AD-A237 605

(2)

DOCUMENTATION PAGE

Form Approved
OMB No 0704 0188

1a REPORT SECURITY CLASSIFICATION: U	1b RESTRICTIVE MARKINGS: NA
2a SECURITY CLASSIFICATION AUTHORITY: NA	3 DISTRIBUTION/AVAILABILITY OF REPORT: Distribution Unlimited
2b DECLASSIFICATION/DOWNGRADING SCHEDULE: NA	
4 PERFORMING ORGANIZATION REPORT NUMBER(S): TSI Mason Research Institute #4193-6	5 MONITORING ORGANIZATION REPORT NUMBER(S): NA
6a NAME OF PERFORMING ORGANIZATION: TSI Mason Research Institute; 6b OFFICE SYMBOL (If applicable): Biochemistry	7a NAME OF MONITORING ORGANIZATION: Office Of Naval Research
6c ADDRESS (City, State, and ZIP Code): 57 Union Street, Worcester, MA. 01608	7b ADDRESS (City, State, and ZIP Code): 800 N. Quincy Street, Arlington, VA 22217-5000
8a NAME OF FUNDING/SPONSORING ORGANIZATION: Office of Naval Research; 8b OFFICE SYMBOL (If applicable): ONR	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: N00014-88-C-0193
8c. ADDRESS (City, State, and ZIP Code): 800 N. Quincy Street, Arlington, VA 22217-5000	10 SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO: 61153N; PROJECT NO: 1113ES; TASK NO: 413K003; WORK UNIT ACCESSION NO:

11 TITLE (Include Security Classification)
Surface-Bound Biomembranes Incorporating Receptors: Electrochemical and Structural Characterization

12 PERSONAL AUTHOR(S)
Nancy W. Downer, Jianguo Li, Elizabeth M. Penniman and H. Gilbert Smith

13a TYPE OF REPORT	13b TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Technical	FROM _____ TO _____	91.6.6	30

16 SUPPLEMENTARY NOTATION
Submitted to Biosensors & Bioelectronics

17 COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	AC Impedance, FTIR, Alkylsilane, Lipid, Receptor Protein, Electrode, Surface Chemistry, Biosensor

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

A method is described for forming stable surface-bound structures that incorporate membrane receptors in a lipid bilayer environment. These structures were formed by dialysis of detergent-solubilized biomembranes in the presence of two alkylsilanated substrates: Si/SiO_2 and glass beads. Electrochemical analysis of the capacitance was used to determine an apparent thickness and the degree of surface coverage at each stage in the deposition process. Similarly, elemental analysis on glass beads was used to quantitate the carbon load. Glass beads substrates were also examined by FTIR spectroscopy to evaluate the alkylsilanated substrate before and after dialysis. A semiquantitative measure of hydrocarbon deposition was derived from the FTIR spectra and compared with the electrochemical and analytical data. In this study, octadecyltrichlorosilane (OTS) and dimethyloctadecylchlorosilane (DMOCS) were used to produce primed substrates with full and partial ... (con't)

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	U
22a NAME OF RESPONSIBLE INDIVIDUAL: Dr. R.J. Nowak/Dr. I. Vodyanoy	22b. TELEPHONE (Include Area Code): (703) 696-4409/4056; 22c OFFICE SYMBOL: ONR

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

U

CONTINUATION OF BLOCK #19:

monolayers, respectively. Reconstitution was performed with vertebrate rhodopsin (Rh) and acetylcholine receptors (AchR). Both receptors could be incorporated in structures with dimensions similar to bilayer membranes at a surface density one order of magnitude less than that for native membranes.

OFFICE OF NAVAL RESEARCH

Contract N00014-88-C-0193

R&T Code 413k003

Technical Report No. 6

Surface-Bound Biomembranes Incorporating Receptors: Electrochemical and Structural Characterization

by

Nancy W. Downer, Jianguo Li, Elizabeth M. Penniman and H. Gilbert Smith

Submitted to

Biosensors & Bioelectronics

TSI Mason Research Institute
Biochemistry Department
57 Union St.
Worcester, MA 01460

June 6, 1991

INTRODUCTION

Integral membrane proteins play a central role in many biological processes that involve transduction, either of environmental stimuli or of signals generated internally by organisms for cell regulation. Because of these roles, integral membrane proteins are prime candidates for incorporation into biosensors that could be used for environmental monitoring, drug development, medical diagnostics, and industrial process control. However, a major hurdle for the development of such biosensors is the coupling of the biological transducing element with an electronic device. This coupling requires an interface that provides an environment supportive of biological function and also allows electrical read-out from the membrane protein.

We report a generic method to accomplish this coupling. It relies on the covalent attachment of an alkylsilane layer to the substrate and the self-assembly properties of membrane lipids and proteins to form the membrane-like structure. This approach to forming a surface-bound biomembrane should allow any membrane protein or receptor amenable to detergent dialysis to be coupled to a substrate surface. The resulting structure has considerable potential for long-term stability and for maintaining receptor function. Successful reconstitution by detergent dialysis has been demonstrated for a wide variety of receptors (Darszon,1983; Levitzki, 1985). Here it is used to form planar structures that are potentially more stable and reproducible than the bilayer lipid membranes (BLM) formed by Langmuir-Blodgett and other techniques (Krull & Thompson, 1985; Montal & Mueller, 1976; Tiede, 1985; Woodbury & Miller, 1990), while still being accessible to electrical measurement.

The emphasis in this report is on the physical characterization of the surface-bound biomembrane structures. Formation of such structures on the surface of glass beads provides samples large enough for

chemical analysis. A variety of techniques, including electrochemical analysis of capacitance, FTIR spectroscopy, and elemental analysis are used to probe the structure and composition of the surface-bound biolayers formed on Si/SiO_2 electrodes and glass microspheres. Our results support the conclusion that the method leads to the formation of a continuous surface-bound matrix consisting of a hydrocarbon/phospholipid bilayer into which proteins have been incorporated during the deposition process. Work reported here involves two integral membrane proteins: the vertebrate visual receptor, rhodopsin (Rh), and the nicotinic acetylcholine receptor (AChR) from the torpedo fish (*T. Nobiliana*). Both membrane receptors can be stably incorporated by use of the silane-based reconstitution technique.

EXPERIMENTAL

Membrane Receptor Preparations. Rhodopsin-containing disk membranes were isolated from bovine retinas (J.A. Lawson, Co., Lincoln, NE) as described by Smith & Litman, 1982. Rhodopsin (Rh) concentration was determined from the ΔA_{500} upon bleaching for octylglucoside-solubilized extracts. Disk membrane preparations employed for dialysis had A_{280}/A_{500} ratios in the range 2.3-2.8. Membranes enriched in nicotinic acetylcholine receptors (AChR) were prepared from the electric organ of torpedo fish, *T. nobiliana*, (Biofish, Assoc., Georgetown, MA) by sucrose gradient density centrifugation (Sobel et al, 1977) and alkaline extraction (Neubig et al, 1979). AChR concentration was determined from the specific binding of [^{3}H-propionyl]-Bungarotoxin (Amersham, Corp., Arlington Heights, IL) measured in a filter assay (Dolly & Barnard, 1977).

