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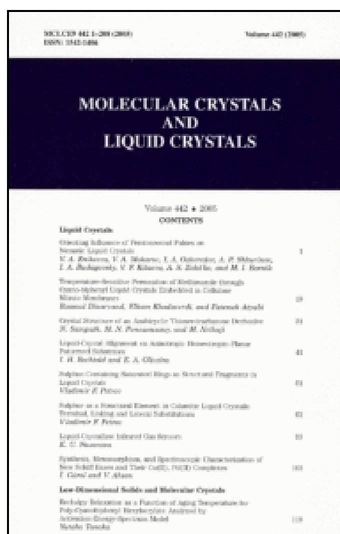

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SYNTHESIS AND LB FILM FORMATION OF FATTY ACID- FAD COMPLEX

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Abstract Flavin adenine dinucleotide can exist in two oxidation states. Switching from one state to the other can be achieved chemically or electrically. The flavin undergoes reduction reversibly by two one electron steps or one two electron step, changing the chemistry of the enediamine subfunction of the isoalloxazine moiety. The adenine moiety is not involved in the catalysis, but helps in anchoring the coenzyme at the active site. The hydrophilicity of FAD has been changed by chemically attaching hydrocarbon chains to the noncatalytic adenine moiety. The modified coenzyme has been verified to retain the electrochemical, optical and the biochemical properties. The amphiphilic molecules can form monolayers at the air-water interface. A film of desired thickness can be formed on a solid support by means of monomolecular deposition using LB film technique. The characterization of the film has been done using spectrophotometric and electrochemical methods.

INTRODUCTION

In recent years, self assembling molecules have attracted interest because of their potential application in the fabrication of transducers, optical devices and sensors[1]. Self assembly of molecules in the form of thin films can be achieved either by physisorption or by chemisorption. Though chemisorption gives more stable monolayers, the packing of the molecules in the layer is better when physisorption techniques are employed. Moreover, the property of the device does not depend on the method of assembly[2]. However, in order to achieve physisorption, the molecule should have well defined hydrophilic and hydrophobic groups of comparable strengths.

Flavin adenine dinucleotide (FAD) acts as a coenzyme for a number of redox flavo enzymes, such as glucose oxidase (GOD), cholesterol oxidase, L - lactate dehydrogenase[3–5]. It undergoes redox reaction from the oxidized state (FAD) to the reduced state ($FADH_2$) during the enzymatic action. The redox reaction can also be achieved electrochemically[6]. The isoalloxazine ring in flavin is involved in the redox action, changing the chemistry of enediamine functional group[7].

The highly hydrophilic nature of FAD poses problems in interfacing of the molecule with appropriate transducer employed in 'dry' electronic applications. Attempts to chemically attach the coenzyme to solid surface have been reported

earlier[8,9]. However, these methods require multi step synthesis and drastic experimental conditions such as treatment with sulfuric acid. We have overcome this by making FAD amphiphilic by attaching long alkyl chains which help to balance the hydrophilicity of the molecule. A three dimensional integration can then be achieved using LB deposition technique.

We report here details of chemical modification of FAD, physico-chemical and biochemical characterization of such a derivative and deposition of monolayers onto solid support.

MATERIAL AND METHODS

Flavin adenine dinucleotide, glucose oxidase (type VII from *A. niger* EC 1.1.3.4) and horse raddish peroxidase (EC1.1.11.7) were purchased from Sigma Chemicals, USA. Fatty acids were obtained from Central Drug House, India. Other chemicals were of analytical grade or better and were used without further purification.

Thin films of modified FAD were prepared by solvent evaporation technique. The solid substrate was dipped in chloroform solution of the substance. A thin film of modified FAD was deposited on solid support (glass plate or platinum strip) on evaporation of the solvent. The Langmuir Blodgett (LB) films were deposited using Alternate trough supplied by Joyce - Loebl, UK.

