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

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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

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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

50 mM Tris, pH = 7.6 buffer and incubated for 10 min in 100 mM ethanolamine at pH = 8.0 to stop the reaction between the amino groups of free lysine residues from His_6-Pfen and the head groups from the NHS-PDA/PDA LB-film [11]. The protein-immobilized film was again rinsed with a 50 mM Tris buffer and air-dried. The immobilized protein concentration was obtained from the difference in absorbance (at 280 nm) of His_6-Pfen before and after incubation with the PDA LB-film. An OD_{280} difference of 0.025 was obtained corresponding to approximately 1 nmol of protein being immobilized on the film. Polymerization was carried out by using a hand-held UV-lamp at 254 nm for 8 min on each side of the slide. A control film was treated in the similar way but without protein. The obtained blue His_6-Pfen – PDA and control – PDA films were used for AFM and colorimetric response studies. A GST-Pfen-PDA film was also prepared in the similar manner except that His_6-Pfen was replaced with GST-Pfen.

3. Results and discussion

3.1. Characterization of PDA, NHS-PDA and the protein purity determination:

Fig. 1 presents the FTIR spectra of PDA and NHS-PDA. The carboxylic acid stretching observed at 1698 cm^{-1} for PDA disappeared in the case of NHS-PDA and a new peak centered at 1729 cm^{-1} was observed due to ester formation. The ^1H and ^{13}C NMR spectra of these compounds are shown respectively in Fig. 2 (A) and (B), respectively. In the ^1H NMR spectrum of NHS-PDA, an additional peak centered at 2.8 ppm is observed, which is absent in the case of PDA. This peak corresponds to the $>CH_2$ resonance of the *N*-hydroxy succinimide moiety as a result of modification of PDA. This was further confirmed by a ^{13}C NMR spectrum. The carboxylic acid peak at 180 ppm in the PDA completely disappeared in NHS-PDA and two new peaks at 170.6 and 170.1 ppm were observed, corresponding to

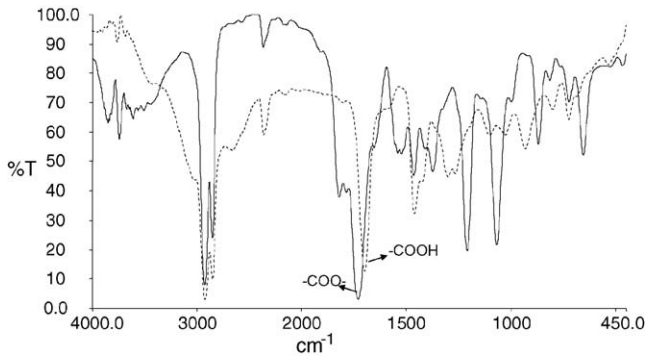

Fig. 1. FTIR spectra of PDA (dotted lines) and NHS-PDA (solid lines).