Substrates. The Si/SiO_2 electrodes were 5 mm square chips cut from n-type and p-type silicon wafers (EG&G Reticon, Inc., Sunnyvale, CA) coated on one side with a layer of SiO_2 960 Å thick. The ohmic contact was provided on the reverse side by a gold layer to which leads were attached with silver epoxy (Epoxy Technology, Inc., Billerica, MA). The electrode assembly was sealed in glass tubing with the oxide surface exposed. Glass beads (37 μm diameter) were purchased from Ferro, Inc. (Jackson, MS).

Alkylsilanization of Substrates. Octadecyltrichlorosilane (OTS) or dimethyloctadecylchlorosilane (DMOCS) solutions (v/v %) were freshly prepared each time by diluting silane reagent in anhydrous solvent I (80:12:8, n-hexadecane/carbon tetrachloride/chloroform) under argon in a plastic glove bag (Atmosbag from Aldrich Chemical Co., Milwaukee, WI). Si/SiO_2 electrodes or glass beads were dried overnight at 100 °C under vacuum before alkylation. Substrates were reacted by immersion in the silane solution at room temperature under dried argon with occasional agitation. Reactions were terminated by

rinses with solvent I followed by chloroform and the substrates were cured at 100 °C under vacuum for 10-12 hr.

Assembly of Biomembranes on Alkylsilanized Substrates. Alkylsilanized substrates (glass beads or electrode surfaces) were placed in one compartment of a custom-built flow dialysis unit containing the detergent-solubilized membrane preparation. The volumes of both the upper chamber, containing substrate, and the lower one for flowing dialysate were 2.5 ml. In a typical experiment, the membrane receptor concentration was adjusted to 0.5-1.0 mg/ml and solubilized in 30 mM octylglucoside, 100 mM KCl, 20 mM HEPES buffer, pH 7.5. Dialysis was across a membrane with 3500 MW cutoff (Spectra/Por 6, Spectrum Medical Industries, Los Angeles, CA) against a continuous flow of HEPES/KCl buffer. A flow rate of 4 ml/min was used to remove detergent for 20 hr.

After dialysis, electrodes were removed from the chamber, rinsed once in buffer, then transferred to an electrochemical cell for capacitance measurements. Glass beads were collected by twice centrifuging through a 50% sucrose solution to separate them from vesicles or membrane fragments, which floated at the sucrose/buffer interface. For detergent extraction or receptor binding assays, the bead samples were washed once in buffer. For FTIR analysis, an additional wash in distilled H_2O was followed with drying at 60 °C for 1 to 2 hr.

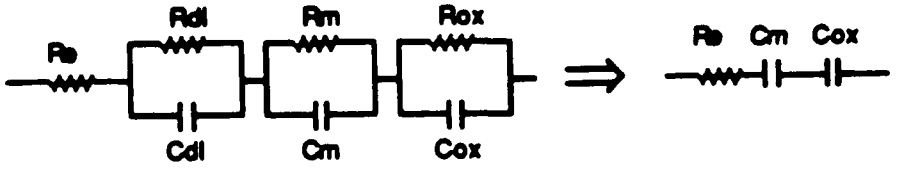
Compositional Analysis. Carbon loading of treated glass bead substrates was assessed by elemental analysis for carbon performed on a 440 CHN/OS Analyzer (Control Equipment/Leeman Labs, Lowell, MA). Samples of approximately 35 mg were run in triplicate or quadruplicate for each substrate type and/or treatment.

FTIR spectra were recorded on a Cygnus 100 Spectrometer (Mattson Instruments, Inc., Madison, WI); UV-Vis spectra, on a Shimadzu UV-265 spectrometer. To obtain FTIR transmission spectra,

pproximately 10-30 mg of beads were pressed between NaCl windows and then infused with CCl_4. Bead pectra were analyzed after subtraction of the CCl_4 spectrum to minimize the solvent peaks at 1540, 1220, nd 980 cm^{-1}. For comparison of hydrocarbon loading between samples varying in size, the parameter R_{3000} was calculated by taking the ratio of the integrated intensity from 3000 - 2750 cm^{-1} to ΔA_{900}, the bsorbance at 900 cm^{-1} corrected for the background at 2300 cm^{-1}. Plots of log R_{3000} vs. ΔA_{900} were fitted vith a linear regression program . All values for R_{3000} reported in tables correspond to a $\Delta A_{900} = 0.5$. The parameter R_{3700} could be analyzed analogously to R_{3000} to monitor the state of surface silanols (Downer *t al*, 1991). The integrated intensity from 3700-2500 cm^{-1} was used with correction for the superimposed ıydrocarbon peak if necessary.

To determine receptor spacing on dialyzed glass beads, samples of about 400 mg of dialyzed beads vere separated from the vesicle fraction as described above. For determination of Rh incorporation, vashed beads were extracted with 30 mM octylglucoside and the ΔA_{500} upon bleaching was used to :alculate the amount of surface-bound Rh per weight of beads extracted. Surface density of AChR ncorporated by dialysis was determined by a modification of the assay for specific α-bungarotoxin binding.

Electrochemical Analysis. Surface structures on the Si/SiO_2 electrodes were analyzed by AC mpedance techniques (MacDonald, 1977) with the instrumentation shown in Figure 1. A small amplitude ≈ 10 mv peak to peak) sinusoidal AC function was superimposed on the DC potential applied to the nembrane electrode and a lock-in amplifier used to measure the phase difference and ratio of peak values ɔetween the input voltage and output current waveforms. With the membrane electrodes studied here, the mpedance was almost purely capacitive and was evaluated from the quadrature component of the total mpedance. The capacitance analysis was based on the following equivalent circuit:



where R_e is the electrolyte resistance; C_{dl}, the double layer capacitance; R_{dl}, the double layer polarization resistance; C_m is the membrane capacitance; R_m, the membrane resistance; C_{ox}, the oxide layer capacitance; and R_{ox}, the oxide resistance. R_{dl}, R_m, and R_{ox} are assumed to be sufficiently large under the conditions employed here that they make a negligible contribution to the measured impedance. Since the double layer capacitance (20-40 $\mu F/cm^2$) is at least an order of magnitude greater than that expected for the membrane or oxide, the resulting capacitive circuit can be reduced to the series capacitors as shown above.

RESULTS AND DISCUSSION

To form surface-bound matrices incorporating AChR or Rh, detergent dialysis was performed on substrates primed by the covalent attachment of long (C_{18}) hydrocarbon chains. In this procedure, the hydrophobic surface can serve as the initiation site for formation of surface-bound bilayer structures containing membrane proteins, while excess lipid and protein will form free-standing vesicles.

Substrates of different hydrophobicity were tested for their efficacy in supporting the formation of surface-bound biomembranes. OTS-reacted substrates have been demonstrated to bear a densely packed monolayer of C_{18}-chains giving effectively complete surface coverage, whereas DMOCS-reacted surfaces carried 30-80% as much carbon and were more accessible to hydrophilic probes (Downer et al, 1991). These various surfaces may show differences in their ability to support receptor incorporation and retention of biological function.

The dialyzed substrates were examined by several physical techniques to provide evidence for

incorporation of membrane material at the silanized surface. Specific determinations of rhodopsin or acetylcholine receptors were used to evaluate the efficiency of incorporation for the protein receptors.

