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Physical and chemical characterization of enolase immobilized polydiacetylene Langmuir–Blodgett film

K. Sadagopan [a], Shilpa N. Sawant [b], S.K. Kulshreshtha [b], Gotam K. Jarori [a,*]

[a] *Department of Biological Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Mumbai 400005, India*
[b] *Novel Materials and Structural Chemistry Division, Bhabha Atomic Research Centre, Mumbai 400085, India*

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Abstract

Hexa histidine tagged recombinant *Plasmodium falciparum* enolase (His_6-Pfen) was covalently immobilized on a Langmuir–Blodgett film of a self assembled mixture of 10,12-pentacosadiynoic acid and its *N*-succinimidyl ester derivative (PDA LB-film). The film was polymerized with a UV-lamp at 254 nm to obtain a blue coloured, protein hooked polydiacetylene film. Atomic force microscopy (AFM) was used to characterize the surface morphology of the protein-immobilized film. The colorimetric response (CR) of the His_6-Pfen hooked PDA LB-film to 2-phosphoglyceric acid (2-PGA), the substrate of enolase, in the presence of magnesium ions was studied spectrophotometrically. The CR of glutathione *S*-transferase-Pfen (GST-Pfen) immobilized film prepared by using similar procedure was also examined. The results suggest that binding of Mg (II) to the enzyme facilitates the interaction of the enzyme with 2-phosphoglycerate. This ligand-binding event could be detected by an observed increase in colorimetric response of the film by ∼10%. Thus the incorporation of enolase on a PDA film resulted in the formation of a novel material, which can serve as a biosensor.

Keywords: Enolase; Covalent immobilization; AFM; Protein–ligand interaction; Colorimetric response

1. Introduction

Among several conjugated polymers (polyacetylene, polyaniline, polyphenylene, polypyrrole, polythiophene, polyfluorene, etc.), which are known to have unique optical, electrochemical and electrical properties [1 and references therein], polydiacetylene-based biosensors are of particular interest for the last decade. This is because self-assembled Langmuir–Blodgett films of polydiacetylene or its derivatives are sensitive to external stimuli [2] in the form of mechanical stress, temperature, pH [3,4] or prolonged UV-irradiation [5,6] and undergo a colour change from blue to red. Huo et al. [6] proposed a mechanism of self-folding process according to which in the blue polymerized form, the head groups are aligned in an ordered form, which became disordered under external stress. This leads to a change in the electronic environment resulting in the colour change to red. The most significant application of polydiacetylenes as sensors for pathogen detections was reported by Charych et al. for detection of influenza virus [7] and cholera toxin [8] using functionalized polydiacetylene. In these biosensors, the polydiacetylene head groups were modified with the receptor molecules viz. sialosides and gangliosides to interact with toxins or viruses and allowed the latter to merge with the polymer backbone to cause the colour change of the film from blue to red. Later, Song et al. [9] developed a polydiacetylene biosensor consisting of *S*-glycosylated instead of a *C*-glycosylated sialo receptor as reported before having comparable affinity to detect toxins and viruses. In the above sensors, a high affinity interaction between the receptor molecules and the targeted macromolecules (toxins or viruses) changes the polydiacetylene backbone and causes the colour change. On the other hand, preferential interaction of the anilide moiety of polydiacetylene Langmuir–Schaefer film with α-cyclodextrin molecule and the resultant colour change of the PDA film was reported by Cho et al. [10]. In this cyclodextrin–anilide system, an inclusion complex is formed by these two entities causing the colour change of the PDA film [10]. A different approach was adopted by Cheng and Stevens [11] where colorimetric

* Corresponding author. Tel.: +91 22 2280 4545; fax: +91 22 2280 4610.
E-mail address: gkj@tifr.res.in (G.K. Jarori).

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detection of glucose was made by using a hexokinase (as a sensor) immobilized polydiacetylene film. Here the enzyme was covalently cross-linked to the PDA head groups, and conformational changes induced by the binding of glucose were reported by changes in CR of the polydiacetylene film. Thus polydiacetylene-based biosensors have emerged as very promising materials to have numerous applications to detect various host-guest interactions.

