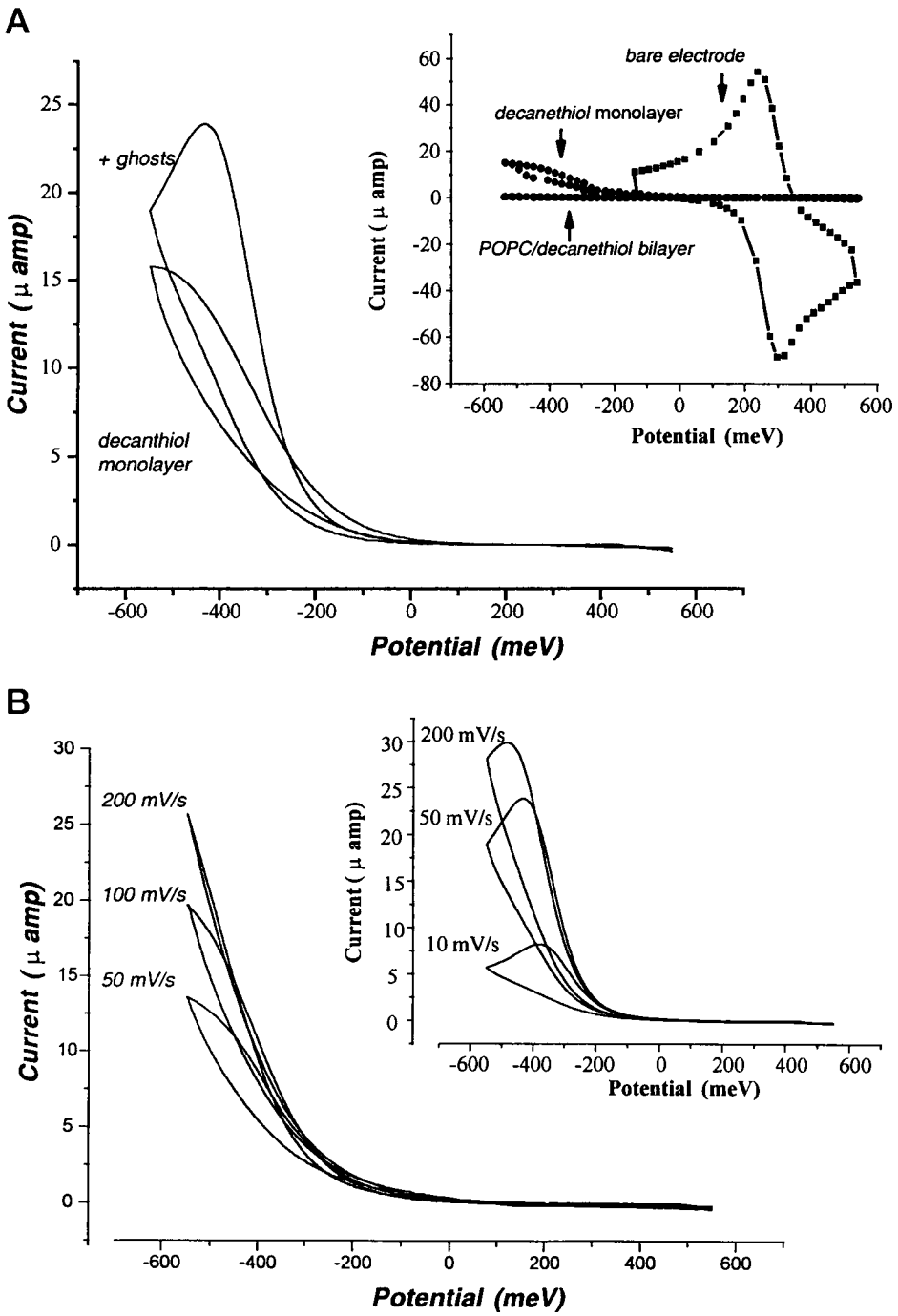

FIGURE 7 Cyclic voltammetry was performed with 1 mM $K_3Fe(CN)_6$ in 1 M KCl. (*A*) Electron transfer at a decanethiol monolayer-covered electrode and after formation of an erythrocyte membrane hybrid by the addition of ghost. The inset shows electron transfer at a bare electrode, a decanethiol-covered electrode, and a POPC/decanethiol hybrid electrode. (*B*) Electron transfer at a decanethiol/erythrocyte hybrid electrode after a 30-min exposure to DIDS and before DIDS (*inset*) at 10, 50, and 200 mV/s.

that erythrocyte membrane components perturb the short-chain alkanethiol layer, as the total membrane capacitance for the hybrid layer is very large. Ellipsometry and SPR data on the hybrid bilayer indicate that apparent thickness of the erythrocyte layer is slightly less on decanethiol compared with octadecanethiol monolayers. However, the large capacitance of the hybrid erythrocyte membrane prepared with decanethiol, and the indication of diffusion-dependent electron transfer, suggests that the decanethiol monolayer is perturbed by the addition of the erythrocyte layer and is no longer the barrier to current that it was before ghost addition.