FTIR and Compositional Analysis

Figure 2A shows FTIR spectra for OTS-reacted glass beads before and after dialysis to incorporate membrane receptors (AChR). The most obvious changes observed in the spectrum after dialysis are 1) an enhancement in the intensity of the complex hydrocarbon stretching bands between 3000 and 2750 cm^{-1} and 2) an enhancement of the very broad band extending from 3700 cm^{-1} down to about 2500 cm^{-1}. This latter band reflects both adsorption of water and surface silanols in various states of hydrogen-bonding (McDonald, 1958) and the increased intensity is consistent with hydration resulting from dialysis. Figure 2 also shows the expanded hydrocarbon region (panel B). For this experiment, there was a greater than two-fold increase in the integrated intensity of the hydrocarbon absorbance after dialysis as well as a change in the overall character of the complex band. The spectrum appears to reflect the superposition of phospholipid hydrocarbon incorporated by dialysis on the C_{18} spectrum of silane hydrocarbon.

Difference spectra reflecting the incorporation of material by reconstitution onto OTS-reacted beads are displayed in Figure 3 for different systems including vertebrate retinal disk membranes containing rhodopsin (Rh), AChR-enriched membranes, and synthetic phospholipid (DMPC). Each difference spectrum was obtained by subtraction of the silanized bead spectrum from the dialyzed bead spectrum to flatten the plateau region resulting from glass absorption below 2000 cm^{-1}. The difference spectra displayed are for a comparable sample size with ΔA_{900} close to 0.3. For each dialyzed sample, the residual hydrocarbon absorption reflecting the deposition of lipid has broad maxima centered at 2943, 2888, and 2835 cm^{-1}. However, there appeared to be more phospholipid incorporated by DMPC-dialyzed beads than for either membrane-dialyzed sample (see also below). The amplitude of the large, broad band centered at

3300 cm^{-1} varied significantly for the three samples, apparently reflecting different amounts of residual hydration after the washing and drying procedure. A meaningful comparison of this feature would require equilibration of the samples at controlled humidity before FTIR analysis.

For the experiments with Rh- and AChR-containing membranes, the difference spectra also suggest there are positive peaks in the region from about 2000 cm^{-1} to 1400 cm^{-1}, which encompasses the frequencies for protein amide bands. These features were not observed in experiments with phospholipid alone. Although specific identification of peaks is complicated by potential artifacts from the broad silica band, from water absorption bands, and from subtraction of the solvent band around 1540 cm^{-1}, the difference spectra suggest that with other sampling techniques, FTIR can be used to monitor protein incorporation from reconstitution.

To quantify phospholipid incorporation, carbon content has been determined by elemental analysis on glass bead samples at various stages in the process of biomembrane formation from Rh-containing photoreceptor membranes. These results are summarized in Table I together with the FTIR parameter, R_{3000}, that is used as a semi-quantitative measure of hydrocarbon incorporation. The first part of Table I records experiments in which substrates were prepared using silane concentrations of $\leq$ 1% and reaction times of 1 hr. Both carbon loading and R_{3000} were determined for control beads, the silanized substrates, and four DMOCS-reacted samples after dialysis with Rh-containing membranes. R_{3000} correlates reasonably well with the elemental analysis although the relationship is not linear above about 250 μg carbon/g beads where the FTIR parameter falls off. The uncertainty is also quite large for the FTIR parameter, as illustrated in the second part of the table.

The second part of Table I shows data from experiments in which conditions of the silanization step were varied in order to optimize the surface coverage. The values of carbon loading by elemental analysis

and R_{3000} reported for 4% DMOCS and 2% OTS substrates are the means from three batches of silanized beads. The carbon loading measured for 2% OTS-reacted beads appears to be a limiting value and is consistent with full monolayer coverage of the surface (Downer *et al*, 1991). Reconstitution of Rh-containing membranes onto the 1% DMOCS-reacted substrate yields almost twice as much carbon as for the 2% OTS-reacted beads, indicating that phospholipid is incorporated to form the equivalent of a hydrocarbon bilayer. As the 1% DMOCS reaction does not provide complete surface coverage, these results suggest that phospholipid and protein are added to fill in the gaps in the silane layer.

Also listed in Table I are the results from FTIR analyses comparing the hydrocarbon incorporation resulting from dialysis with photoreceptor membranes vs. synthetic phospholipid onto 2% OTS and 4% DMOCS substrates. Figure 4 shows representative plots used to determine values of R_{3000} for Table I. These data indicate an approximately 3-fold increase in hydrocarbon content for dialysis of photoreceptor membrane onto substrates with incomplete silane layers (DMOCS at $\leq$ 1% in the silanization step). For the substrates with enhanced surface coverage (4% DMOCS and 2% OTS reactions) the increase in carbon content upon dialysis was 1.5 to 1.6-fold. By the R_{3000} measure, substantially more hydrocarbon appeared to be incorporated when the dialysis was conducted with phospholipid (DMPC) alone. A comparison of the FTIR data with capacitance measurements discussed later in the paper suggests that this results from vesicles adhering to the beads rather than formation of multiple lipid layers.

Capacitance Analysis of Biomembrane Formation

Electrochemical techniques provide a means for acquiring detailed information about the molecular structure at an electrode surface. To take advantage of this for characterizing the membrane-like structures produced by dialysis, semiconductor/oxide electrodes (Si/SiO_2) were chosen as a substrate with surface

chemistry similar to that of glass beads.

Electrodes were subjected to the same silanization reactions and dialysis procedures as were glass beads. Capacitance values of the separate deposited layers were determined from the quadrature component of the impedance at a bias potential chosen such that the silicon substrate is in the accumulation condition and, therefore, conductive. For this condition, the space charge capacitance of the semiconductor is eliminated and the measured capacitance reflects the oxide layer in series with the various deposited layers in the presence of electrolyte.

Figure 5 shows traces of the capacitance for the bare Si/SiO_2 electrode surface, AChR-dialyzed OTS-treated surface, and finally the electrode washed with chloroform and/or octylglucoside to remove membrane components (hydrated OTS-treated surface = $Si/SiO_2/OTS$). The capacitance of each layer was calculated from the relationship for series capacitors:

$$\frac{1}{C_T} = \frac{1}{C_{SiO2}} + \frac{1}{C_{layer}}$$

where C_T is the total measured capacitance for the silane- or membrane-coated electrode, C_{SiO2} is the capacitance measured for the bare (oxidized) electrode, and C_{layer} is the capacitance of the structure incorporated at the surface after silanization alone or after dialysis. The thickness of each layer was calculated from the capacitance by use of the relationship $C = \epsilon\epsilon_0/d$ by assuming a value of 3 for the dielectric constant. A similar capacitive system has been used to detect antigen binding to substrate-bound antibodies (Bataillard et al, 1988).

