Available online at www.sciencedirect.com

SCIENCE DIRECT®

Bioelectrochemistry 66 (2005) 89–94

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

Surface immobilized optical probes: Pyrene molecules covalently attached to silica and indium-doped tin oxide

Maciej Mazur[a], G.J. Blanchard[b,*]

[a]*Laboratory of Electrochemistry, Department of Chemistry, University of Warsaw, 02-093 Warsaw, Pasteura 1, Poland*
[b]*Department of Chemistry, Michigan State University, East Lansing, MI 48823-1322, USA*

Received 22 December 2003; received in revised form 15 April 2004; accepted 27 April 2004
Available online 8 August 2004

Abstract

We report on the covalent attachment of pyrene to quartz and indium-doped tin oxide (ITO) surfaces, and its characterization by steady-state and time-resolved emission spectroscopy. We estimate the chromophore surface coverage on the basis of their electrochemical oxidation mechanism in acidic solution. Electrochemical oxidation of immobilized pyrene results in the formation of radical cations, which are converted into hydroxypyrene and, after a second oxidation step, are transformed into isomeric forms of dihydroxypyrene/pyrenedione redox couples. Our findings demonstrate that surface-bound pyrene can be used as a spectral probe in environments that are isolated from oxidizing conditions and agents.

Keywords: Optical probe; Pyrene; Covalent attachment; Lipid bilayer; Surface transformation

1. Introduction

Supported lipid bilayer mimics have been investigated extensively because of their ability to function in a biomimetic manner. Interest in these systems has ranged from their use in understanding basic physical properties of lipid bilayers to supporting transmembrane proteins in their active forms. This family of molecular interfaces has also been the focus of recent attention owing to their potential utility in the construction of biosensors [1–7]. Lipid bilayer films represent a relatively well-controlled environment, but the interrogation of these systems has proven to be challenging. Optical spectroscopies of chromophores imbedded in bilayer films have been used to gain molecular-scale insight into the dynamics and average environments contained within these structures [8,9]. Our interest here lies in the characterization of an optical probe molecule, especially with regard to its stability and propensity for oxidative degradation.

Pyrene is a chromophore that has been used extensively to probe intermolecular interactions in bulk solutions and selected other environments [10]. The motivation for using pyrene as a probe is that its optical response is anomalously sensitive to local environment polarity, a result of strong vibronic coupling between the S_1 and S_2 electronic states. We have devised an experimental means to covalently immobilize pyrene on solid substrates such as quartz and indium-doped tin oxide (ITO). Using this immobilization technique, we are poised to use pyrene for the characterization of hybrid bilayer assemblies. In this report, we characterize surface-bound pyrene spectroscopically and electrochemically. Our data show that surface-bound pyrene readily forms an electroactive hydroquinone/quinone redox couple, and we are able to determine surface coverage by measurement of the charge exchanged during the electrochemical cycling between dihydroxypyrene and pyrenedione. These electrochemical experiments demonstrate that pyrene can undergo stepwise oxidation and that its use as an

---

* Corresponding author. Department of Chemistry, Michigan State University, East Lansing, Michigan 48824, USA. Tel.: +1 517 355 9715x224; fax: +1 517 353 1793.

E-mail address: Blanchard@chemistry.msu.edu (G.J. Blanchard).

1567-5394/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2004.04.005

interfacial probe is limited under conditions of oxidizing agents being present.

2. Experimental

2.1. Chemicals

All chemicals used in this work were of the highest quality commercially available: 1-aminopyrene (Aldrich, >98%), adipoyl chloride (Aldrich, 98%), 4-methylmorpholine (Aldrich, 99%), 1-hydroxypyrene (Aldrich, 98%), 1,6-pyrenedione (Aldrich), sodium borohydride (Aldrich, 98%), acetonitrile (Aldrich, anhydrous, 99.8%), ethyl acetate (Spectrum Chemical, ACS grade), sulfuric acid (Aldrich, 99.999%), hexane (Aldrich, 95+%), 1-pentanol (Aldrich, 99+%). Aqueous solutions were prepared from water distilled in-house.

2.2. Steady-state emission spectroscopy

Emission and excitation spectra were recorded with a JY-Spex Fluorolog 3 spectrometer using a 3-nm band-pass for excitation and a 3-nm band-pass for emission collection.

