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Molecular recognition between a biotinylated polythiophene copolymer and phycoerythrin utilizing the biotin–streptavidin interaction

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Abstract

The interaction of photoactive proteins with an electroactive matrix is valuable in leading to potential technological applications in opto-electronic signal transduction for optical displays, color mimicking, biosensor, and protein research applications. In this study, the Langmuir–Blodgett technique is used to couple these components through the well-known biotin–streptavidin complexation. Biotinylated copolymers of 3-substituted thiophenes have been synthesized both to enhance mechanical film integrity and to provide the electroactive matrix for attachment of the antennae protein, phycoerythrin. It has been determined that biotinylation of the thiophene copolymers improves their film forming properties and results in stable monolayers. Pressure–area isotherms indicate that protein interaction with the polymer monolayer is occurring with the streptavidin and phycoerythrin proteins. Fluorescence spectroscopy of transferred films confirms the presence of phycoerythrin in the final molecular assemblies. Each stage of protein binding to the biotinylated copolymer monolayer could be monitored through fluorescence microscopy at the air–water interface. These results suggest that this copolymer system is a promising material for integrating virtually any biotinylated macromolecular system to an electroactive matrix. In addition, this copolymer system may be "fine tuned" to maximize protein integration by varying the distance between biotin functionalities and/or the length of the biotin spacer arm.

1. Introduction

The primary focus of this research is the coupling of a photoactive protein to an electroactive polymer material where the polymer would provide both a rugged template for the organization and immobilization of protein as well as serve in a signal transduction role for potential biosensor, optical display and color mimicking applications. Such biosensor applications require the optimization of signal transduction to maintain sensitivity and low detection limits. Currently, researchers are investigating a variety of approaches to address this need, including thin layers (Langmuir–Blodgett (LB) sandwiches) of preformed conjugated polymers [1, 2], embedding sensing elements (*e.g.* enzymes [3] or DNA [4]) in conductive polymers and the incorporation of electron mediators such as ferrocene into an LB conducting matrix [5] to provide close contact between sensing and transduction matrices. Our approach is to use direct covalent linkage between the sensing element and conductive polymer where one may control the surface organization, density, orientation, size and spatial distribution of the elements in the assembly to optimize biosensing.

Polythiophene is a well known electroactive polymer which is of interest for its high conductivity and environmental stability [6–8]. Substitution of long alkyl chains to the polythiophene backbone has shown that processible, functional polymers for electronics applications may be formed [9–12]. LB films having well-defined composition, structure and thickness have been prepared by mixing alkylated polythiophenes with various surface-active materials such as stearic acid [13, 14]. Here we discuss the characterization and manipulation of a monolayer of a novel surface-active alkylated polythiophene which has been specifically derivatized with the functional group biotin for protein attachment.

Although photoactive biological proteins have been extensively investigated for their light-transducing properties, only recently has the possibility of using such proteins as active components of photonic devices actually been explored. This research has already yielded great promise as spatial light modulators [15], artificial photoreceptors [16], holographic media [17] and optically bistable devices [18] have all been demonstrated using the well-known protein bacteriorhodopsin. Our photoactive protein of choice is the light-harvesting phycobiliprotein found in blue-green and red algae,

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phycoerythrin. Phycobiliproteins are stable and highly water-soluble proteins which are uniquely assembled into complex "antennae" arrays which serve to collect and filter light in a unidirectional energy pathway to chlorophyll for photosynthesis with greater than 90% efficiency [19–21]. Phycoerythrin is the outermost phycobiliprotein and exhibits remarkable optical properties *in vivo*, in solution and in thin film form including an unusually high Stoke's shift (81 nm, 495 nm excitation and 576 nm emission), intense fluorescence (roughly 20× that of fluorescein) and quantum yields as high as 0.98 [22, 23]. Time-resolved fluorescence properties and the molecular environments of the multiple tetrapyrrole chromophores found in this protein are also well understood [24, 25]. In thin film form, phycoerythrin has demonstrated the ability to retain these optical properties as well as to exhibit interesting photovoltaic and photoconducting behavior [26]. It is expected that such proteins, organized and immobilized in a conducting matrix, should yield interesting electronic and optical properties.