Table II summarizes values of silane layer and biomembrane thickness derived from capacitance data for electrodes reacted with OTS or DMOCS and subsequently dialyzed with receptor-containing

membrane (Rh or AChR) or with phospholipid (DMPC) alone. The results are presented as mean values of the apparent layer dimensions from measurements on a set of n electrodes. The dimensions tabulated in Table II assume full coverage of the surface with an insulating layer (hydrocarbon or hydrocarbon/protein). A value for the fractional coverage, $\Theta = d/d_0$, is also calculated where d is the apparent thickness determined as described and d_o is the thickness of an ideal, complete layer, 25.7 Å and 50 Å for a C_{18} monolayer (silanized substrate) or bilayer (dialyzed substrate), respectively. Although this simple model cannot be expected to strictly describe the complex case of the receptor/lipid matrix, O values provide a useful way to compare the systems.

First, it can be seen from the calculated dimensions of the silane layers reported in Table II, that OTS-reacted beads generally carried a surface layer equivalent to a complete C_{18} monolayer whereas DMOCS-reacted beads were fractionally covered. After dialysis with membrane lipids and receptors (AChR and Rh), apparent surface coverage ranged from 0.7 to 1.3 depending upon the substrate. That the total structures generally yield calculated surface coverages less than one may reflect inaccuracies in the assumed electrode areas, dielectric constant, or ideal layer thickness. They may also indicate defects in the layers arising from the insertion of receptor proteins. However, these capacitance measurements provide support for the picture, developed from data shown in Table I, that reconstitution by dialysis onto a silane-primed substrate surface leads to the formation of a relatively complete surface-bound biomembrane with dimensions similar to that of a natural membrane.

In most experiments, there was good correspondence between the degree of surface coverage estimated from the FTIR parameter, R_{3000}, and that measured by capacitance for parallel experiments on glass beads and Si/SiO_2 electrodes. However, there is an discrepancy between the high phospholipid incorporation indicated for DMPC dialysis by FTIR analysis (Table I) and the incomplete coverage

observed with the capacitance analysis. This suggests that the phospholipid detected by FTIR may be in vesicles adhering to the surface rather than in a surface-bound multilayer. The vesicles would not electrically insulate the surface, but would contribute to the hydrocarbon absorption in FTIR spectra. This finding raises the possibility that the presence of integral membrane proteins such as Rh and AChR during the dialysis procedure influences the membrane formation process and may enhance the formation of single bilayer structures.

Evaluation of Receptor-containing Biomembranes.

The focus of studies reported here is to characterize surface-bound biomembranes and determine factors that influence both receptor incorporation and intactness of the bilayer. Experiments comparing the efficiency of membrane receptor and phospholipid incorporation for different DMOCS- and OTS-reacted substrates are summarized in Tables III and IV.

The surface density of receptors incorporated after detergent dialysis was determined for AChR by assaying the α-bungarotoxin binding sites and for Rh by use of the absorbance difference produced by bleaching of a total extract from the glass bead surface. Results in Table III are the mean values of the intermolecular spacing from all experiments on OTS vs. DMOCS substrates. Although the spacings determined for reconstitution on OTS-reacted substrates are smaller than those for DMOCS-reacted beads, the error in these determinations is $\pm$ 30%. Thus there is not a statistically significant difference between the two substrate types. Nor could these measurements discriminate differences between particular reaction conditions used to prime substrates with either reagent.

The data were also used to calculate a surface density that could be compared with that for the receptor in its natural membrane. Our results indicate that for both Rh and AChR, incorporation by

dialysis results in densities that are roughly 3-10% that of the original membranes. For the case of AChR, this is at least an order of magnitude greater than the density of receptors achieved by adsorbing receptor-containing vesicles onto a lipid coated, planar substrate (Eldefrawi, M.E. et al, 1988).

The results on phospholipid incorporation are summarized in Table IV. They are reported as the ratio of R_{3000} values for dialyzed sample relative to the appropriate silanized substrate. Thus a value close to 2 is expected for substrates with full monolayer coverage and a slightly higher value would result if gaps in the substrate silane layers were to be filled in with membrane phospholipid. Control dialysis with octylglucoside (OG) alone did not lead to enhancement of this ratio. For reconstitution with DMPC alone, high levels of phospholipid incorporation were observed (ratios greater than 3.0), but these were reduced to values close to 1.0 if the glass beads were spun twice on a sucrose gradient and subjected to 4 or 5 buffer washes (DMPC,stripped). The more rigorous treatment did not reverse the phospholipid incorporation observed for reconstitution with receptor-containing membrane preparations.

Experiments reported for dialysis with AChR and Rh illustrate some general observations on the reconstitution procedure for forming surface-bound biomembranes. Although incorporation of membrane components, receptor and/or phospholipid, was detected in all experiments, there is considerable variability from run to run. A probable cause for some of this is the variation in silanization of substrates. Of 3 experiments with AChR, one indicated no phospholipid incorporation by the R_{3000} measure, although there was still evidence for surface bound-receptor within the limits of uncertainty reported in Table III. In experiments with the vertebrate photoreceptor system, there is a suggestion of more efficient incorporation of phospholipid (Table IV) and Rh with DMOCS-reacted substrates prepared at lower concentration of silane reagent. However, this difference between DMOCS and OTS has not been observed for the conditions that provide more complete silanization.

SUMMARY AND CONCLUSIONS

Two substrate systems that are amenable to analogous silane chemistry provided the basis for characterizing the properties of a novel membrane-like matrix that can be used generically to incorporate membrane receptors at biosensor surfaces. Both rhodopsin and nicotinic acetylcholine receptors could be incorporated into these structures at surface densities that are 3- 10% of those found for native membranes.

Elemental analysis applied to the stepwise process carried out with glass bead substrates showed that self-assembly of membrane components by detergent dialysis adds phospholipid carbon to roughly the equivalent of bilayer coverage on the substrate surface. Electrochemical analysis of the Rh and AChR-containing biomembranes formed at the surface of Si/SiO_2 electrodes provides a structural picture of the surface-bound matrix that is complementary to the chemical analysis. The dimensions of the surface layer (35 -65 Å) determined from capacitance measurements were compatible with a bilayer model. OTS-based systems resulted in a thicker or more intact insulating layer at both stages of the procedure.

FTIR spectroscopy proved useful as a means of qualitatively assessing the substrate and the surface-bound matrix. Significant differences could be observed in surface hydration depending on receptor type, sample history and whether the silane layer was formed from DMOCS or OTS. FTIR spectroscopy also proved to be a convenient technique for providing semi-quantitative estimates of the C_{18} silane and/or phospholipid hydrocarbon incorporation at each stage of the procedure.

By bringing to bear a variety of techniques, we have demonstrated surface-bound biomembranes structures that incorporate receptor proteins. The dialysis-based reconstitution method for forming these structures appears to have potential as a generic means for coupling membrane receptors to planar substrates in biosensor development. The correlation of FTIR data with capacitance measurements and

compositional analysis establishes that FTIR analysis of glass beadscan be used as a rapid and practical method for monitoring the deposition of membrane components for biosensor method development.

Although there was not a significant difference between DMOCS- and OTS-reacted substrates in terms of their ability to support receptor incorporation for the two membrane systems studied here, preliminary results have suggested that DMOCS-reacted substrates have an advantage for the reconstitution of rhodopsin's physiologic function, to mediate the light-activation of cGMP-phosphodiesterase. The system described here can be applied to a wide variety of membrane receptors to elucidate the factors that are important for functional reconstitution in biosensor configurations.

