THE JOURNAL OF PHYSICAL CHEMISTRY B

ARTICLE

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Real-Time Monitoring of Invertase Activity Immobilized in Nanoporous Aluminum Oxide

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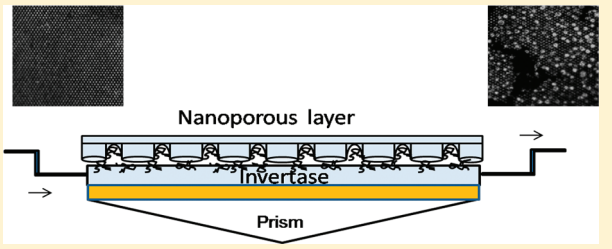
ABSTRACT: In this work, we demonstrate the activity of enzyme invertase immobilized in the pores of nanoporous anodized 3 μm thick aluminum oxide (AA). The porous anodic alumina has uniform nanosized pores with an interpore distance of p = 100 nm, with pore diameters on the order of 60−65 nm. The pores trap the enzyme and continuous monitoring of the activity is carried out in a flow cell where the substrate is made to flow and the product is detected. The activity of the immobilized enzyme has been determined for the different concentrations of sucrose and for pH ranging from 3 to 6.5. Maximum activity was found for pH 4.5. Adsorption of the enzyme followed by its interaction with the substrate have been analyzed using confocal laser scanning microscopy (CLSM) and surface plasmon spectroscopy (SPR) and the results obtained show excellent correlation. SPR results show a biphasic kinetics for the adsorption of the enzyme as well as its interaction with the substrate with rates of adsorption for the enzyme at $k = 2.9 \times 10^5\ M^{-1}\ s^{-1}$ and $1.17 \times 10^5\ M^{-1}\ s^{-1}$. The rate of interaction of the substrate with the invertase is initially rapid with $k = 4.49 \times 10^5\ M^{-1}\ s^{-1}$ followed by a slower rate $1.43 \times 10^4\ M^{-1}\ s^{-1}$.



INTRODUCTION

Immobilization of enzymes and proteins to solid substrates is required for a variety of applications in catalysis and biosensing.[1−6] In all these applications, stability and activity of the enzymes is an important issue, and developing reusable and stable devices is thus a key factor. In this work, a simple adsorption technique has been used to study immobilized invertase on nanoporous aluminum oxide substrate. Invertases are an important class of enzymes that catalyze the hydrolysis of sucrose into glucose and fructose. Invertase is the main enzyme that is used for commercial production of invert sugar.[7] In many of the industrial applications, immobilized enzymes are used and among the different immobilization techniques, physical adsorption of enzymes to solid matrices is a well established method. This is characterized by its simple and easy to use gentle procedures and generally the enzyme activity does not change.[8] A number of research papers have appeared in the literature which deals with the activity of free versus immobilized enzyme in the different supports.[9−13]

This paper presents the immobilization of invertase in nanoporous anodic alumina (AA) and its use in a flow cell to monitor in real time the formation of the product glucose and fructose. Anodized aluminum oxide substrate can be fabricated by anodizing high purity Al film in an acidic solution.[14] During anodization, a hexagonal array of cylindrical pores of uniform size is formed perpendicular to the film surface. The pore diameter ranging from 10 nm to hundreds of nm with a pore density of about 10^8−10^{11} count cm^{-2} can be obtained by controlling the anodization conditions.[15] The pore dimension is proportional to the anodization time varying from tens of nm to mm.[16] Such designed AA has been used for patterning nanocomposites, in optical, electronic and magnetic devices and also in the preparation of nanowires and nanoparticles of semiconductors or metals.[17,18] In many of the recent applications, the nanoporous AA has been used for controlled molecular release in biology, for example, in controlled drug release, to provide nutrition media in cell growth, selective separation of drug enantiomers or even DNA oligos.[19,20] Research has been carried out with such templates to design nanoribbons and linear arrays of nanoparticles.[21,22]

In this work, we use a reactor system based on a micrometer thick aluminum oxide layer on which uniform pores of diameter 60 nm with a interpore distance of 100 nm have been prepared. A hexagonally ordered porous alumina matrix has been prepared following a two step anodization process and the surfaces have been imaged in scanning electron microscope. The activity of the immobilized enzyme in nanopores of AA has been studied for different concentrations of the substrate sucrose. A quantification of the amount of enzyme trapped has been made using surface plasmon resonance spectroscopy (SPR). Our strategy involves the immobilization of the enzyme on an ultrathin Au film (<50 nm), followed by monitoring sucrose binding to the enzyme by SPR. This allows the monitoring of a label free enzyme in real time, and also provides quantitative estimates of the influence of the solid on protein stability.The overall characteristics of the immobilized enzyme, storage stabilities, and turn over characteristics have been studied.