Streptavidin (ST) is a well-known tetramer protein which contains four identical sites specific for biotin binding [27]. The biotin–ST complex, once formed, is essentially irreversible with a stability comparable with that of a covalent bond [28, 29]. Biotinylated biomolecules may be bridged together with ST to serve in a wide variety of biotechnology applications [30, 31]. It has been shown that the highly specific molecular recognition of biotin to the tetramer protein ST may be used to form oriented two-dimensional protein domains in the plane of a lipid monolayer [32, 33]. Recent studies have demonstrated extension of this approach to a cassette attachment methodology or triple-layer formation with the monoclonal antibody Fab [34] and the light-harvesting protein phycoerythrin [35].

In this paper, we report the cassette attachment of biotinylated phycoerythrin (B-PE) to biotinylated poly(3-undecylthiophene-*co*-3-thiophenemethanol) (B-PUMT) using ST as a linker protein. Pressure area measurements were used to characterize monolayer formation of the copolymer. Expansion of these curves after ST and B-PE exposure indicated protein interaction with the biotinylated copolymer monolayer. Fluorescence microscopy at the air–water interface was used to monitor protein attachment at each stage of assembly and fluorescence spectroscopy confirmed the presence of protein in transferred films.

2. Experimental details

The synthesis of the B-PUMT was carried out by attaching biotin to the copolymer of 3-undecylthiophene and 3-thiophenemethanol (PUMT) [36]. ST and *R*-phycoerythrin, biotin-XX conjugate were purchased from Molecular Probes, Eugene, OR. The streptavidin was received as a lyophilized powder which was reconstituted to a stock solution in Millipore water. The purity was confirmed from a single band by Coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis. The biotinylated phycoerythrin was shipped in 0.1 M sodium phosphate containing 0.1 M NaCl stock solution and was used as received. The XX represents a 14 atom "spacer" attached to enhance the ability of biotin derivatives to bind to the relatively deep binding sites of ST. Biotinylation was carried out by covalently binding biotin to random amide sites on the protein, resulting in three to four biotin functionalities per molecule.

Pressure–area measurements and film transfers were carried out on a Lauda MGW Filmwaag Balance. A Microscope Mountable Micro-Trough MT-100 from Ultrathin Film Technology Ltd., Mountain Lakes, NJ, was used for fluorescence microscopy studies. An argon ion laser, excitation wavelength 495 nm, was used to probe protein interaction with the B-PUMT monolayer. All monolayer protein assemblies were prepared by spreading a 0.5 mM chloroform solution of B-PUMT onto a 0.1 mM sodium phosphate and 0.1 M NaCl, pH 6.8, aqueous (Millipore water) subphase at 30 °C. ST from a prepared buffered stock solution was injected as uniformly as possible under the expanded B-PUMT monolayer to yield a final subphase concentration of approximately 1×10^{-9} M. The film was then allowed to incubate for times ranging from 2 to 24 h. Prior to injection of B-PE, the subphase was flushed with protein-free buffer to remove excess, unbound protein. B-PE was then introduced in a similar manner under the monolayer and allowed to incubate for 2 h before characterization and film transfer. For transfer studies, the monolayers were compressed to an annealing surface pressure of approximately 15 mN m^{-1} and then deposited either vertically (approximately 100% transfer on the upstroke) or horizontally onto various solid supports for characterization. Fluorescence spectra were obtained using an argon ion laser (coherent, Innova 90E), with 10 mW laser power as the pumped light source. The sample was excited at 496.4 nm which is close to the absorption peak of the native phycoerythrin and the emission was scanned from 500 to 700 nm.

3. Results and discussion

A series of biotinylated thiophene copolymers have been synthesized in our laboratory to provide electroactive, mechanically rugged and environmentally stable templates upon which various optically active proteins

may be organized and immobilized. Biotinylation of the PUMT copolymer was confirmed from IR spectroscopy and nuclear magnetic resonance and the resulting material was found to be soluble in chloroform with $M_n = 3351$ and $M_w = 3492$ [37]. UV visible spectroscopy showed that both the PUMT and the B-PUMT copolymers exhibited a broad λ_{max} around 400–450 nm with an absorption edge past 600 nm indicative of extended π conjugation along the polymer backbone. The B-PUMT gave a slight blue shift believed to be due to disruption of the π conjugation on introduction of biotin to the backbone.