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The absorption studies of FAD derivative in the form of thin film on glass plate were done on CARY-17 spectrophotometer using a glass plate of identical thickness as reference. Cyclic voltammetric (CV) experiments were done using the normal three probe technique[10], with a Potentiostat/Galvanostat Model 273 of EG & G Princeton Applied Research. A thin film of the sample deposited on platinum strip was used as the working electrode. The reference and the counter electrodes used were saturated calomel and platinum mesh, respectively. 0.1M sodium phosphate buffer of pH 6.8 served as electrolyte. The buffer was presaturated with Argon for an hour to remove possible interference due to dissolved oxygen. The voltage was varied from 0.0 to -0.8 V with a scan rate of 50mV/s.

SYNTHESIS OF FATTY ACID DERIVATIVES OF FAD

FAD is a hydrophilic molecule, which contains polar groups such as phosphate and amine. Our strategy to impart amphiphilicity to FAD is based on covalently attaching a long alkyl chain through the amino group of the adenine ring. Adenine ring is known not to partake in redox reactions. Hence modification of adenine ring by attaching fatty acid, is expected to leave electrochemical and biochemical properties of FAD unchanged.

Stearic acid has been converted to acyl chloride using thionyl chloride. Appropriate amount of fatty acid and thionyl chloride have been refluxed in a round bottomed flask fitted with a water cooled condenser kept in water bath maintained at 340 K for an hour. Excess of thionyl chloride has been distilled off from the reaction mixture. The acyl chloride and FAD have been heated in a round bottomed flask fitted with an air condenser at 340 K for 2 hr. The reaction mixture has been treated with excess of water and then extracted to ether. The ether solution of fatty acid derivative of FAD [Flavin (6-stearoyl amido) adenine diphosphate -SFAD] has been washed again with excess of water to remove unreacted FAD.

Then the reaction mixture has been washed with hot acetonitile to remove the unreacted fatty acid. It is envisaged that the linkage between FAD and fatty acid is formed through amide bond formation between NH_2 of adenine ring and COOH of fatty acid, which has been confirmed by ^{1}H NMR.

CHARACTERIZATION

The attachment of fatty acid chains to FAD changes its solubility characteristics. SFAD is found to be soluble in ether and chloroform and insoluble in water. The absorption spectrum of SFAD in chloroform exhibits peaks at 352 nm and 447 nm. FAD in aqueous solution has absorption maxima at 376 nm and 449 nm respectively[3]. The observed blue shift of the peak at 376 nm is due to the change in polarity of the solvent.

Additional support for the formation of complex has been obtained from hydrolysis of amide bond between NH_2 of FAD and COOH of fatty acid. Hydrolysis of the amide bond of the complex can be effected by adding dilute hydrochloric acid. The characteristic absorption maxima of amide bond at 190 nm is used as a monitor. Breaking of the bond is reflected as decrease in the intensity of the absorption peak. Thus amide bond formation between FAD-NH_2 and COOH of fatty acid gets unequivocally established.

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BIOCHEMICAL CHARACTERISTICS

The biochemical function of the modified FAD namely to form coenzyme to redox enzymes such as glucose oxidase has been assayed using standard assay procedures[11]. The procedure consists of following steps. (1) preparation of apoenzyme (2) formation of holoenzyme with modified FAD as coenzyme and (3) assay of enzyme activity. The apoenzyme has been prepared by the method described by Sawoda[12]. The apoenzyme has been then incubated with SFAD in 0.1 M sodium phosphate buffer of pH 6.1 for 24 hr. 100μl of the apoenzyme-coenzyme mixture was taken out at time t=0 of the incubation and introduced into a solution containing 50 μg of horse raddish peroxidase and 100 μl of 2M glucose in 3ml of o-dianisidine solution (10mg/100ml) in 0.1 M sodium phosphate buffer of pH 6.1. After 24hr, the activity of the protein solution has been measured with glucose as substrate. The oxidation of glucose has been monitored by observing breaking of hydrogen peroxide formed during the oxidation of glucose using peroxidase and oxidizing o-dianisidine and noting absorbance at 500 nm[11]. The activity of the reconstituted enzyme has been found to be 2100 U/g, which though low, is significant. The smaller value of activity may be due to incomplete conversion of total mass of apoenzyme into holoenzyme. One may recall that modified FAD is insoluble in aqueous medium and use of organic solvents is generally detrimental to enzymatic activity. These facts prevent bringing together large quantities of modified coenzyme in contact with apoenzyme. However, our results clearly indicate that the incorporation of modified coenzyme in GOD has not nullified the catalytic activity of the enzyme.