2.3. Time-resolved emission measurements

The time-correlated single-photon counting (TCSPC) system used to acquire time-resolved spectroscopic data has been described elsewhere [11] and we present a brief overview of the system here. The second harmonic of the output of a mode-locked CW Nd:YAG laser (Coherent Antares 76-S) is used to pump a cavity-dumped dye laser (Coherent 702-2) operated at 560 nm using Pyromethine 567 laser dye (Exciton Chemical). The output of the dye laser was typically 100-mW average power at a 1-MHz repetition rate with ~5-ps pulses. A fraction of each pulse is used as a temporal reference beam with the remaining light being frequency doubled using a Type II KDP crystal to provide a 280-nm light for chromophore excitation. The system is operated in reverse time mode to ensure the operation of the counting electronics in a linear regime, and is characterized by an instrument response function that is typically 35-ps fwhm. The instrument is controlled using National Instruments LabVIEW® v. 7.0 software.

2.4. Electrochemical measurements

Electrochemical measurements were conducted with a PC-controlled Electrochemical Workstation (CH Instruments Model 650A), using a small volume three-electrode cell with Pt wire as the counter electrode. All potentials are quoted versus Ag/AgCl/1 M KCl_{aq} reference electrode.

2.5. Substrate preparation

Quartz slides (NSG Precision Cells, P/N 10040 UV fused silica windows–non-fluorescent) and gold electrodes (Au, 99.99%; 0.2 cm^2) were cleaned by immersion in piranha solution (3:1 v/v ratio of concentrated sulfuric acid and hydrogen peroxide) for ca. 20 min. Indium-doped tin oxide (ITO) films (Bayview Optics, Dover-Foxcroft, ME) deposited on the same quartz slides were used for spectroscopic measurements and ITO films on glass substrates (Delta Technologies) were used for electrochemical measurements.

2.6. Bonding 1-aminopyrene to quartz and ITO

Quartz and ITO substrates were reacted with adipoyl chloride (0.3 ml) in dry acetonitrile (10 ml), using 4-methylmorpholine (0.3 ml) as a Lewis base, under reduced pressure for 1 h. The reacted substrates were removed from the reaction vessel, rinsed with dry acetonitrile and ethyl acetate, dried under a stream of nitrogen and the terminal acid chloride functionalities were reacted with 1-aminopyrene by exposure to a 30-mM solution of the compound in dry acetonitrile for 1 h. The substrates were then removed from the reaction vessel, washed with dry acetonitrile and ethyl acetate, and dried under a stream of nitrogen.

2.7. Preparation of thin films 1-hydroxypyrene and 1,6-pyrenedione on gold

1-Hydroxypyrene and 1,6-pyrenedione thin films on gold were prepared by immersing the substrates into an acetonitrile solution of the compound (50 mM), followed by removal from the solution and drying under ambient conditions. The modified electrodes were then characterized electrochemically in aqueous solution.

2.8. Preparation of 1,6-dihydroxypyrene

1,6-Dihydroxypyrene was prepared by reduction of 2 ml 10^{-4} M 1,6-pyrenedione in 1-pentanol with 2 ml of saturated sodium borohydride in 1-pentanol. The yield for this reaction was quantitative.

2.9. Preparation of N-(1-pyrenyl)-acetamide

1-Aminopyrene (0.5 g) was reacted with excess acetic anhydride (20 ml) in dry acetonitrile (20 ml) for ca. 20 h, resulting in the precipitation of *N*-(1-pyrenyl)-acetamide. The product was collected in 95% yield by filtration.

3. Results and discussion

The goal of this work is to characterize surface-bound pyrene chromophores. 1-Aminopyrene was attached covalently to silica and indium-doped tin oxide (ITO) surfaces by an established immobilization procedure using adipoyl chloride as a molecular tether [9]. Pyrene is a well-known fluorescent probe that has been used widely to study local polarity in the solution phase. The spectral response of surface-bound pyrene is also useful for characterizing mono- and multilayer structures [12,13].

We show in Fig. 1a the excitation and emission spectra of amidopyrene bound covalently to an ITO-coated substrate. The excitation spectrum reveals an intense band at ca. 350 nm and a weak band at 285 nm. Comparison of this spectrum to the solution phase excitation spectrum of *N*-(1-pyrenyl)-acetamide (Fig. 1c) shows that the intensity ratio of the bands at 350 and 285 nm is different for the two systems. The weakness of the 285 nm band in Fig. 1a may be due to optical absorption by ITO in the UV, although the exact mechanism of attenuation of this band remains to be understood fully. The intensity ratio of pyrene immobilized on quartz (Fig. 1b) is close to that of solution phase *N*-(1-pyrenyl)-acetamide. This observation underscores the fact that the intensity of the excitation bands for immobilized species depends on the optical characteristics of the substrate.