Acknowledgements

This work was supported in part by the U.S Army Research Office and the Office of Naval Research. We thank Leslie DeLuca for technical assistance.

References

Bataillard, P., Gardies, F., Jaffresic-Renault, N., Martelet, C., Colin, B. and Mandrand, B. (1988). Direct detection of immunospecies by capacitance measurements. Anal. Chem., 60, 2374-2379.

Darszon, A.(1983). Strategies in the reassembly of membrane proteins into lipid bilayer systems and their functional assay. J. Bioenerg. Biomemb., 15, 321-334.

Dolly, J. O. and Barnard, E.A. (1977). Purification and characterization of an acetylcholine receptor from mammalian skeletal muscle. Biochemistry, 16, 5053-5060.

Downer, N.W., Li, J., DeLuca, L.W., Penniman, E.M., and Smith, H.G. (1991) Surface-Bound Alkyl monolayers: Electrochemical and structural characterization. *Manuscript submitted.*

Eldefrawi, M.E., Sherby, S.M., Andreou, A.G., Mansour, N.A., Annau, Z., Blum, N.A., & Valdes, J.J. (1988). Acetylcholine receptor-based biosensor. Anal. Lett., 21, 1665-1680.

Krull, U. & Thompson, M. L. (1985) Lipid membrane technology for chemical and biosensor development. Trends in Anal. Chem., 4, 90-96.

Levitzki, A.(1985). Reconstitution of membrane receptor systems. Biochim. Biophys. Acta, 822, 127-153.

Heuser, J.E. & Salpeter, S.R. (1979). Organization of acetylcholine receptors in quick-frozen, deep-etched, and rotary-replicated *Torpedo* postsynaptic membrane. J. Cell Biol., 82, 150-173.

Liebman, P.A., Parker, K.R. & Dratz, E.A. (1987) The molecular mechanism of visual excitation and its relation to the structure and composition of the rod outer segment. Ann. Rev. Physiol., 49, 765-791.

MacDonald, D.D. (1977). "Transient Techniques in Electrochemistry", Plenum Press, New York.

MacDonald, R.S. (1958). Surface functionality of amorphous silica by infrared spectroscopy. J. Am. Chem. Soc., 62, 1168-1177.

Montal, M. and Mueller, P. (1972) Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. Proc. Natl. Acad. Sci. USA, 69, 3561-3566.

Neubig, R.R., Krodel, E.K., Boyd, N.D. and Cohen, J.B. (1979). Acetylcholine and local anesthetic binding to Torpedo nicotinic postsynaptic membranes after removal of nonreceptor peptides. Proc. Natl. Acad. Sci. USA, 76, 690-694.

Smith, H.G. & Litman, B.J. (1982). Preparation of osmotically intact rod outer segment disks by ficoll flotation. Methods Enzymol., 81, 57-61.

Sobel, A. Weber, M. and Changeux, J.-P. (1977). Large scale purification of the acetylcholine-receptor protein in its membrane-bound and detergent-extracted forms from Torpedo marmorata Electric Organ. Eur. J. Biochem., 80, 215-224.

Tiede, D. M. (1985) Incorporation of membrane proteins into interfacial films: model membranes for electrical and structural characterization. Biochim. Biophys Acta, 811, 357-379.

Woodbury, D.J. and C. Miller. (1990) Nystatin-induced liposome fusion. Biophys. J., 58, 833-839.

FIGURE LEGENDS

Figure 1. Block Diagram of Experimental Set-up

Figure 2. FTIR spectra of OTS-reacted glass beads before (lower trace) and after (upper trace) dialysis with AChR-containing membranes. Panel A shows the spectrum from 4000 cm^{-1} to 1000 cm^{-1}. In panel B the region containing hydrocarbon stretching frequencies is expanded.

Figure 3. FTIR difference spectra. Difference spectra for the dialyzed bead sample vs. the OTS-reacted control substrate are shown for dialysis with A) DMPC, B) AChR-containing membranes from Torpedo fish electric organ, and C), Rh-containing photoreceptor membranes.

Figure 4. Plots of Log R_{3000} vs. Absorbance Difference used to compare surface coatings. The solid line is the best fit calculated from a linear regression on data points shown. The samples include optimized OTS- and DMOCS-reacted control beads and Rh-dialyzed OTS-reacted beads.

Figure 5. Capacitance curves for different layers at the electrode surface for an experiment with AChR. Shown are the bare oxidized electrode surface, -.- ; OTS-reacted electrode, -..- ; and AChR-dialyzed electrode.

TABLE I

CARBON INCORPORATION AFTER SILANIZATION AND RECONSTITUTION

	Elemental Analysis	FTIR
	μg carbon / g beads	R_{3000}
Clean beads	13 ± 6[a]	0.36
DMOCS (≤ 1%, 1hr)	53 ± 7[a]	0.74
OTS (≤ 1%, 1hr)	143 ± 26[a]	2.06
Rhodopsin/DMOCS (≤ 1%)	381 ± 9[a]	1.80
	429 ± 7	2.97
	360 ± 1	2.04
	411 ± 25	2.19
DMOCS (4%, 6hr)	147 ± 40[b]	1.39 ± 0.45[b]
OTS (2%, 3hr)	244 ± 13[b]	2.31 ± 0.64[b]
Rhodopsin/DMOCS (4%)		2.21
Rhodopsin/OTS (2%)		3.38
DMPC/DMOCS (4%)		10.87
DMPC/OTS (2%)		8.96

[a] Mean ± s.d for n = 4 determinations on the sample

[b] Mean ± s.d. for determinations on 3 silanized samples

TABLE II

DIMENSIONS OF SURFACE-BOUND STRUCTURES DERIVED FROM CAPACITANCE MEASUREMENTS

			Silane Layer		Total Structure	
			d (Å)	O	d (Å)	O
Dialysis Addition	Substrate					
DMPC	OTS	(n = 5)	40.7 ± 5	1.58	49.6 ± 5.3	0.99
	DMOCS	(n = 5)	9.3 ± 2.9	0.36	16.6 ± 5.0	0.33
AChR	OTS	(n = 4)	27.5 ± 7.2	1.07	64.8 ± 14	1.30
	DMOCS	(n = 3)	7.3 ± 2.5	0.28	40.3 ± 23	0.81
Rhodopsin	OTS	(n = 5)	24.1 ± 6.9	0.94	36.8 ± 4.9	0.74
	DMOCS	(n = 6)	20.7 ± 4.7	0.81	34.7 ± 8.1	0.70

TABLE III

SURFACE DISTRIBUTION OF RECEPTORS

Receptor	Silane	Spacing (Å)	Density (#/m^2)	Receptor Density in Native Membrane
AChR	DMOCS	726	1.9×10^{14}	
	OTS	332	4.2×10^{14}	1.0×10^{16} [a]
Rhodopsin	DMOCS	240	1.7×10^{15}	
	OTS	213	2.2×10^{15}	2.5×10^{16} [b]