Received: December 23, 2010
Revised: April 6, 2011
Published: April 29, 2011

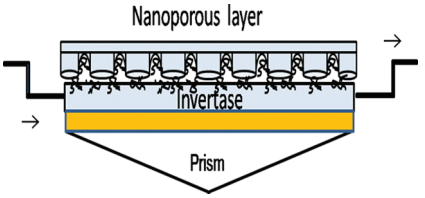


Figure 1. Scheme for the flow cell.

EXPERIMENTAL SECTION

Preparation of Ordered Nanopores. For the preparation of the ordered nanoporous alumina, high purity aluminum foil has been used (Goodfellow, thickness 0.5 mm, purity 99.999%). Highly ordered nanopores with an average inter pore distance of 100 nm and diameter around 60−65 nm have been prepared according to the procedure described by Gösele and co-workers.[16] we used the anodic aluminum oxide (AAO), which was produced by anodizing aluminum in 0.3 M oxalic acid under a constant potential of 40 V.

This procedure resulted in anodic alumina (AA) substrates with an interpore distance of p = 100 nm and pore diameters that were enlarged to D_0 = 65−75 nm, with a chosen thickness of l = 3.5−4.0 μm

Scanning electron microscopy (SEM) measurements were performed with a LEO Supra-35 SEM from Zeiss with acceleration voltages between 6 and 10 kV. Here, 50 nm of Au were evaporated onto the samples before measuring. Pore diameter and spacing have been determined by software-assisted analysis (Scion Image, Scion Corporation; Adobe Photoshop.)

Immobilization of the Enzymes in the Pores. Invertase from baker's yeast (grade VII) has been used in this study. To prepare immobilized invertase in the ordered nanopores, the AA has been first cleaned by dipping in isopropyl alcohol (IPA) and then the solution sonicated for about 10 min. Then the AA layers have been washed several times with deionized water, dried under a stream of nitrogen and finally dipped in high performance liquid chromatography (HPLC) grade ethanol to exclude any air and to ensure complete adsorption of the enzyme in the pores. The AA layers have been then incubated in invertase at the defined pH for about 1 h at T = 22 °C and then washed with buffer, then with deionized water before setting it up in the flow-cell (Figure 1). The activity of the immobilized enzyme has been tested at pH 4.5, 5.9, and 6.5. and for different concentration of invertase and substrate concentration. In all the experiments, the sample collected from the outlet of the flow cell has been tested for the enzyme activity using Fehling's solution for formation of glucose. Normally, for the free enzyme, one unit of enzymatic activity (U) corresponds to the quantity of enzyme that produces one micromol of glucose and fructose in the hydrolysis of a 5% (w/v) sucrose solution, at 55 °C and pH 5.0. The substrate sucrose, acquired from Sigma Chemical USA was 99.9% pure. This sucrose has been used in acetate buffer (pH = 4.5) and phosphate buffer (pH = 5.9, 6.5) for the different concentrations used in this study.

Confocal Laser Scanning Microscope (CLSM) Analysis. Using an inverted confocal microscope (TCS SP5, Leica, Solms, Germany) with an oil immersion objective HCX PL APO, 1003 magnification and numerical aperture 1.4 micrographs of the enzyme immobilized in AA and after reaction have been acquired with the all micrographs prefocused at the oil/glass substrate interface with the microscope operating in reflection mode. Micrographs have been treated with Image J Analysis Software version 1.34S (National Institutes of Health, Bethesda, MD).