The emission spectra of surface-bound and solution phase pyrene reveal some interesting differences (Fig. 1a and b). For both solution and surface-bound chromophores, there is an intense emission response with vibronic features at 385 and 405 nm, the broad band at 475 nm seen for solution phase *N*-(1-pyrenyl)-acetamide in hexane is absent for the surface-bound species. We assign the 475-nm band to excimers. The lack of excimer formation for the surface-bound pyrene can be explained in a number of ways. The most obvious explanation is that the chromophore surface loading density is too low for excimer formation to proceed. It is also possible, but less likely, that the surface attachment places steric limits on the alignment of the chromophores which would serve to preclude the formation of excimers. We can use electrochemistry to help resolve which of these possibilities is more likely (vide infra).

Further differences in the behavior of the immobilized and solution phase chromophores are elucidated by time-resolved emission spectroscopy. We show in Fig. 2 the emission transients for *N*-(1-pyrenyl)-acetamide in hexane and the same chromophore bound to silica, with the silica substrate immersed in hexane. For the solution phase chromophore, the emission signal decays very slowly, over multiple nanoseconds, while the surface-bound pyrene decays much more rapidly, with lifetime(s) under 1 ns. The molecular structure of the two chromophores is essentially identical and the remarkable difference in lifetimes must be the direct result of their local environments. Substrate-induced quenching cannot account for these data because the substrate is a dielectric material. One possible explanation for these findings is that there is an anomalous concentration effect for O_2 and/or H_2O at the silica substrate relative to that found in liquid phase hexane. Alternatively, we can suggest that oxygen quenching of the pyrene singlet excited state on a solid surface is more effective than in the bulk solution. We also note that the surface-bound chromophore exhibits a non-exponential decay, characteristic of either multiple emitting species, efficient collisional