[a] Heuser & Salpeter, 1979

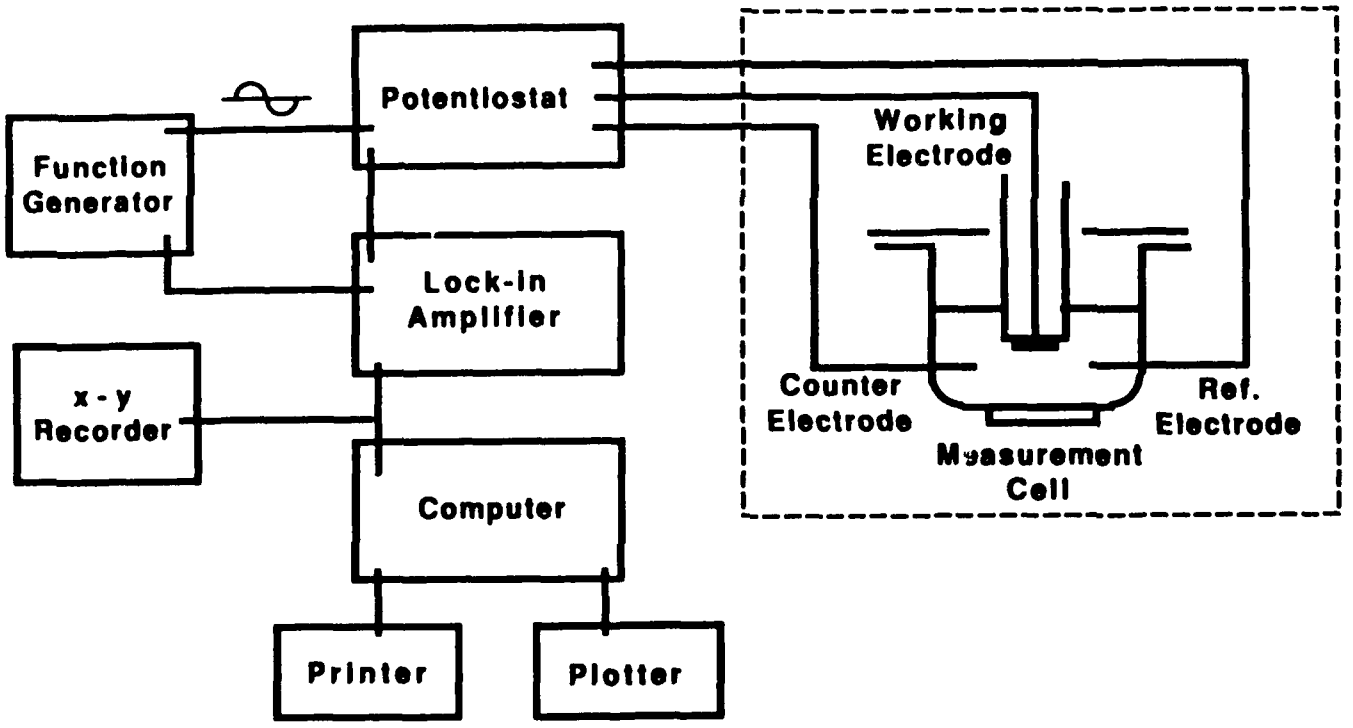
[b] Liebman *et al*, 1987

TABLE IV

RELATIVE PHOSPHOLIPID INCORPORATION BY DIALYSIS

	$R_{3000,dialyzed} : R_{3000,silanized\ control}$	
	DMOCS Substrate	**OTS Substrate**
Additions for dialysis		
none (OG only)	0.9 - 1.2	
DMPC	5.8 6.1	3.0 3.8
DMPC (stripped)	0.95	0.89
Acetylcholine Receptor	2.6 2.1 0.94	5.6 1.8 0.89
Vertebrate Rhodopsin		
reacted @ ≤ 1% silane	2.1 1.4 2.7 2.5 2.3	1.9
reacted @ 4% DMOCS	1.2	
reacted @ 2% OTS		1.4

Figure 1



Block Diagram of Experimental Set-Up

Figure 2

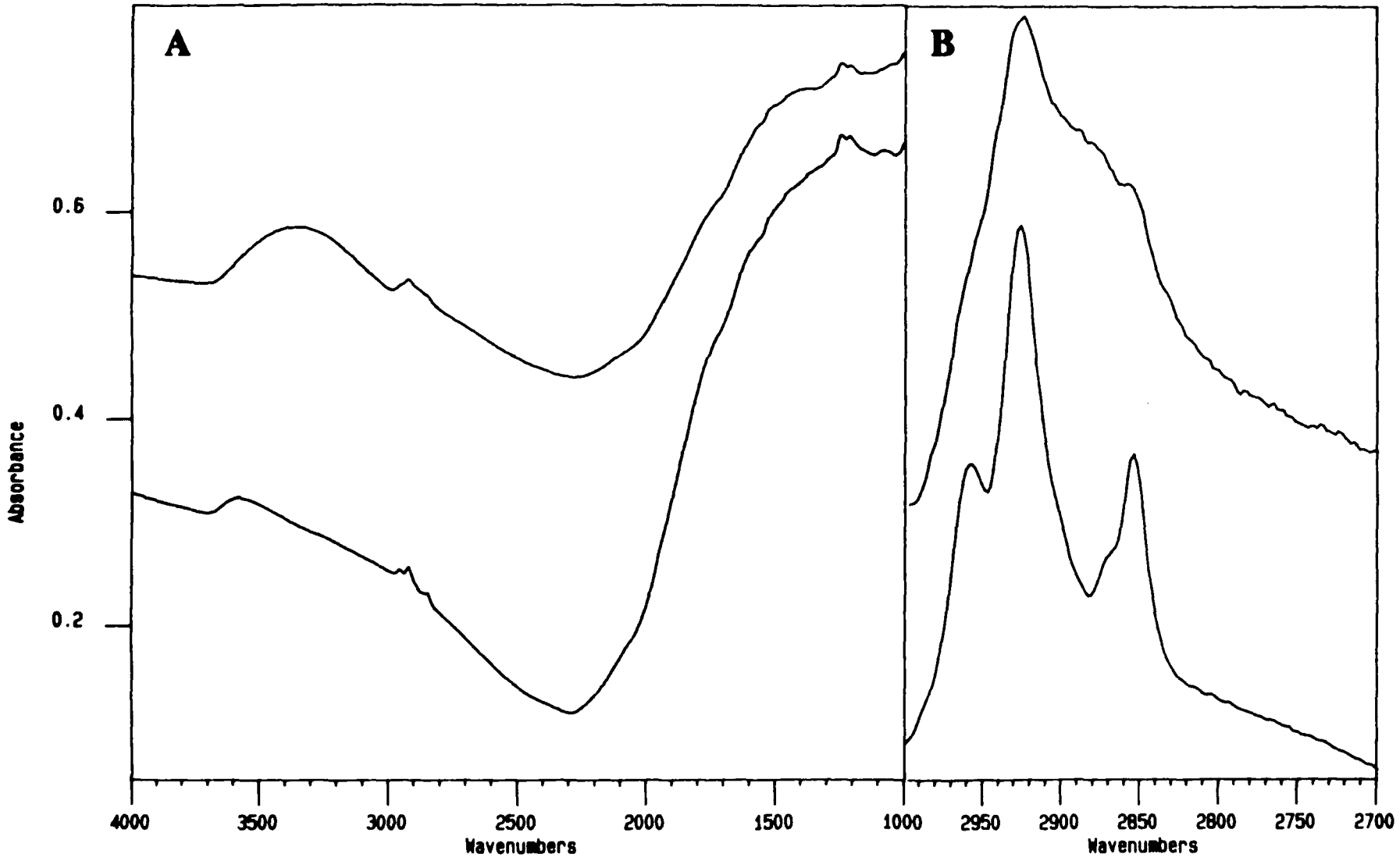

A
B
0.6
0.4
0.2
Absorbance
4000
3500
3000
2500
2000
1500
1000
Wavenumbers
2950
2900
2850
2800
2750
2700
Wavenumbers