LB FILMS OF MODIFIED FAD

Surface pressure - area isotherm of the fatty acid and amphiphilic FAD

100 μl of amphiphilic FAD has been dissolved in chloroform (1mg/ml) and spread on one of the compartments of the alternate trough containing 0.2mM cadmium chloride solution. Chloroform has been evaporated from the surface by air current and the modified FAD molecules have been allowed to equilibrate. The surface area is then compressed with the help of a teflon barrier using a compression rate of 1sq cm per minute while the pressure exerted at the Wilhelmy plate is measured with the help of the electro microbalance, connected to the units which control the barrier movement.

A plot of surface pressure π against the molecular area (A) is shown in figure 1. Decrease in the area of the surface increases intermolecular interactions taking the molecule from the two dimensional gaseous state to a two dimensional solid phase through an intermediate liquid phase. The π-A isotherms of stearic acid and stearoyl derivative of FAD, i.e., SFAD have been compared in figure 1. One observes that the size of the hydrophilic part of the molecule influences the isotherm. The solid phase appears around 15mN/m for SFAD. The stability of the monolayer has been checked and found to be excellent at a surface pressure of 20mN/m. The monolayer of SFAD is stable over prolonged periods of time (over 15 min).

The deposition of the multilayers have been done on glass plate and platinum strips which have been pretreated to remove oil contamination. The glass plate has been treated with chromic acid followed by alkali and then thoroughly washed with double distilled water before drying. Platinum plate has been cleaned with dilute nitric acid, washed with acetone and dried. The monolayers have been deposited onto solid substrates at a surface pressure of 20mN/m in a centro symmetric Y-type fashion.

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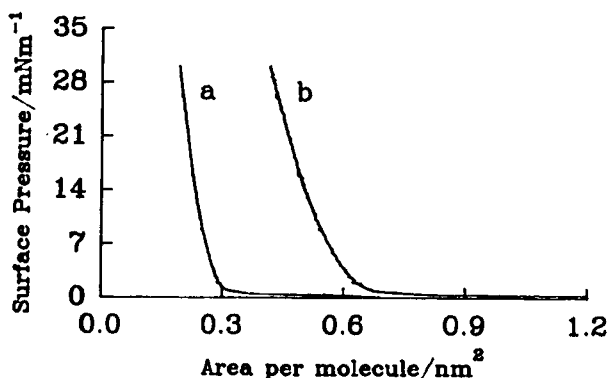

Figure 1 Surface pressure (π - Molecular area (A) isotherms of (a) stearic acid, (b) SFAD at the air-water interface.

Optical Properties of the modified FAD films
The absorption maxima characteristic to isoalloxazine ring of FAD in aqueous solution appear at 376 and 449nm. SFAD in chloroform solution exhibit maxima at 352 nm and 447 nm, which are shifted with respect to those of native molecule. These shifts are arising due to the changes in the polarity of the solvents. The visible range absorption spectrum of LB film of SFAD on glass plate is shown in figure 2.