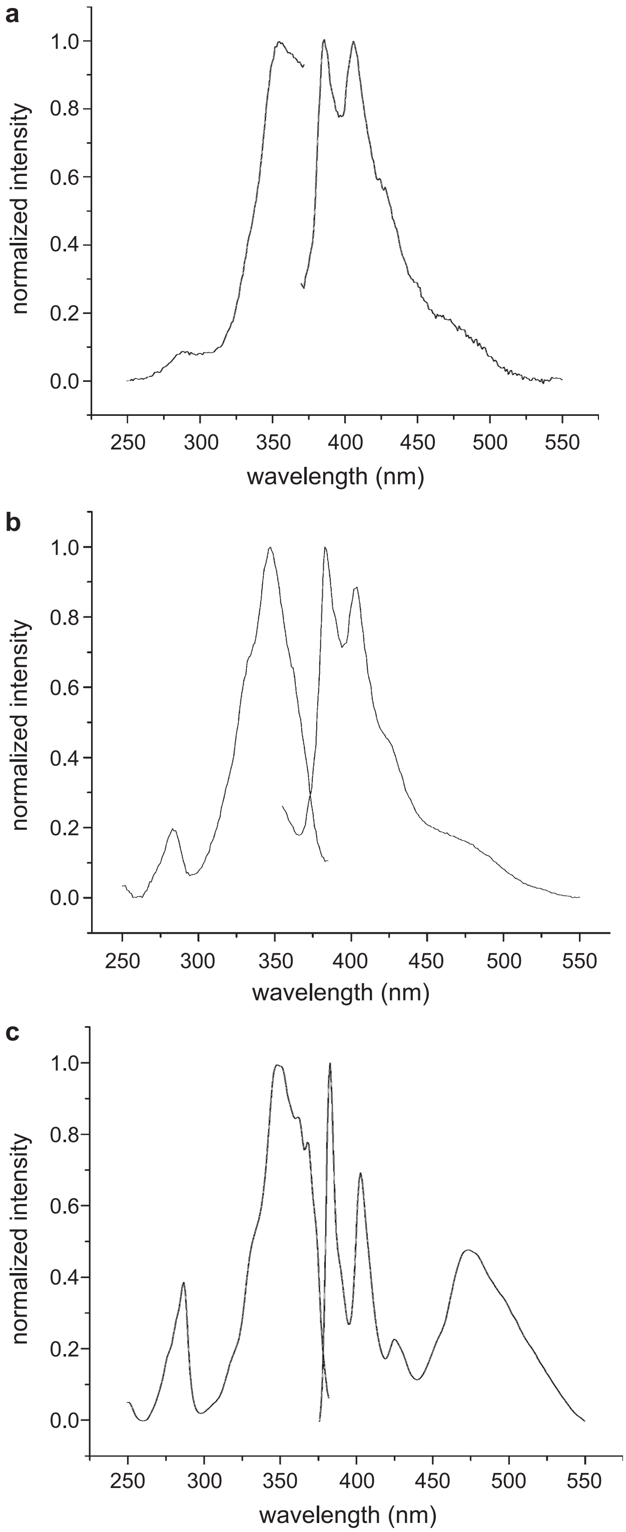

Fig. 1. (a) Excitation and emission spectra of aminopyrene attached to an adipoyl chloride adlayer on ITO and (b) quartz. (c) Solution phase spectra of *N*-(1-pyrenyl)-acetamide in hexane.

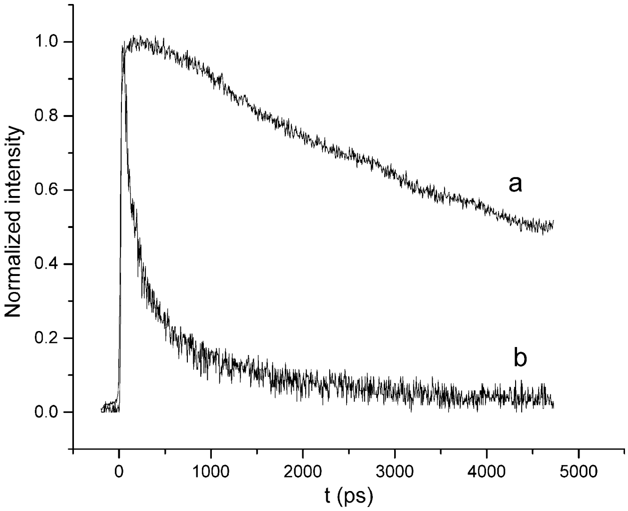

Fig. 2. (a) Fluorescence intensity decay at 405 nm for *N*-(1-pyrenyl)-acetamide in hexane (280-nm excitation), (b) the same data for aminopyrene bound covalently to ITO, with the sample immersed in hexane.

quenching or Förster excitation transport [13]. It is clear that further experimentation will be required to distinguish between these several possibilities.

The steady-state and time-resolved experiments show that the surface-bound pyrene is amenable to optical detection, indicating their utility as probes of structure and dynamics in thin films systems. These spectroscopic measurements do not, however, address quantitatively the areal concentration of these chromophores on the surface. Electrochemical methods are much better suited to a quantitative examination of surface loading density. We attached pyrene to the surface of ITO and examined its voltammetric behavior (Fig. 3). The cyclic voltammetry data for the pyrene-coated ITO electrode in sulfuric acid solution shows that, in the first scan, there is an irreversible oxidation peak with maximum at ca. 1.05 V (labeled as I). We assign this signal to the oxidation of pyrene to form a radical cation. In the subsequent scans, this signal rapidly decreases, suggesting the oxidized pyrene reacts to form another species. During the first scan, after reversing the electrode polarization, we observe a new redox signal; three pairs of peaks at 0.6 V (II), 0.35 V (III) and 0.2 V (IV). The peaks labeled II gradually decrease in subsequent scans, suggesting they are intermediate species. At the same time, peak(s) III increase during scanning and peak IV remains constant. Peaks III and IV are associated with the final products of pyrene oxidation.