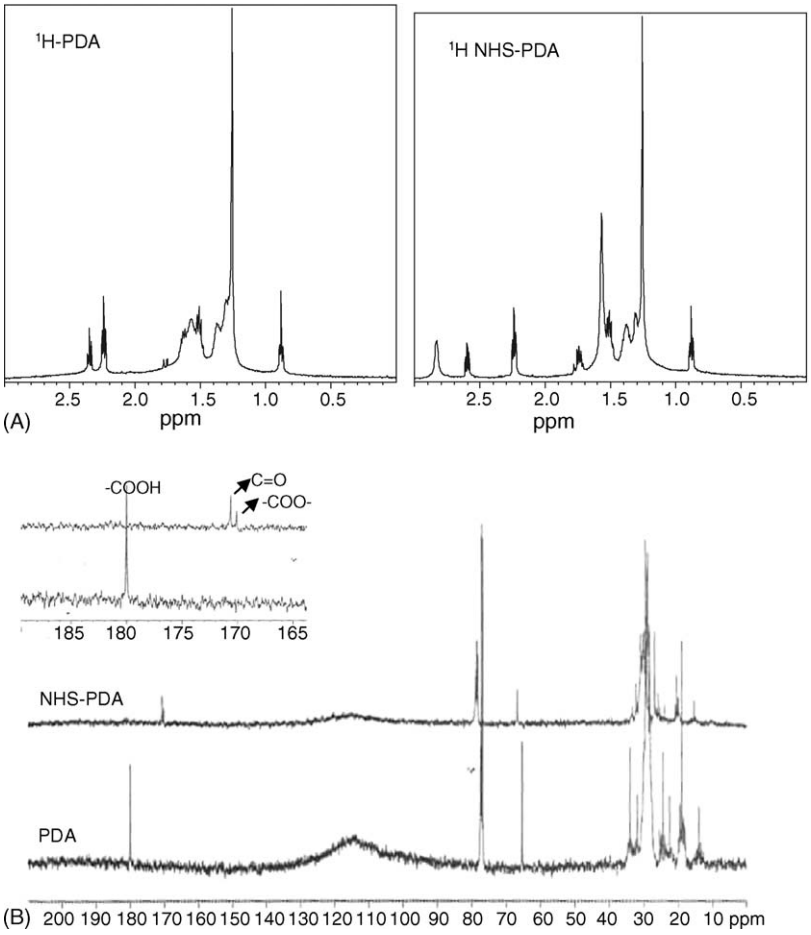

Fig. 2. (A) ^1H and (B) ^{13}C NMR spectra of PDA and NHS-PDA.

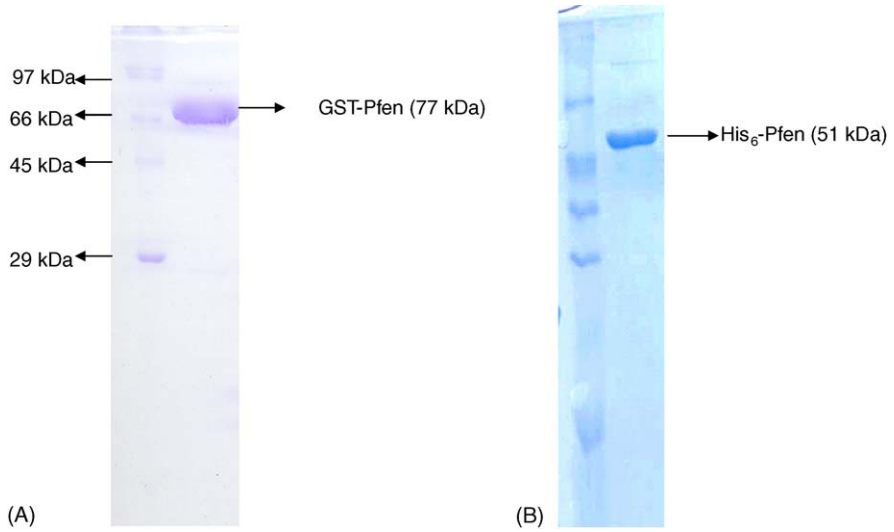

Fig. 3. SDS-PAGE analysis of His_6-Pfen and GST-Pfen (A) lane 1: molecular weight markers; lane 2: purified GST-Pfen (B) lane 1: marker; lane 2: purified His_6-Pfen.

the keto and ester groups in NHS-PDA, respectively. Thus the structures of PDA and NHS-PDA were confirmed. Fig. 3(A) and (B) show the 12% SDS-PAGE of purified GST-Pfen and His_6-Pfen, respectively. The protein used in these experiments were >95% pure as evident from the gel.

3.2. LB films of PDA

The surface pressure–area isotherm of a 1:1 molar mixture of PDA/NHS-PDA is shown in Fig. 4. The isotherm shows an abrupt increase in surface pressure at around 37 $Å^2$. In the high-pressure region, the curve is steep and shows a mean molecular area of 25 $Å^2$, corresponding to the true molecular cross-section of an alkyl diacetylene molecule. These values are in accordance with the report by Sasaki et al. for the monolayer of PDA self-assembled over an aqueous sub-phase at a temperature of 15 °C [5]. The collapse pressure of the film under the current study was observed around 55 mN/m. The collapse pressure is critically dependent on the sub phase temperature.