Monolayer studies with these copolymers demonstrated that biotinylation greatly enhances the film-forming properties. Stable reproducible, transferrable monolayers were obtained with significantly better isotherms than the unbiotinylated copolymer. It is believed that the biotin functionality not only provides the necessary binding sites for protein attachment, but also serves to enhance the hydrophilic character of the copolymer enough to allow for stable monolayer formation. The mechanical integrity of the B-PUMT copolymer was implied by the formation of a plastic-like fiber which could be drawn up from the collapsed monolayer [38].

Figure 1 gives a series of pressure–area curves for the B-PUMT, B-PUMT after ST injection and B-PUMT after ST and then B-PE injection. As shown, in each case of protein introduction, a significant expansion in area of the B-PUMT was observed throughout the compression. This suggests that protein interaction is occurring at each stage of the assembly formation. Such an expansion was not observed in control experiments where PE (unbiotinylated) was injected, implying that specific molecular recognition of biotin to ST is the driving force behind protein attachment.

Figure 2 gives an idealized schematic of the different stages which are occurring in this proposed cassette methodology which immobilizes B-PE onto the B-PUMT copolymer monolayer. The first stage illustrates a monolayer of just the B-PUMT, inverted, to reveal the randomly exposed biotin groups. Stage 2 shows complexation of ST to the exposed monolayer biotin groups leaving accessible biotin binding sites on the reverse side of the tetramer protein. Stage 3 shows the final complexation of randomly biotinylated phycoerythrin to the free