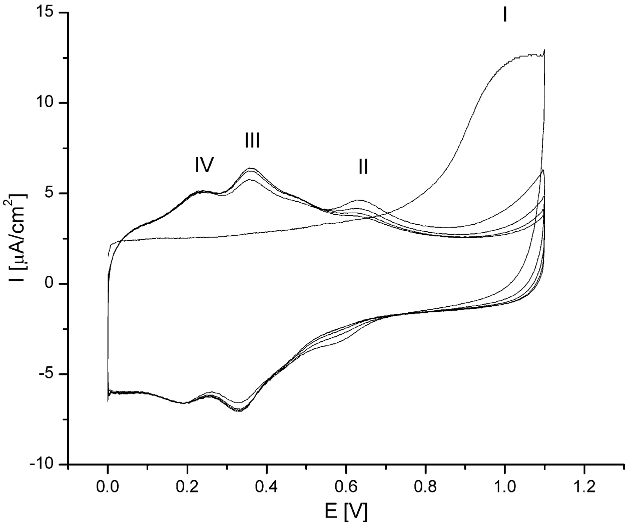

Fig. 3. Cyclic voltammograms of 1-amidopyrene adipate bound to ITO in aqueous 0.5 M H_2SO_4. Sweep rate 0.1 V/s.

We have used emission spectroscopy to identify these products. Fig. 4a shows the emission spectrum of a chromophore adlayer on ITO following electrochemical oxidation in sulfuric acid solution. While the broad emission band centered around 400 nm is present, similar to that seen for bound pyrene, the vibronic structure in this spectral region has changed. New bands appear at 372, 382, 393 and 404 nm. Based on literature data on the oxidative degradation of pyrene, dihydroxy and dione derivatives are the most likely final products [14–17]. We show in Fig. 4b and c the solution phase spectra of 1,6-dihydroxypyrene and 1,6-pyrenedione, respectively. 1,6-Dihydroxypyrene exhibits vibronic bands at 372, 385 and 392 nm, in agreement with the spectrum seen for our electrochemical oxidation products of surface-bound pyrene. The differences in the band intensities may result from the details of the chromophore surface bonding, in analogy to the spectral differences seen for solution phase and surface-bound pyrene (Fig. 1a and b). The spectrum of 1,6-pyrenedione reveals a strong band at 404 nm, matching a spectral feature of the pyrene oxidation product. The electrochemical

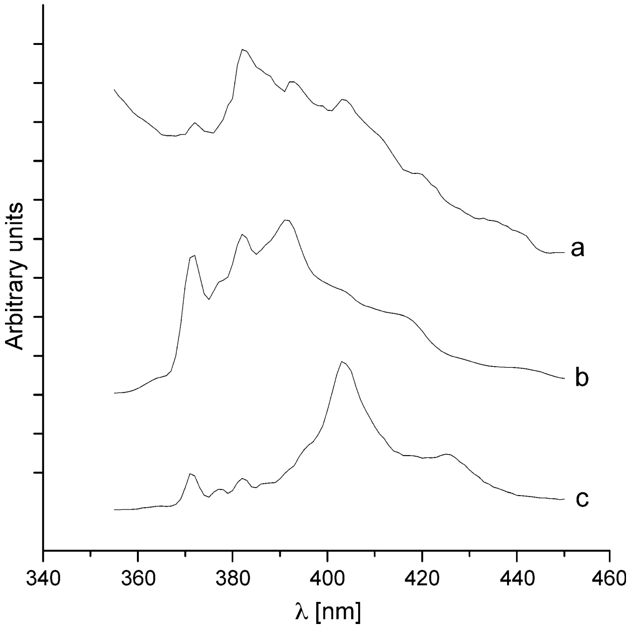

Fig. 4. (a) Emission spectra of electrochemical oxidation products of amidopyrenehexanoate bound to ITO; (b) 1,6-pyrenedione in 1-pentanol; (c) 1,6-dihydroxypyrene in 1-pentanol.

oxidation of pyrene results in its transformation to the dihydroxypyrene/pyrenedione redox couple. We show in Fig. 5a the cyclic voltammogram of 1,6-pyrenedione physisorbed on a gold electrode. The reduction peak for dihydroxypyrene is seen at 0.27 V, and the corresponding oxidation peak at 0.33 V. This voltammetric behavior is very similar to the redox peaks III seen for the oxidation of surface-bound pyrene (Fig. 4). A slight shift in the formal potential may be associated with the amide substitution of the pyrene ring system. Our findings indicate that the electrochemical signals III are associated with the 1,6-dihydroxy/1,6-pyrenedione redox couple on the ITO surface. Moriconi et al. [18] have reported the formal potential of 1,8-dihydroxypyrene/1,8-pyrenedione to be shifted 100 mV cathodically with respect to that of the corresponding 1,6 isomers. The signals labeled as IV in Fig. 3 are most likely associated with 1,8-dihydroxypyrene/1,8-pyrenedione redox couple. The origin of the redox pair II remains unassigned. We show in Fig. 5b the cyclic voltammogram of 1-hydroxypyrene, characterized by an irreversible oxidation peak at ca. 0.7 V which decreases in sequential scans in correspondence with the appearance of a new redox pair at ca. 0.3 V. The similarity of the redox pair at 0.3 V to 1,6-dihydroxypyrene/1,6-pyrenedione couple is evident (Fig. 5a). Because the formal potential of the species II is relatively close to the potential associated with the oxidation of 1-hydroxypyrene, it is most probable that the peak II in Fig. 3 is associated with the monohydroxy derivative(s) of surface-bound pyrene.