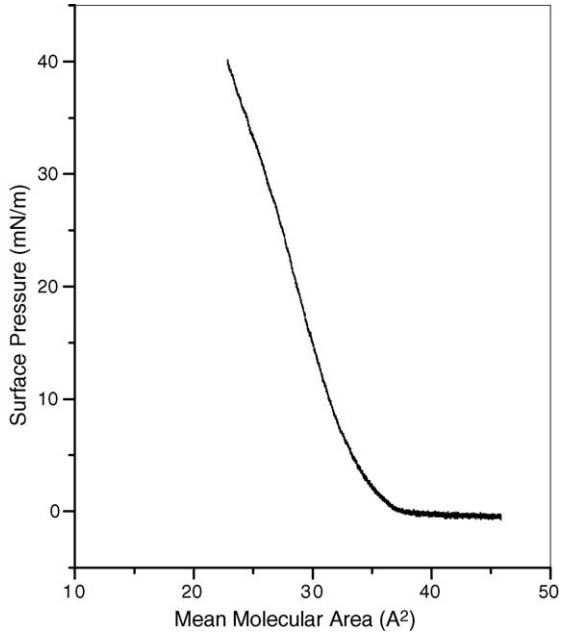

Fig. 4. The surface pressure–area isotherm of a 1:1 molar mixture of PDA/NHS-PDA.

3.3. AFM studies on the immobilized Pfen

3.3.1. Topography of His_6-Pfen PDA film

Fig. 5 represents the topography of the immobilized His_6-Pfen on a PDA film observed by AFM at (A) 2 μm × 2 μm and (B) 5 μm × 5 μm scale area. The arrow in each image represents a particle among the group of spherical clusters. Multiple cross-sections of the individual protein images were made, and the mean diameter and height of each single particle were measured. Generally AFM is known to overestimate the diameter of biological samples due to the geometry of the tip, which induces broadening effects in the image. The diameter at half-maximal height of the individual molecule was measured as this method is reported to compensate for the artificially induced overestimation of the protein width [16].

Enolase molecules were observed as spherical clusters having a diameter of ~40 nm and a height of ~5–8 nm. Recently Almeida et al. [17] studied the adsorption of yeast enolase (Yen) molecules on silicon wafers in liquid environment with time, and imaged the topography by AFM. The image obtained by us for His_6-Pfen suggested that its spherical nature was similar to the image of Yen adsorbed on silicon wafers where a protein concentration of 0.5 g/l in a NaCl (0.1 mol/l) solution was used. This observed similarity in topography was in spite the fact that the Yen adsorption on silicon surfaces is driven mostly by electrostatic means [17,18], whereas, His_6-Pfen was immobilized on PDA by covalent linkage. Further, the observed height of a spherical particle was ~5–8 nm, which was in good agreement with that observed for Yen (7 nm) by Almeida et al. As both of these proteins (r-Pfen and Yen) are highly homologous (~60% homology) and exist as homodimers under physiological (near neutral pH) conditions, it is not surprising to observe similar particle

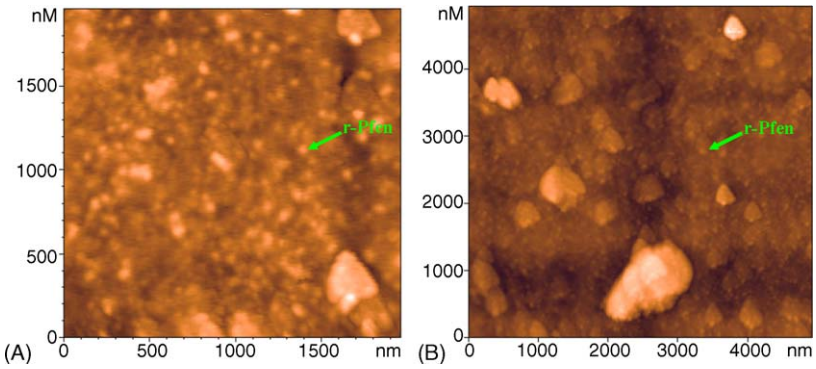

Fig. 5. Atomic force microscopy images of His_6-Pfen immobilized on a PDA film. Scan area (A) 2 μm × 2 μm and (B) 5 μm × 5 μm.

shape and size. In our laboratory, we made another recombinant form of Pfen tagged with glutathione *S*-transferase (GST-Pfen). This protein has a molecular mass of 77,648 Da. To examine the sensor properties of this heavy version of Pfen, we immobilized this protein on a PDA film and characterized it.