CONCLUSIONS

Spontaneous fusion of phospholipid vesicles to alkanethiol SAMs offers a convenient way to prepare stable lipid bilayer membranes on solid supports. We demonstrate in this communication that native erythrocyte membrane ghosts

appear to associate with C10- and C18-alkanethiol monolayers in a similar fashion. Our morphological observations are consistent with the addition of a single layer of cell membrane. Measurement of thickness of the adsorbed erythrocyte ghost membrane by ellipsometry, SPR, and AFM suggest that the thickness of the upper leaflet of the hybrid structure is probably between 30 and 40 Å. This is not an unreasonable estimate for the thickness of a single leaflet of an erythrocyte membrane, given the extensive glycocalyx, the conformation of which is not clear, and the presence of transmembrane proteins, all of which contribute to the total thickness of the layer (Vitala and Jarnefelt, 1985). Other data, such as the increase in wettability of the surface and the decrease in the capacitance, also indicate the addition of membranous material to the alkanethiol monolayer surface. Microscopies indicate that the coverage of the surface is apparently continuous.

AChase in erythrocytes is associated with the outer leaflet of the membrane and retains its activity in the alkanethiol/erythrocyte hybrid. This observation suggests the potential for developing this as an approach for sensor fabrication, as this enzyme is a target protein for several classes of inhibitors of commercial value, including pesticides. The sharp decrease in the activity in the first 2 days could be due to denaturation and also degradation of the protein.

As the AChase is an extracellular enzyme, the presence of its activity suggests that, on interaction with the SAM, the outer leaflet of ghost membranes opens up and spreads out, incorporating in this process the enzyme molecules exposed at the cell surface. The SPR evidence of the effect of neuraminidase supports this hypothesis, as the glycocalyx is found only on the external leaflet of the cell membrane. Mechanisms have been suggested for the spreading of liposomes on phospholipid monolayers (Kalb et al., 1992), and for vesicle spreading on a hydrophilic surface, such as glass (Contino et al., 1994). Future experiments directed to determining the composition of the hybrid layer should indicate whether only the outer leaflet or whether both leaflets of the cell membrane associate with the surface. In this study, we aimed only to demonstrate the presence of membrane components and activity in an alkanethiol/cell membrane hybrid. To establish the sidedness of the two membrane leaflets during fusion, and also the role of the cytoskeleton in fusion, additional biochemical characterization will be needed. As erythrocyte membranes have native transmembrane asymmetry in protein composition, they may prove to be a convenient experimental tool to understand the details of fusion of vesicles and cell membranes to a hydrophobic surface. More detailed studies will be needed to confirm that the surface energy associated with the formation of these asymmetric hybrid bilayers in aqueous buffers is sufficient to open up cell membrane bilayers, which have an excess of 50% of protein by weight, and that the extensive network of cytoskeleton present in erythrocyte membranes is not a hindrance for membrane-alkanethiol monolayer fusion.

The observation of electron transfer in the erythrocyte-hybrid membrane, and its blockage by DIDS, suggests that Band 3 protein activity may be associated with these layers. Details of how a transmembrane protein such as Band 3 might be located in this film need to be further explored. We intend to pursue future studies with an alkanethiol derivative that is more accommodating to transmembrane proteins. Successful incorporation of the transmembrane chloroplast ATPase was achieved in a lipid bilayer at a gold surface by in situ derivatization of a sulfur-terminated pentaalanine (Naumann et al., 1995). Other approaches include thiol-derivatized phospholipid molecules (Lang et al., 1994; Duschl et al., 1994, 1996).

Our observations suggest that stable biomimetic surfaces can be produced directly from isolated cell membranes. The presence of AChase activity after extensive washing and handling suggests that the association of the erythrocyte membrane with these surfaces is very strong. Even deliberate passage of these surfaces through the air-water interface did not result in the loss of activity (data not shown). Post-fusion recovery of the enzyme activity, which is sensitive to the physical properties of the surrounding lipid bilayer, is a promising result in an attempt to fuse and transfer membrane-associated activities on to solid supports. The simplicity and versatility of this method may enable devices to be made from many molecules that are difficult to manipulate once removed from their environment. Such methodology would be of practical interest as the tedium of membrane protein purification could be circumvented, and the membranes could be enriched with designed proteins in their native lipid matrix. For studies of multicomponent systems, such as energy- or signal-transducing systems in membranes and in fabricating biosensors based on these principles, such a model system would be well suited given the amenability of performing electrical and optical measurements directly.

Ellipsometric measurements were performed with the courtesy of Dr. Robert Baier, State University of New York at Buffalo.

This work was supported partially by grant GM 30969 from the National Institutes of Health (to S.W. Hui). N.M. Rao is supported by an Indo-U.S. Science and Technology Fellowship administered by the Academy for Educational Development during his stay in the U.S.

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