From these data, the surface oxidation chemistry of surface-bound pyrene emerges. The electrochemical oxidation of pyrene results in the formation of radical cations which react with water to form a monohydroxy derivative. This monohydroxy derivative can undergo further oxidation to yield a mixture of isomeric forms of the 1,6- and 1,8-dihydroxypyrene/pyrenedione redox couples (the 1,6- and 1,8-notation used here does not follow IUPAC nomenclature, and is employed to make the comparison between the non-substituted and substituted compounds more clear; in fact the position 1 in the pyrene molecule is already occupied by the amido group used for linking so the systematic names of the compounds are different).

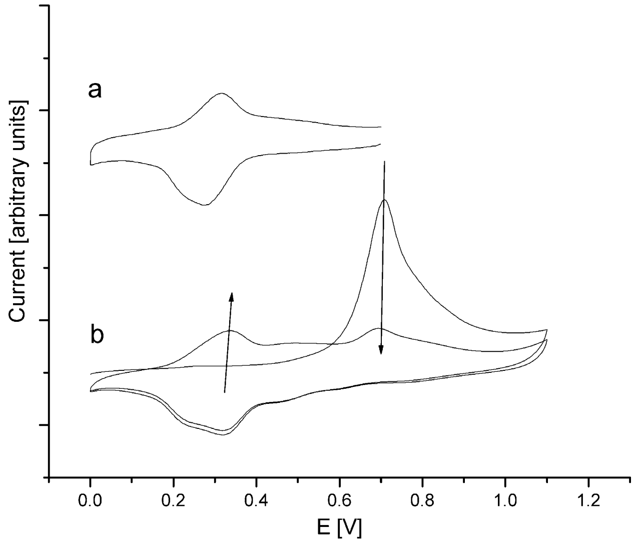

Fig. 5. Cyclic voltammograms of thin layers of (a) 1,6-pyrenedione and (b) 1-hydroxypyrene physisorbed on gold, taken in aqueous 0.5 M H_2SO_4.

Based on the information we have gained, we can estimate the surface chromophore concentration based on the amount of charge consumed by pyrene oxidation (peak I) or the charge exchanged during the dihydroxy/dione reaction (peaks III and IV). Owing to the stability of the dihydroxy/dione redox couple, it is simpler and more accurate to use this system for quantitation. We integrate the charge under the voltammetric peaks for a steady-state curve (after all hydroxypyrene has been oxidized to dihydroxypyrene(s)), and assuming two-electron reaction, we calculate the surface concentration Γ from Eq. (1)

$$\Gamma = Q_{III,IV}/(2F) = 5.3 \times 10^{-11}\ \mathrm{mol/cm^2} \qquad (1)$$

where $Q_{\mathrm{III,IV}}$ corresponds the integrated charge under voltammetric peaks labeled III and IV, F—Faraday constant.

The calculated surface concentration yields an area of 312 Å^2 per molecule. This value suggests that the molecules are not tightly packed on the surface and explains why we do not observe excimer emission from the surface-bound chromophores (Fig. 1b). This relatively low surface coverage operates to our advantage here. The surface-bound chromophores do not change the properties of the substrate surface significantly but their presence is detectable by emission spectroscopy. The high detectability of the surface-bound chromophores allows their ready use as a probe of the formation process of complex thin film structures without causing undue perturbation.

4. Conclusions

We have shown that pyrene can be covalently bound to ITO and quartz surfaces. The pyrene chromophore is useful for probing local environments within molecular films. The reactivity of pyrene under oxidative conditions indicates that, while this chromophore is of great utility in probing molecular-scale phenomena, it should be used in environments that are free of oxidizing species. With this caveat in mind, it will be possible to use such tethered chromophores for signal transduction in a variety of chemical and biological sensors, and we are active in investigating this issue.