Here, we report our initial investigations aimed at developing a sensor for the detection of a substrate of an enzyme. We have used enolase from *Plasmodium falciparum*, as a macromolecular receptor immobilized on a PDA film to monitor the interaction of Mg (II) ions and 2-phosphoglycerate in solution by using a colorimetric sensor. Enolase (EC 4.2.1.11) is a glycolytic enzyme, which catalyzes the inter-conversion of 2-phosphoglyceric acid (2-PGA) and phosphoenolpyruvate (PEP). His_6-Pfen is a homodimer of molecular weight 102 kDa under physiological pH condition [12]. For activity, enolase requires binding of 2 mol of divalent cations (Mg^{+2}) per subunit. Binding at site I (conformational site) leads to conformational changes at the enzyme active site and enables the binding of a substrate. Following the binding of the first metal ion and a substrate, the second metal ion binds (catalytic site) and initiates the catalytic reaction [13]. Since the protein undergoes conformational changes upon the binding of metal ions as well as the substrate, we assumed that this interaction would be capable of producing conformational stress on the PDA moiety to cause the colour change of the film from blue to red. Since solid state based responsive biomolecular assemblies have been of extensive importance for biomedical and bioengineering applications, it would be prudent to demonstrate the response of the PDA self-assembly to such protein–ligand(s) interaction. This will help widen the scope of PDA-based colorimetric biosensors. For this purpose, the recombinant protein enolase from the malarial parasite *Plasmodium falciparum* (His_6-Pfen) was immobilized on a PDA film and the colorimetric response of this film upon binding of 2-PGA (substrate for enolase) was studied by using a UV–vis spectrophotometer. It is also interesting to study the topography in the immobilized films to get structural information of the immobilized protein. AFM was used for this purpose because it can give topography of single protein molecules in nanometer scale due to its exceptional signal-to-noise ratio [14].

2. Experimental

2.1. Materials

10,12-Pentacosadiynoic acid (PDA) was purchased from Fluka and recrystallized from chloroform prior to use to remove the traces of red polymer present as an impurity. *N*-hydroxysuccinimide (NHS from Sigma), octadecyl trichlorosilane (OTCS from Aldrich) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (NDAEH from Sigma) were used as received. Water from a MilliQ deionizer unit was used for preparation of the LB film and dialysis buffers.

2.2. Protein purification

The cloning, expression, purification and the specific activity data of His_6-Pfen have recently been reported by us [12] and that of GST-Pfen (manuscript to be submitted) will be reported elsewhere. For the current study these proteins were purified by affinity methods and purity was assessed by SDS-PAGE. Activity of the enzyme preparations was measured by a spectrophotometric assay as described earlier [12].

2.3. Characterization methods

Infrared spectra of PDA and NHS-PDA were recorded on a Fourier transform infrared (FTIR) spectrometer (Perkin-Elmer, Model 1650). The sample was mixed with KBr, pelletized and the spectrum was taken.

^1H and ^{13}C NMR spectra of PDA and NHS-PDA were recorded on a 500 MHz FT NMR instrument (Brucker Avance-500) with deuterated chloroform as solvent. Spectra were calibrated by using tetramethylsilane (TMS) as an internal standard.

AFM measurements were carried out by using a scanning probe microscope (SPM-Solver P47, NT-MDT, Russia) in a non-contact mode.

A Perkin-Elmer (Model Lambda 40) UV–vis spectrophotometer was used for optical absorbance studies.

2.4. Protein immobilization on self-assembled PDA LB-film

NHS-PDA was synthesized from PDA [15] and characterized by FTIR, ^1H and ^{13}C NMR for their structure. NHS-PCDA FTIR (KBr, cm^{-1}): 2925, 2853, 1729, 1465, 1209, 1068; ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, 3H, $J = 6.6$), 1.26 (br, s, 26H), 1.5 (m), 1.71 (m), 2.24 (t, 2H, $J = 6.9$), 2.60 (t, 2H, $J = 7.5$), 2.84 (br, s); ^{13}C NMR: δ 15.5 ($–CH_3$), 20–33 ($–CH_2–$ groups), 170.6 (–C=O), 170.1 (–COO–).

His_6-Pfen was immobilized on PDA-LB films by using the method reported by Cheng and Stevens [11]. A Langmuir–Blodgett trough (KSV5000, Finland) was used for deposition of LB films. Surface pressure was measured with a platinum Wilhelmy plate. A 1:1 molar PDA/NHS-PDA solution in chloroform was used as the spreading solution. The aqueous sub phase temperature was maintained at 15 °C. After allowing 20 min for the chloroform to evaporate, the surface was compressed slowly to record the pressure–area isotherm. A compression rate of 5 mm/min was used for studying the pressure–area isotherm. When the desired surface pressure was attained, the monolayers were transferred from the air–water interface to a solid substrate (glass/quartz slides pre-coated with OTCS) by a vertical dipping method. A surface pressure of 40 mN/m was maintained for deposition of LB films and three monolayers were deposited. Dipping and raising speed was fixed at 5 mm/min and a waiting time of 10 min was given between dips to dry the LB film.

Purified His_6-Pfen [12] (1.4 mg/ml) in a 50 mM Tris, pH 7.6 buffer containing 150 mM NaCl was used for covalent immobilization. The LB-film as obtained above was incubated in the protein solution for 1 h at 4 °C. The slide was rinsed with