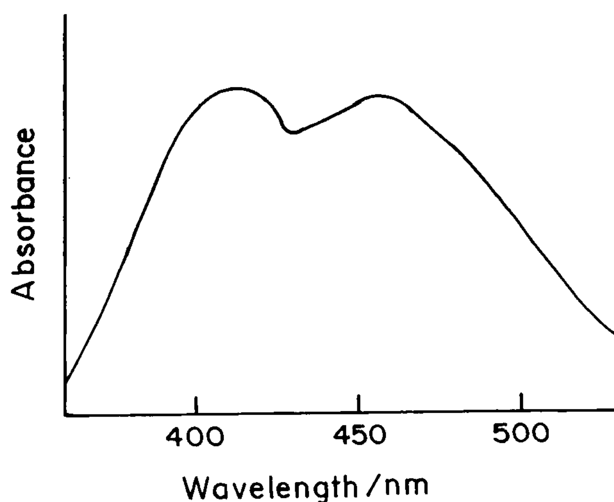

Figure 2 Absorption spectrum of SFAD multilayers (20 layers) at the glass plate with respect to a glass plate of the same thickness.

The absorption characteristics are identical to that observed in chloroform solution. Thus, optical spectroscopy indicates that assembling SFAD in solid state leaves absorption characteristics unchanged.

Cyclic voltammetry
It is known that FAD in aqueous solution exhibits redox potential around -0.44 volts with respect to saturated calomel electrode (SCE)[6,13]. The cyclic voltammogram of the SFAD coated on platinum strip is shown in figure 3(a). One observes that the redox characteristics of the molecule remain unaltered as the cyclic voltammograms of the SFAD resembles that of free FAD. Figure 3(b) gives the cyclic voltammogram of the SFAD film deposited on platinum strip by the method of LB film deposition technique containing 20 monomolecular layers. The redox characteristics are not as prominent as those of the thin film (figure 3(a)). This may be so because of the low concentration of the redox species in contact with the electrode surface. One may recall that the current is proportional to the concentration of the redox species in contact with the electrode surface[6]. One observes that the shapes of

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cyclic voltammograms in two cases are not exactly identical. The observed change may be due to the possible unstacking of the flavin and the adenine moieties in the process of deposition of the LB film.

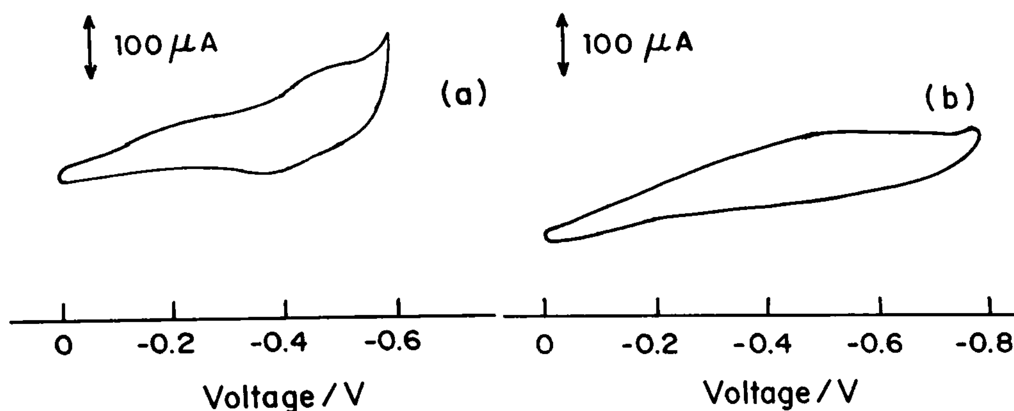

Figure 3 Cyclic voltammogram of SFAD on platinum plate (a) thin film coated by evaporation of solvent (chloroform) and (b) LB film of SFAD (20 layers), with saturated calomel electrode as the reference and platinum mesh as the counter electrode. Scan rate 50mV/sec. 0.1M sodium phosphate buffer of pH 6.8 presaturated with argon was the electrolyte.

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CONCLUSIONS

Coenzyme FAD can be made amphiphilic by chemical modification of adenine ring by attaching fatty acid chains. The modified compound has been found to display chemical and biochemical characteristics, which are similar to those of native FAD. Modification facilitates formation of ordered three dimensional organization using LB deposition technique. Such films have great potential in fabrication of molecular electronic devices.

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