3.3.2. *Topography of GST-Pfen-PDA film*

In order to determine the oligomeric state of GST-Pfen in the immobilized form and to have a comparative account with respect to the His_6-Pfen immobilized form, we performed AFM imaging of GST-Pfen immobilized on a PDA film. Fig. 6 shows the topography of the immobilized GST-Pfen on a PDA film observed by AFM at (A) 2 μm × 2 μm and (B) 5 μm × 5 μm scale area. As can be seen from this figure, GST-Pfen forms elliptical clusters, whereas, His6-Pfen is spherical in nature (Fig. 5). The dimension of the GST-Pfen from the image was measured as described above. The elliptical clusters had a height of 15 nm and widths of 125 nm for the longer axis and of 40 nm for the shorter axis. The observed height is around three times larger than the His_6-Pfen film, suggesting that the GST-Pfen is probably existing as a hexamer and His_6-Pfen as a dimer in the immobilized film.

3.4. *Colorimetric response of r-Pfen-PDA film upon 2-PGA binding*

In order to examine the colorimetric response of a PDA film upon the binding of Mg (II) and a substrate on the immobilized His_6-Pfen, the His_6-Pfen-PDA film was placed in a cuvette containing 50 mM Tris–HCl of pH = 7.6, and its UV–vis absorption spectrum was recorded in the range of 450–800 nm. The spectrum obtained in this buffer solution was considered as blank. As can be seen from Fig. 7(A) (curve (a)), the protein immobilized film (blank) has an absorbance maximum centered around 640 nm, corresponding to the blue form of the film. To the cuvette, 2-PGA was added to a final concentration of 1.5 mM and the absorbance spectra were recorded after 60, 90 and 180 min after addition. The spectrum observed after 180 min of incubation is presented in Fig. 7(A) (curve (b)). This spectrum is very similar to the background spectrum but with a faint absorption peak at 550 nm. As Mg (II) is known to be essential for the binding of 2-PGA, we added $MgCl_2$ to a final concentration of 1.5 mM to the film and incubated for 5 min and then the spectrum was recorded. The observed spectrum showed a significant change in the absorption intensity at different wavelengths (solid line, curve (c), Fig. 7(A)). The observed spectral changes support the view that binding of Mg (II) and 2-PGA to enolase leads to conformational changes. Further, it demonstrates that the fabricated film is able to provide a read out of induced conformational changes as the observed changes in CR. Exposing this film to higher concentrations of Mg (II) (incubated for 15 min in 10 mM Mg (II)) at this stage did not induce any further change. It is important to note that under these conditions the enzyme can catalyze the conversion of 2-PGA to phosphoenolpyruvate. Thus observed responses are attributed to binding of Mg (II) and 2-PGA (or PEP) to enolase. In three separate experiments, the

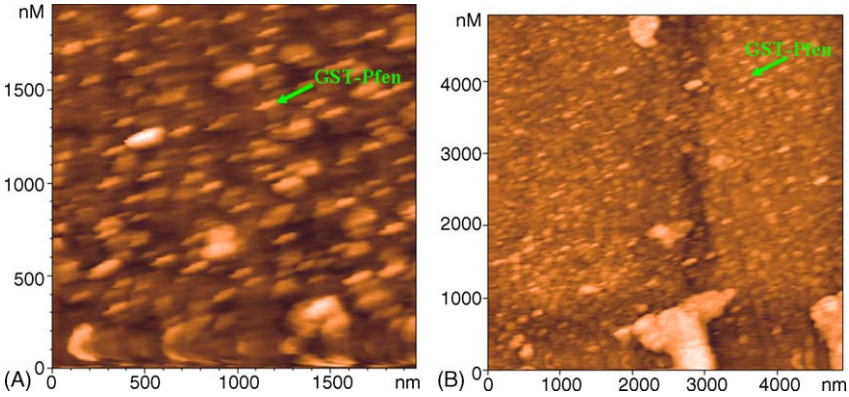

Fig. 6. Atomic force microscopy images of GST-Pfen immobilized on a PDA film. Scan area (A) 2 μm × 2 μm and (B) 5 μm × 5 μm.