Figure 3

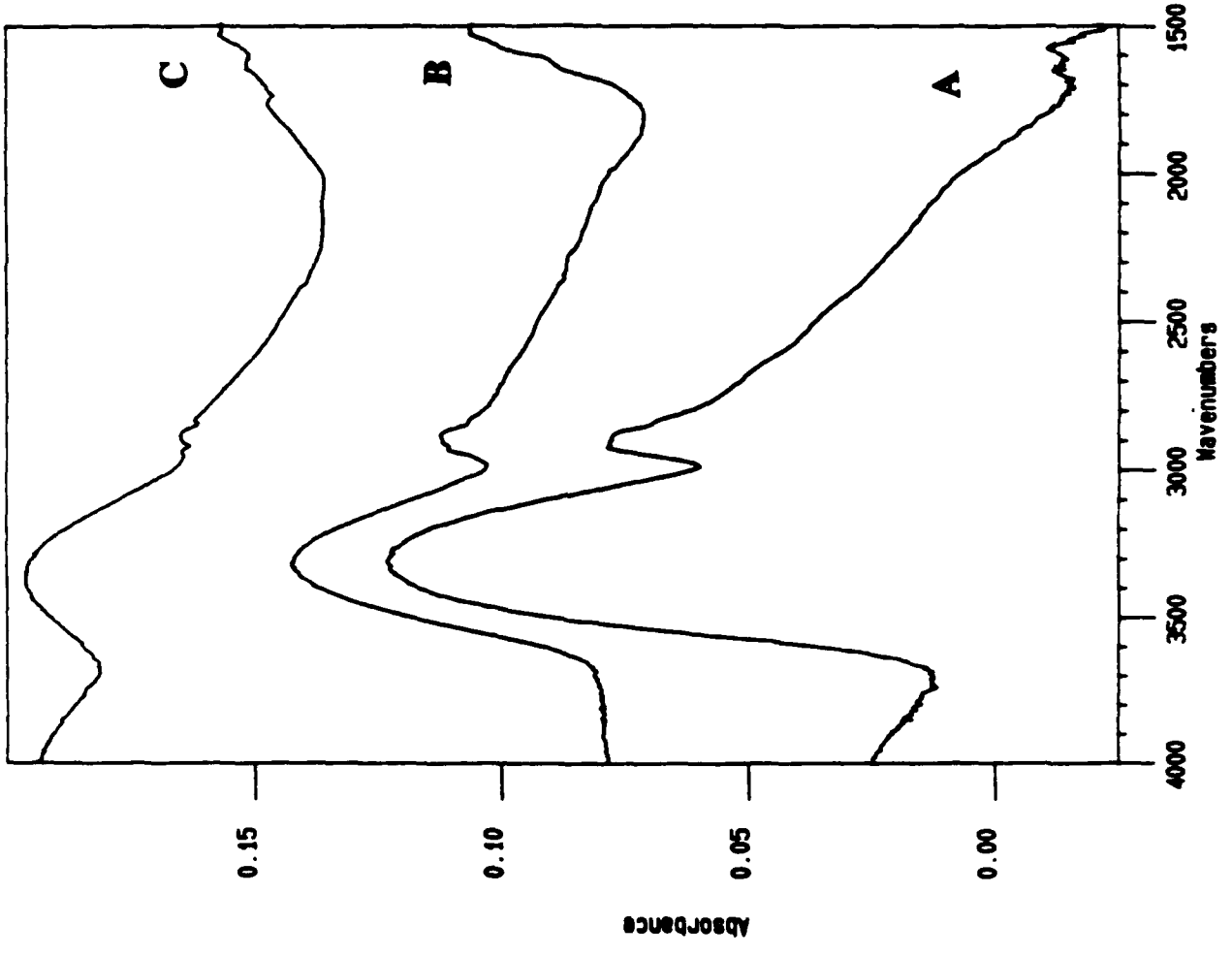

C
B
A
0.15
0.10
0.05
0.00
Absorbance
4000
3500
3000
2500
2000
1500
Wavenumbers

Figure 4

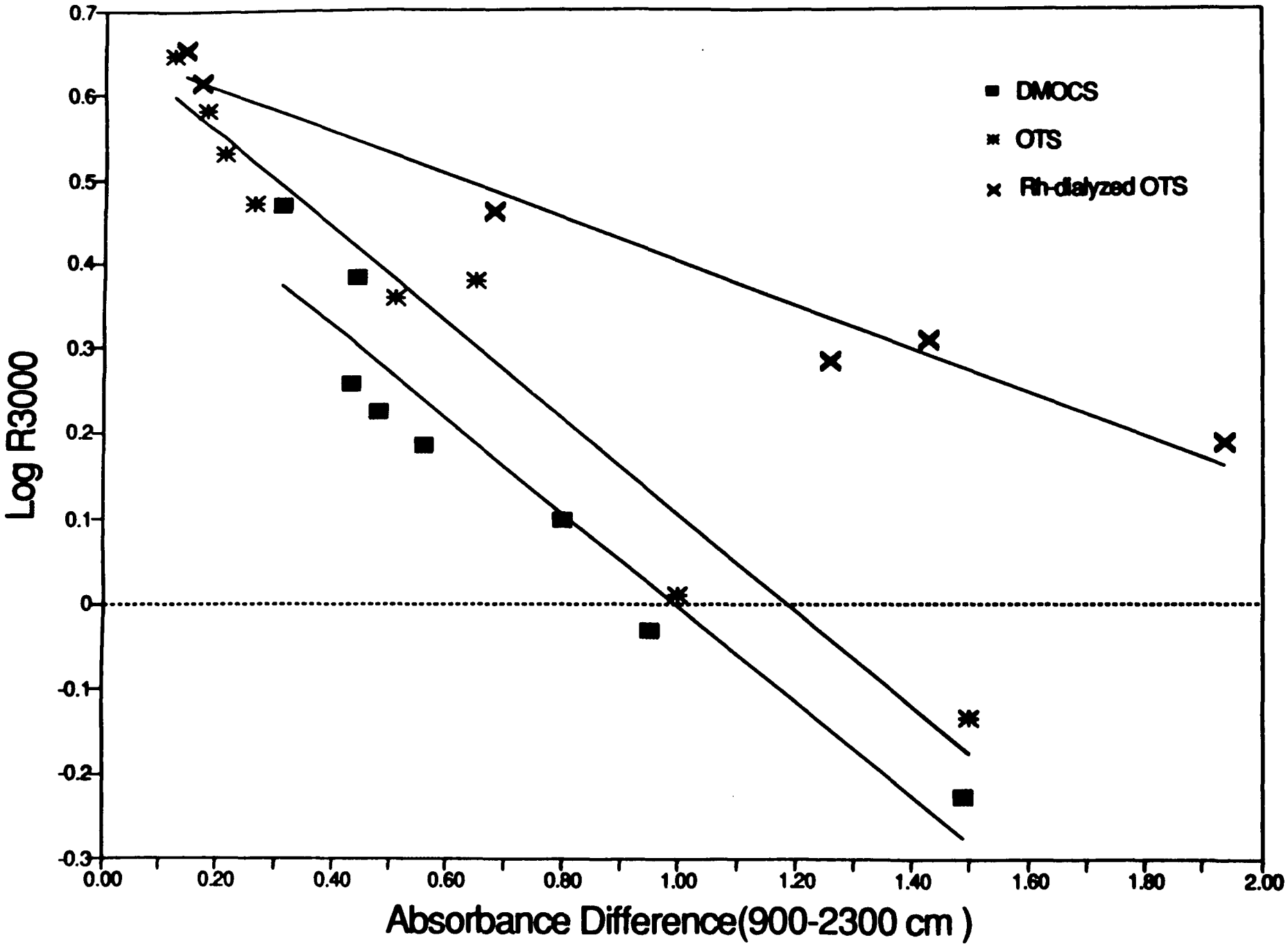
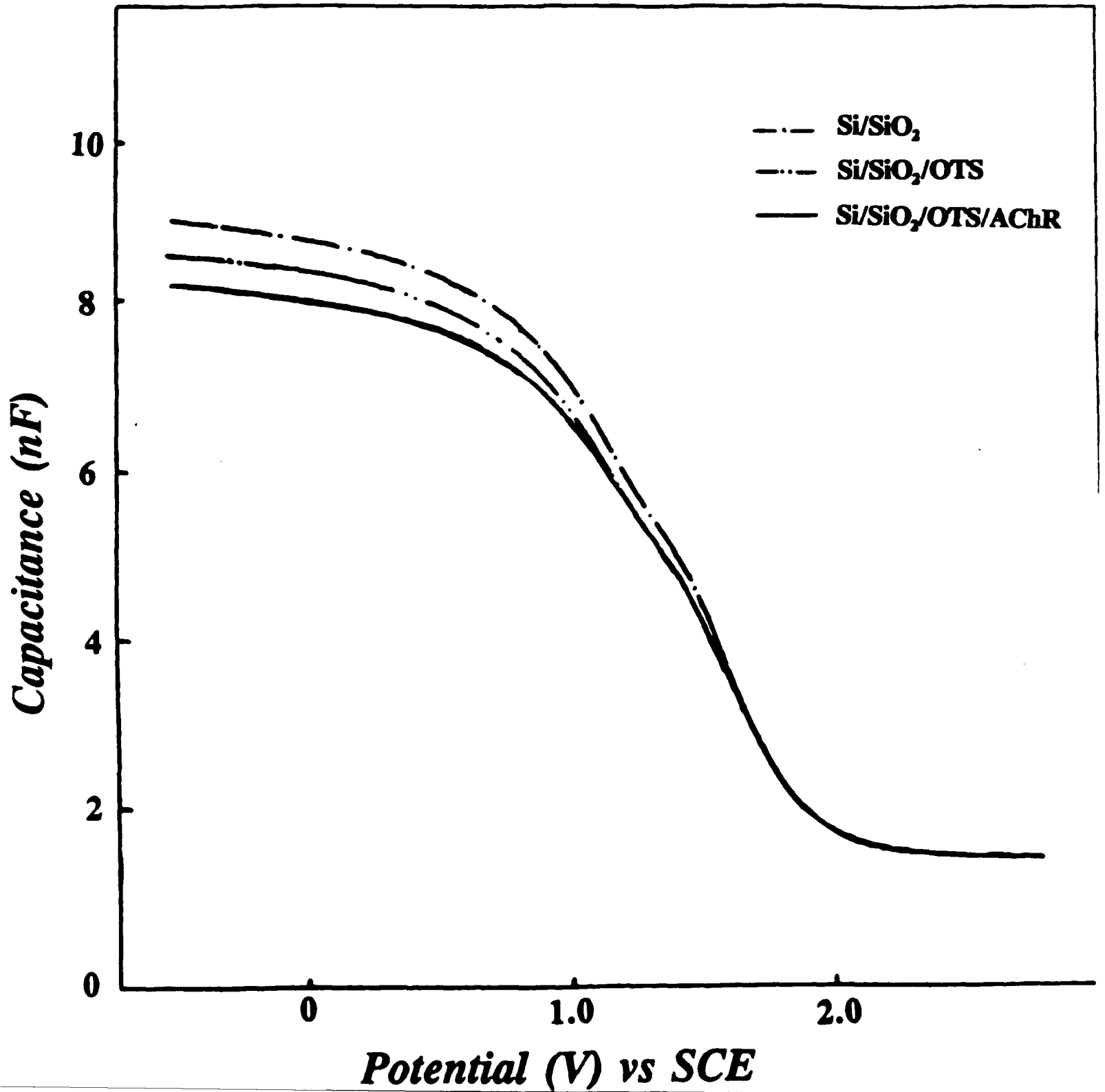

DMOCS
OTS
Rh-dialyzed OTS
Log R3000
Absorbance Difference(900-2300 cm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
2.00

Figure 5


Si/SiO2
Si/SiO2/OTS
Si/SiO2/OTS/AChR
10
8
6
4
2
0
0
1.0
2.0
Capacitance (nF)
Potential (V) vs SCE

TECHNICAL REPORT DISTRIBUTION LIST - GENERAL

Office of Naval Research (2)
Chemistry Division, Code 1113
800 North Quincy Street
Arlington, Virginia 22217-5000

Dr. James S. Murday (1)
Chemistry Division, Code 6100
Naval Research Laboratory
Washington, D.C. 20375-5000

Dr. Robert Green, Director (1)
Chemistry Division, Code 385
Naval Weapons Center
China Lake, CA 93555-6001

Dr. Eugene C. Fischer (1)
Code 2840
David Taylor Research Center
Annapolis, MD 21402-5067

Dr. Elek Lindner (1)
Naval Ocean Systems Center
Code 52
San Diego, CA 92152-5000

Commanding Officer (1)
Naval Weapons Support Center
Dr. Bernard E. Douda
Crane, Indiana 47522-5050

Dr. Richard W. Drisko (1)
Naval Civil Engineering
Laboratory
Code L52
Port Hueneme, CA 93043

Dr. Harold H. Singerman (1)
David Taylor Research Center
Code 283
Annapolis, MD 21402-5067

Chief of Naval Research (1)
Special Assistant for Marine
Corps Matters
Code 00MC
800 North Quincy Street
Arlington, VA 22217-5000

Defense Technical Information
Center (2)
Building 5, Cameron Station
Alexandria, VA 22314

Office of Naval Research (1)
Biological Sciences Division
ATTN: Dr. Igor Vodyanoy, Code 1141SB
800 N. Quincy St.
Arlington, VA 22217-5000