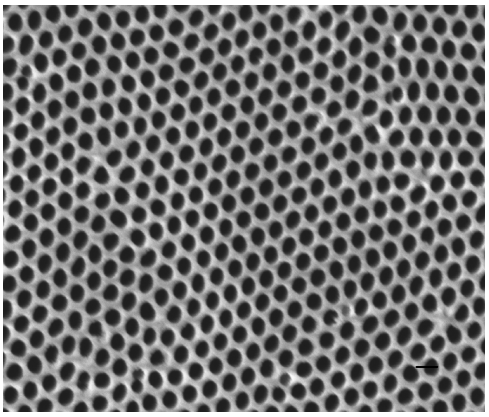


Figure 2. SEM image of porous anodic alumina (scale 100 nm).

SPR Measurement. The SPR measurements have been carried out using the Kretchmann configuration. An incident light from a 30 mW diode laser at 658 nm (LDCU5/4853, Power Technologies, p-polarized) has been focused onto the Au substrate, and the intensity of the reflected light measured by a photodiode (201/579−7227, Thorlabs Inc.) attached to an oscilloscope. The glass substrate coated with an ultrathin film of Au is in contact with a right-angled SF10 prism (Schott glass technology) through an index matching fluid (n = 1.730 ±0.0005, Cargille Laboratories, Inc.). A Teflon cell, with an O ring of inner 0.6 cm, is used to confine the solution in contact with the Au film. Both the prism and the cell have been mounted on a goniometer to control the angle of incidence.

The enzyme adsorption has been first monitored by SPR in real time and the angular dependence analyzed initially for the adsorption of enzyme and then for its interaction with sucrose. Kinetics data for the adsorption studies of the pure invertase and its interaction with sucrose have been analyzed by measuring the change in reflectivity as a function of time. When a continuous flow of protein solution flows over the AA substrate, adsorption of protein occurs. If the flow proceeds until a steady state is reached, adsorption and desorption rates are equal. After the sample solution is replaced with buffer, the bound protein desorbs from the surface and the bare AA is regenerated. To effectively analyze the kinetic data obtained for enzyme substrate interactions, sequential fitting of the desorption and adsorption phases has been employed to measure values of k_d and k_a.

All adsorption and desorption measurements reported in this work have been analyzed based on a simple 1:1 interaction between surface-immobilized enzyme and its substrate (i.e., A + B ↔ AB). In terms of SPR response the rate of desorption of the complex AB can be described by change in reflectivity R (ΔR) at any time t as $\Delta R(t) = \Delta R \exp(-k_d t)$ where ΔR corresponds to the maximum change in SPR signal measured at each protein concentration. These data has been fitted using a Langmuir adsorption isotherm, assuming the invertase monolayer is uniform and sucrose adsorption to each immobilized invertase molecule is independent of sucrose adsorption to neighboring molecules. A series of adsorption curves observed for invertase concentrations ranging from 10 to 300 nM to AA substrate have been made and the adsorption rate constants estimated.