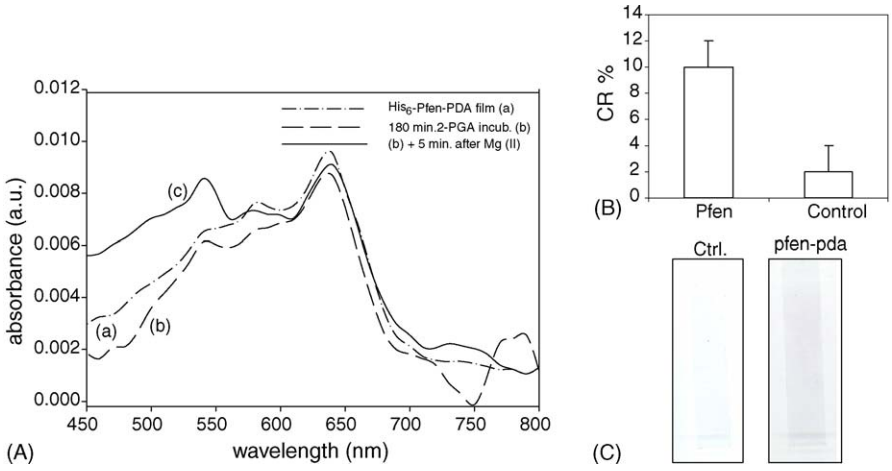

Fig. 7. UV–vis spectra of His_6-Pfen-PDA film upon binding Mg (II) and 2-PGA. (A) (a) A His_6-Pfen-PDA film in 50 mM Tris of pH = 7.6 (background); (b) at 180 min after the addition of 1.5 mM 2-PGA; (c) 1.5 mM Mg (II) added to the film in (b) and incubated for 5 min. (B) Plot of CR % vs. r-Pfen and control. (C) Colour change of a Pfen film with respect to a control film. A slight red tinge is observable with naked eyes for Pfen-PDA film.

percentage colorimetric response (CR%), as defined by Charych et al. [7], was found to be 10 ± 2 for the films used in this study. For the control PDA film (where protein was not immobilized), the spectra were acquired before and after the addition of 2-PGA and Mg (II) at same time intervals as above. The CR% was found to vary in the range of 2–4 (for two such control slides) (spectra not shown). The observed CR% has been plotted for the His_6-Pfen-PDA and control PDA films in Fig. 7(B). From Fig. 7(C), the colour change of the His_6-Pfen-PDA film can be visualized compared to the control PDA film. The observed CR for His_6-Pfen led us to believe that presence of bulky groups attached to this protein might induce more stress on PDA during ligand(s) interaction and hence may result in higher CR. Further, as in the hybrid protein (GST-Pfen) both domains are known to dimerise, it is likely that GST-Pfen may form higher oligomers (>dimers). Coupling of such oligomers to the PDA film may impart greater receptor density, leading to higher CR. However, it should be realized that such response can be a complex function of the oligomeric structure of the sensor and the mode in which it gets attached to the PDA film during covalent labeling. In order to test the impact of the change in oligomeric structure of a sensor protein, we used GST-tagged Pfen (GST-Pfen). As both domains of this recombinant protein (GST and Pfen) are capable of forming dimers, it is expected that the hybrid protein may associate to form multi-subunit oligomers. Our gel filtration studies indicate that it is indeed the case (data not shown). We have immobilized this protein on a PDA film and investigated the colorimetric response to ligand interaction.