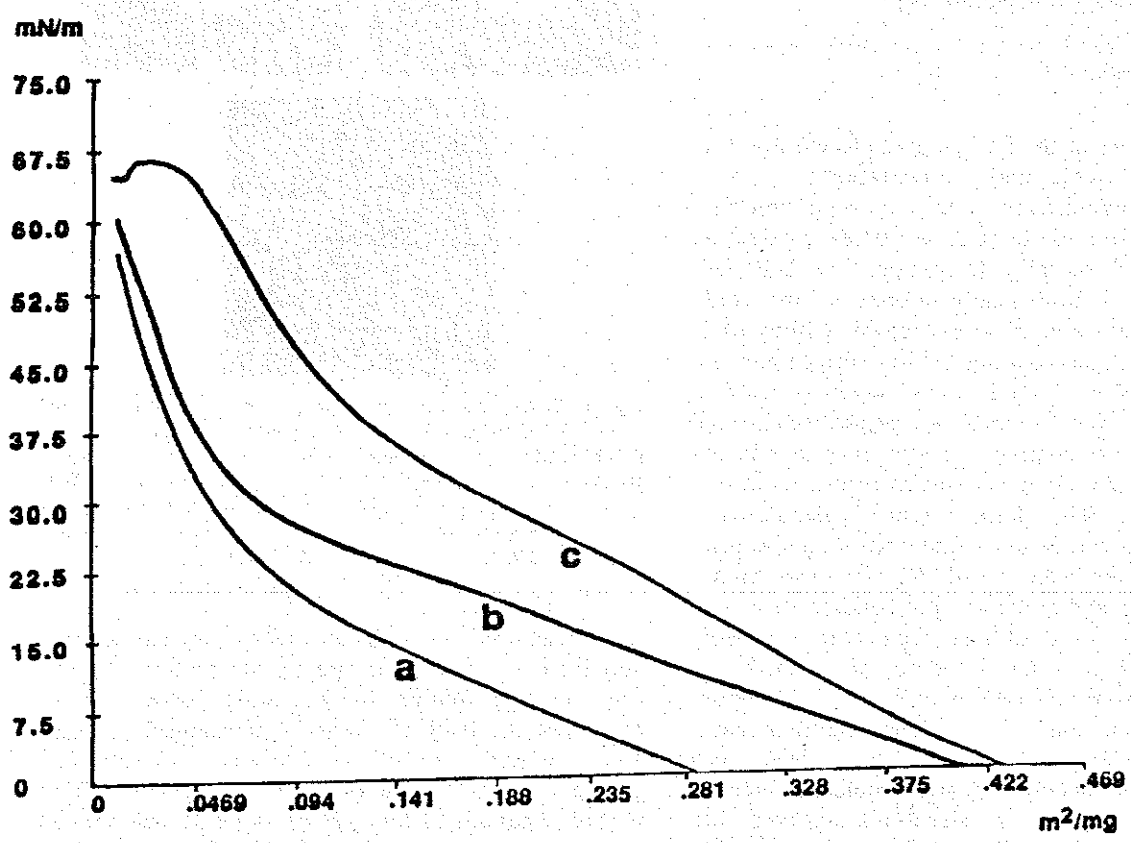

Fig. 1. Pressure–area curves for the three stages of protein attachment: curve a, B-PUMT; curve b, B-PUMT/ST; curve c, B-PUMT/ST/B-PE.

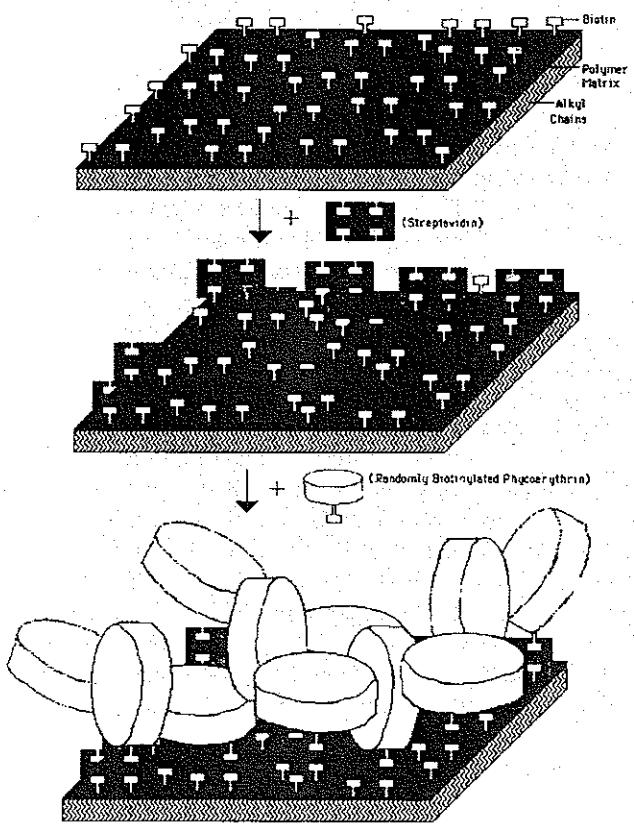

Fig. 2. Idealized schematific illustrating the sequential stages of the cassette attachment methodology.