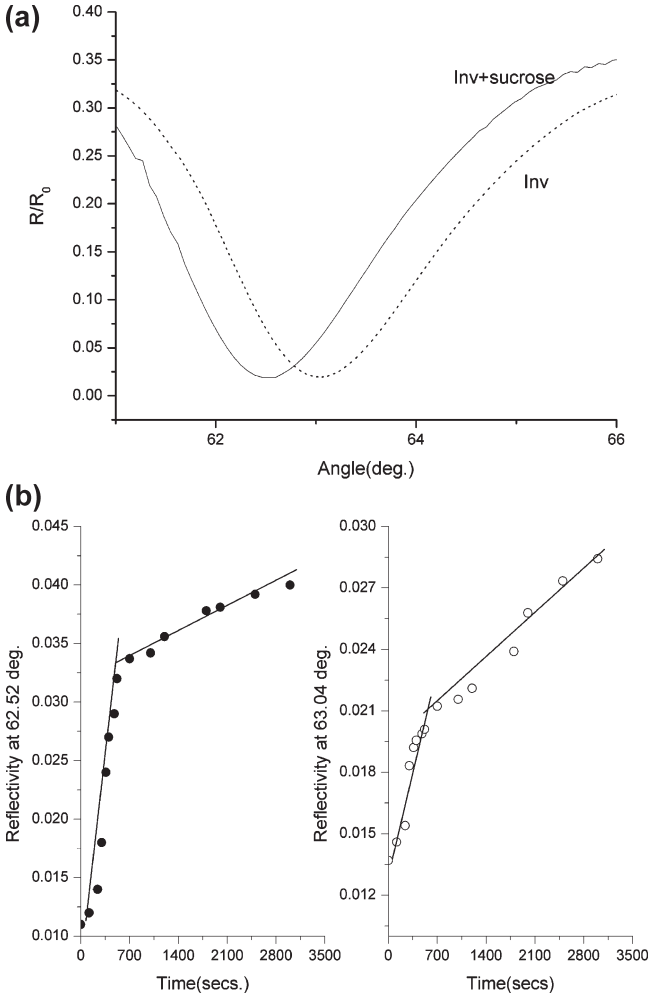


Figure 3. (a) Change in SPR intensity versus incident angle for a invertase adsorption and b sucrose interacting with the enzyme. (b) Kinetic analysis of a adsorption of invertase and b interaction of sucrose with invertase.

Activity Measurements. The flow cell has been used as a reactor in combination with the enzyme immobilized on AA with the enzyme immobilized at a particular pH and the sucrose made to flow through the cell at a defined particular flow rate. Data processing has been done by following the method of initial velocities.[23,24] The activity has been tested by taking 0.5 mL samples of the reactor solution at regular intervals. These samples have been warmed to 37 °C, cooled to room temperature and stocked in the refrigerator for later assay of the glucose and fructose produced during hydrolysis.[25] Activity tests have been carried out at the temperature of 37 °C and pH values: 4.5, 5.9, and 6.5 for the immobilized enzyme.

RESULTS AND DISCUSSION

Figure 2 shows the SEM picture of the porous anodic alumina before immobilization of the enzyme. After incubating the AA layer with the enzyme, the corresponding angular-dependent SPR profiles were recorded. In the enzymatic assay, initially the dark (background) signal is determined. Then, a buffer of pH = 4.5 is injected into the flow cell serving as the reference, for several minutes in order to establish a baseline, The second step involved

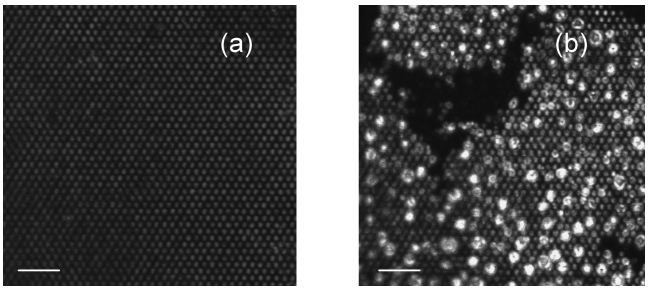


Figure 4. CLSM images of a bare porous anodic alumina and b Invertase adsorbed substrate (scale 0.5 μm).