3.5. Colorimetric response of GST-Pfen-PDA film

Fig. 8 gives the CR of GST-Pfen upon binding of 2-PGA and Mg (II) (each 1.5 mM final concentration). Trace (a) (dotted line) represents the spectrum of GST-Pfen in 50 mM Tris–HCl of pH = 7.6 in the absence of ligands (control). Traces (b) and (c) show CR for 30 and 60 min after incubation in the aforementioned concentrations of 2-PGA and Mg (II), respectively. The spectrum did not change with a further increase in incubation

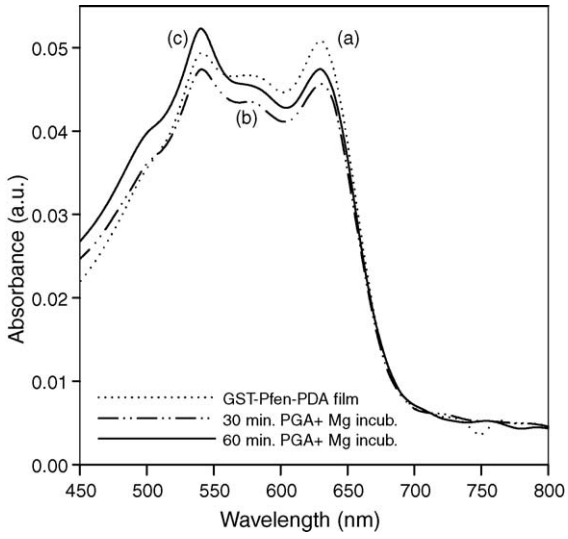

Fig. 8. UV–vis spectra of a GST-Pfen-PDA film upon Mg (II) and 2-PGA binding; (a) GST-Pfen-PDA film in 50 mM Tris of pH = 7.6 (background); (b) at 30 min; (c) at 60 min after the addition of 2-PGA and Mg (II) (1.5 mM final concentration).

time to 90 min. The control spectrum of the GST-Pfen-PDA film had slight absorbance at 550 nm (Fig. 8 trace (a)). This may be due to the association of heavy oligomers in a small surface area of the film, creating enough stress to generate a slight red tinge in the film. In spite of this background, the CR changed by ~7% for the 60 min incubated film. It appears that incorporation of dimers (relatively lighter form of the sensor) with higher coverage density may be a better strategy to improve CR, rather than attaching a heavy oligomer to the polydiacetylene LB-film.

4. Conclusions

In this study, we have characterized the physical and chemical properties of a Pfen immobilized PDA LB-film and the stress induced colour change of the PDA film as a result of conformational change induced in Pfen upon its interaction with metal

ions and substrates. This study is in accordance with the earlier conclusions drawn on Yen that binding of Mg (II) is essential for subsequent interaction of substrates or substrate analogs with enolase [19,20]. Spherical and elliptical clusters were observed by AFM for the immobilized r-Pfen and GST-Pfen on PDA-film, respectively. The observed topography of His_6-Pfen (dimer) is similar to those reported for yeast enolase (dimer) adsorbed on silicon surfaces in liquid environment. Thus from AFM studies, we could get the molecular level details of the immobilized proteins. Also it is inferred that the immobilized proteins retain their intact oligomeric form, as known from the gel filtration studies in buffer solutions [12], which is the primary requirement for designing the biomolecular based solid-state sensor materials. The observed percentage CR of a His_6-Pfen-PDA film during the binding of ligand(s) is less than that observed by Cheng and Stevens [11] for their glucose-hexokinase induced conformational stress on PDA LB-film by around 5–7%. The less CR% observed can be attributed to the optical property of the self-assembled films as the sub-phase temperature used in this study is 15 °C, and also it can be attributed to the difference in the nature of conformational changes between hexokinase and Pfen upon their respective ligand(s) binding and the resultant stress on the PDA backbone. It is noteworthy to mention here that films free of background red absorbance would be more suitable than those with slight red phase absorbance for such protein–ligand interaction based colorimetric sensors. Detailed effect of increasing the amount of Mg ions or 2-PGA and that of other divalent metal ions on the CR of the PDA-enolase film will give an insight into the specificity of the sensing mechanism and the inhibition effect of higher concentration on the enzyme activity on the CR of the PDA LB-film. Since the observed CR under the condition reported in this study was less, no further attempts were made to test the aforementioned possibilities. Also as pointed out by Cheng and Stevens [11], one needs to have an additional unit of interaction attached to such sensor designs to amplify the small CRs resulting from protein–ligand interactions. From the present study, it is also inferred that it will be interesting to study PDA-based colorimetric sensors designed on antigen-antibody interactions, where interaction is between two macromolecules which are expected to give higher CR's resulting in to the design of highly specific and sensitive immunosensors.

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