Acknowledgement

We are grateful to the National Science Foundation for their support of this work through Grant 0090864. MM is grateful to the National Science Foundation and NATO for their support of a post-doctoral fellowship (DGE-0209459).

References

[1] E. Sackmann, Supported membranes: scientific and practical applications, Science 271 (1996) 43–48.

[2] M.M. Lipp, K.Y.C. Lee, J.A. Zasadzinski, A.J. Waring, Phase and morphology changes in lipid monolayers induced by SP-B protein and its amino-terminal peptide, Science 273 (1996) 1196–1199.

[3] I. Willner, E. Katz, Integration of layered redox proteins and conductive supports for bioelectronic applications, Angew. Chem., Int. Ed. 39 (2000) 1180–1218.

[4] E.K. Sinner, W. Knoll, Functional tethered membranes, Curr. Opin. Chem. Biol. 5 (2001) 705–711.

[5] G. Wiegand, N. Arribas-Layton, H. Hillebrandt, E. Sackmann, P. Wagner, Electrical properties of supported lipid bilayer membranes, J. Phys. Chem., B 106 (2002) 4245–4254.

[6] P. Krysiński, A. Żebrowska, B. Pałys, Z. Łotowski, Spectroscopic and electrochemical studies of bilayer lipid membranes tethered to the surface of gold, J. Electrochem. Soc. 149 (2002) E189–E194.

[7] P. Krysiński, A. Żebrowska, A. Michota, J. Bukowska, L. Becucci, M.R. Monelli, Tethered mono- and bilayer lipid membranes on Au and Hg, Langmuir 17 (2001) 3852–3857.

[8] C.E. Talley, R.C. Dunn, Single molecules as probes of lipid membrane microenvironments, J. Phys. Chem., B 103 (1999) 10214–10220.

[9] P. Krysiński, G.J. Blanchard, Synthesis and characterization of amphiphilic biomimetic assemblies at electrochemically active surfaces, Langmuir 19 (2003) 3875–3882.

[10] D.S. Karpovich, G.J. Blanchard, Relating the polarity dependent fluorescence response of pyrene to vibronic coupling. achieving a fundamental understanding of the py polarity scale, J. Phys. Chem. 99 (1995) 3951–3958.

[11] L. Dewitt, G.J. Blanchard, E. Legoff, M.E. Benz, J.H. Liao, M.G. Kanatzidis, Determination of ground and excited state isomerization barriers for the oligothiophene: 3′,4′-dibutyl-2,2′:5′,2″-terthiophene, J. Am. Chem. Soc. 115 (1993) 12158–12164.

[12] L. Kelepouris, P. Krysiński, J. Blanchard, Gauging molecular interactions between substrates and adsorbates. Substrate mediation of surface-bound chromophore vibronic coupling, J. Phys. Chem., B 107 (2003) 4100–4106.

[13] D.S. Karpovich, G.J. Blanchard, Dynamics of a tethered chromophore imbedded in a self-assembled monolayer”, Langmuir 12 (1996) 5522–5524.

[14] A.J. Fatiadi, Effects of temperature and of ultraviolet radiation on pyrene absorbed on garden soil, Environ. Sci. Technol. 1 (1967) 570–572.

[15] Y. Mao, J.K. Thomas, Photochemical reactions of pyrene on surfaces of γ-alumina and silica-alumina, Langmuir 8 (1992) 2501–2508.

[16] C.A. Reyes, M. Medina, C. Crespo-Hernandez, M.Z. Cedeno, R. Arce, O. Rosario, D.M. Steffenson, I.N. Ivanov, M.E. Sigman, R. Dabestani, Photochemistry of pyrene on unactivated and activated silica surfaces, Environ. Sci. Technol. 34 (2000) 415–421.

[17] M.E. Sigman, P.F. Schuler, M.M. Ghosh, R.T. Dabestani, Mechanism of pyrene photochemical oxidation in aqueous and surfactant solutions, Environ. Sci. Technol. 32 (1998) 3980–3985.

[18] E.J. Moriconi, B. Rakoczy, W.F. O’Connor, Oxidation-reduction potentials and absorption spectra of polycyclic aromatic quinones, J. Org. Chem. 27 (1962) 2772–2776.