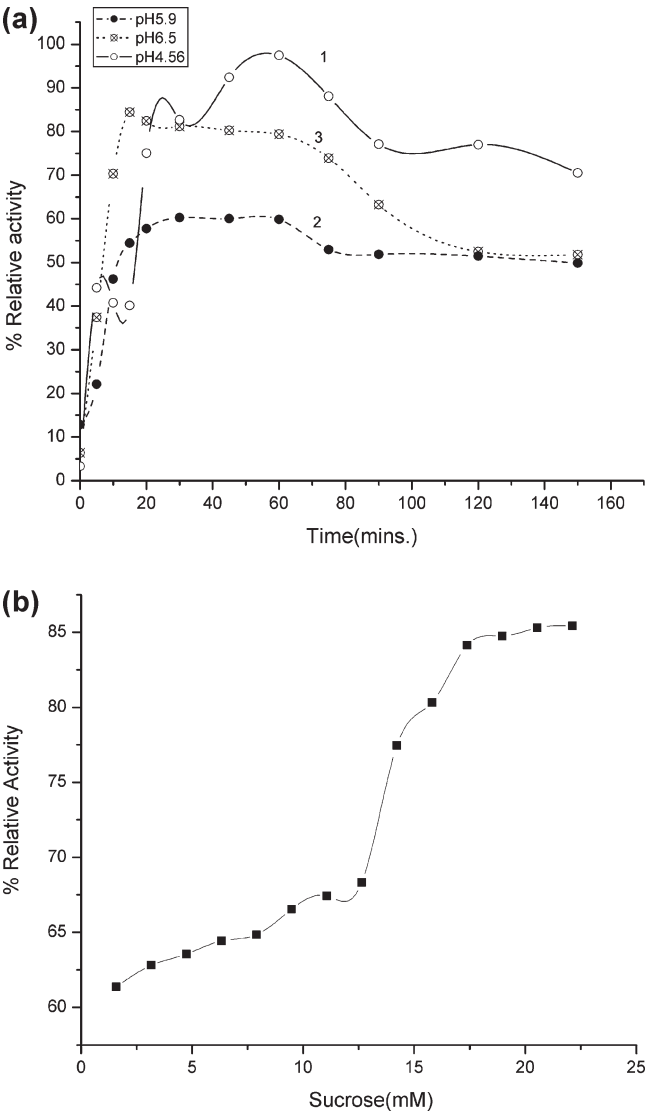


Figure 5. (a) Relative activity plot of the invertase immobilized on AA as a function of pH. (b) Relative activity plot of the enzyme against sucrose concentration.

SPR signal measured on invertase immobilized AA substrate after which the sucrose solution is allowed to run through the SPR flow cell. The substrate changed to product by the catalytic action of invertase immobilized on the SPR sensor surface. A plot of SPR with the immobilized enzyme in the AA with and without the

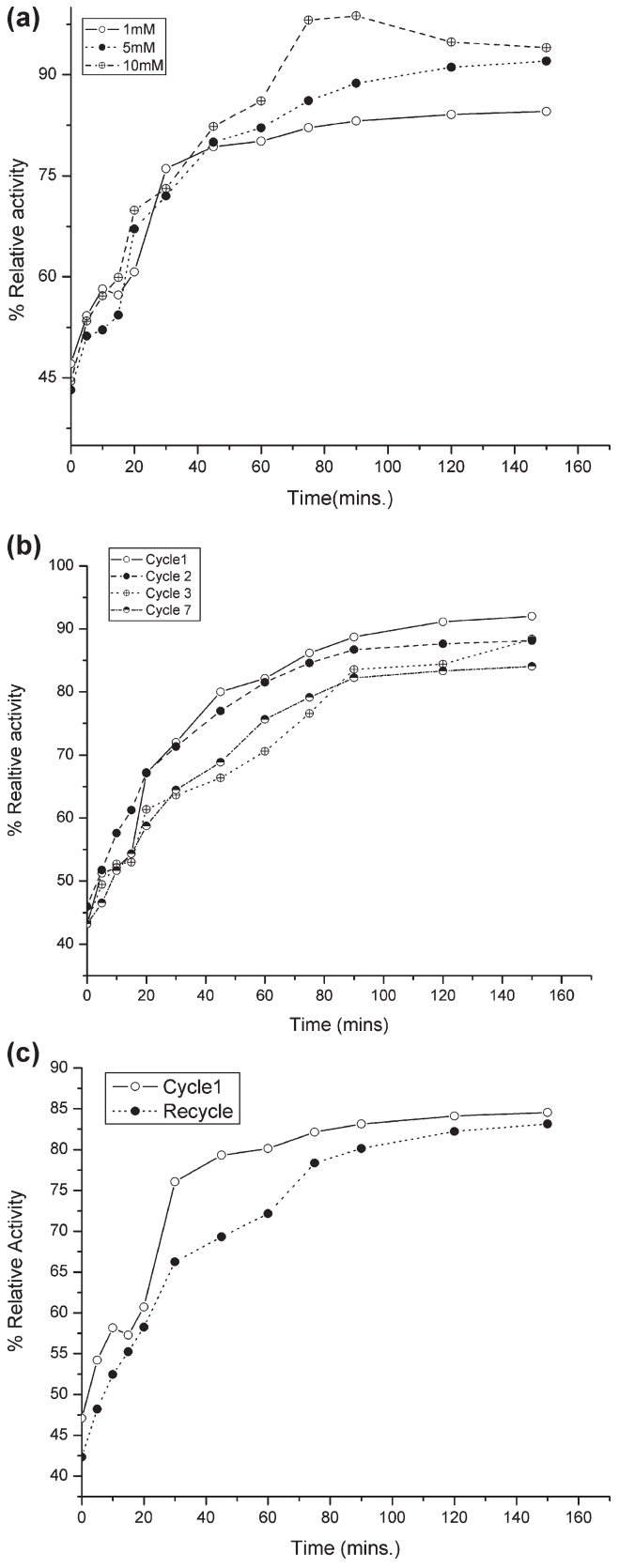


Figure 6. (a) Relative activity for different concentrations of enzyme immobilized in AA. (b) Activity over 1 week for the immobilized enzyme. (c) Recycle and reuse of porous anodic alumina for adsorption of the enzyme.

introduction of sucrose is shown in Figure 3a. This figure shows that SPR minimum shifted from 63.04 to 62.52°. Figure 3b presents the SPR results showing a biphasic kinetics for the adsorption of the enzyme as well as its interaction with the substrate with rates of adsorption for the enzyme at $k = 2.9 \times 10^5\ M^{-1}\ s^{-1}$ and $1.17 \times 10^5\ M^{-1}\ s^{-1}$. The rate of interaction of the substrate with the invertase is initially rapid with $k = 4.49 \times 10^5\ M^{-1}\ s^{-1}$ followed by a slower rate $1.43 \times 10^4\ M^{-1}\ s^{-1}$.

Initially a rapid response has been observed because of the refractive index difference between the enzyme and sucrose. This is followed by a slower increase that indicated adsorption of sucrose to the enzyme and subsequent product formation. This is accompanied by a further decrease.

Parts a and b of Figure 4 shows the CLSM image of bare porous anodic alumina and that with freshly adsorbed invertase in the pores. Figure 5a represents the relative activity plot of the immobilized enzyme as a function of pH. The optimal activity of the immobilized enzyme is seen at pH = 4.5. Free enzyme is known to remain fully active between pH 4 and 8.5. However in the case of the immobilized enzyme on AA, pH values below 4.5 and above 7 can damage the pore structure of AA. Therefore, immobilization studies have been carried out only between pH 4.5 and 7.

Figure 5b presents the relative activity of the enzyme plot against sucrose concentration. It is seen that the maximum activity is around 15 to 22 mM concentration of sucrose. This concentration is higher compared to the usual substrate concentration required for the free enzyme suggesting that lower substrate concentrations are not enough to reach to the active site of enzyme inside the support. It is seen that immobilization rate is high with activity retention as high as 95% in some cases.

The activity for different concentration of enzyme immobilized in AA has been studied and the plot is presented in Figure 6a. It is seen that as concentration increases activity also increases and above 10 mM there is only a marginal increase in the acitivity.

Figure 6b shows the number of cycles that can be applied for the immobilized enzyme and the corresponding activity. In order to check the activity and stability of the immobilized enzyme, the porous anodic alumina substrate has been stored under buffer and aged. The activity has been tested over a period of 1 week. It is found that initially there is a small decrease in activity that could possibly arise from desorption of the enzyme. The quality and recyclability of porous anodic alumina substrate has been tested by cleaning the enzyme coated substrate after 1 week completely. Saturated solutions of guanidine have been used to release the entire enzyme from the support. This support could be reused for 5 cycles without any decrease in enzyme loading capacity.

Figure 6c shows recycled substrate after initial cleaning and subsequent adsorption of the enzyme. It is seen that from the plots the AA layer can be recycled for atleast 5 to 6 times without much change in the activity of the enzyme.

Even though AA has been used as a template to attach small molecules as well as lipid membranes and some biomolecules, this procedure presented here uses the simple natural adsorption for immobilization of invertase and other enzymes

The economic feasibility of the process is directly related to the costs of the reagent. This investigation indicates that immobilization process is safe and of economic interest, especially in the application of continuous hydrolysis of sucrose on pilot or industrial scale.

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ACKNOWLEDGMENT

A.D. would like to thank the DAAD, FRG, for reinvitation and the award of a fellowship under which this work was carried out. She would like to thank Prof. Dr. C. Steinem, Head, host laboratory, Gottingen, FRG for useful discussions and Mr. T. D. Lazzara, for help with the preparation of the substrates.

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