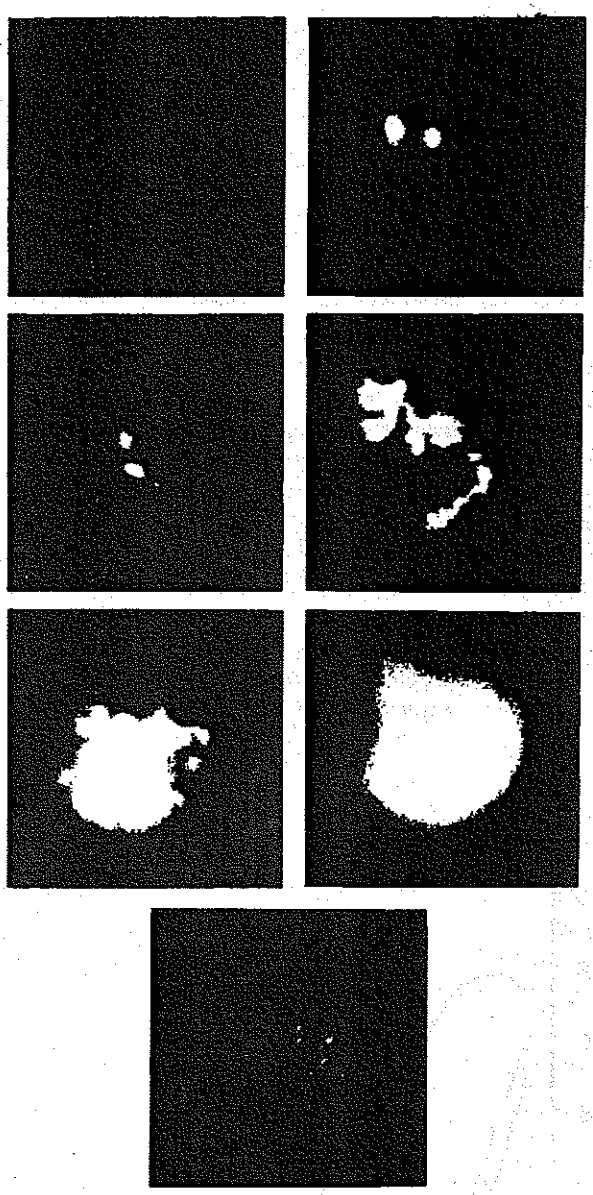

Fig. 3. Fluorescence microscope images obtained during assembly formation: (a) B-PUMT expanded monolayer (less than 2 mN m^{-1}); (b) B-PUMT compressed monolayer (15 mN m^{-1}); (c) expanded B-PUMT monolayer within 2 h after ST injection; (d) expanded B-PUMT monolayer 24 h after ST injection; (e) expanded B-PUMT monolayer 24 h after ST injection; (f) expanded B-PUMT monolayer after ST and B-PE injection; (g) transferred film of (f).

biotin binding sites of the ST bridging protein resulting in a variety of possible binding orientations.

Fluorescence microscopy at the air–water interface provided the *in situ* visualization of protein interaction at each stage of assembly formation. The B-PUMT monolayer itself is intrinsically fluorescent and could therefore be observed via fluorescence microscopy without the use of a fluorescent tag. At low surface pressure (less than 2 mN m^{-1}) small, dark, regularly shaped, circular domains were observed in a bright background (Fig. 3(a)). During compression, the reverse was observed, where bright circular domains formed in a dark background (Fig. 3(b)). These bright circular domains gradually became larger, until finally reaching a stage at collapse where the majority of the view was bright. These observed transformations are believed to be due to the dispersion of crystalline regions formed from tightly packed alkyl chains of the polymer dispersed in an amorphous matrix caused by disorder of the biotin-containing regions. Since the B-PUMT synthetic route used here results in a random copolymer system, such irregularity of the functional groups would be expected.

A dramatic change in the structures observed with the B-PUMT monolayer occurred on injection of ST. First, immediately after injection, the B-PUMT monolayer began moving simultaneously as the surface pressure increased, indicating direct protein interaction with the monolayer. Within 2 h, the circular domains characteristic of the B-PUMT monolayer changed to larger, bright, irregular, dendritically shaped formations dispersed in a dark background (Fig. 3(c)). If left for extended periods of time (24 h), these irregular formations remained but became distributed in a brighter, grainy-looking background (Figs. 3(d) and 3(e)). It is

speculated here that the copolymer acts as a non-crystallizable contaminant in a similar way that the introduction of avidin to ST was shown to shift the crystal morphology of ST bound to a biotinylated lipid monolayer [39]. In this case, the inherent amorphous nature of the random copolymer may dictate the morphology and thus act as "the contaminant" to inhibit crystalline formation of ST. After flushing with protein-free subphase (2 h ST incubation period) and injection of B-PE to the monolayer, the domains could no longer be observed and only a uniform, extremely bright film was present. After repeated flushing to remove any excess B-PE, the brightness diminished still leaving a uniform, bright film with no observable domain structures (Fig. 3(f)). This was direct observation of binding and complete coverage of the strongly fluorescent phycoerythrin protein to the monolayer film. It should be noted that phycoerythrin is a large (molecular weight, 240 000), bulky, disc-shaped molecule (60 Å by 120 Å) [23] with random sites of biotinylation. These factors play an important role in the final organization of protein and may also inhibit the formation of crystalline regions. It is important to stress that these fluorescence studies are of main interest in that they directly show distinct organizational changes which are occurring in the B-PUMT monolayer on each stage of protein injection through the intrinsic fluorescence of the polymer monolayer and that further insight regarding these structures is purely speculation at this time.

The monolayer assemblies were transferred onto hydrophobic solid glass supports using the horizontal dipping technique. Figure 3(b) shows the fluorescence image of the final B-PUMT/ST/B-PE assembly after rinsing in Millipore water to remove any residue salt. Little change is observed in this image suggesting that the mechanical integrity of the assembly is strong. The binding of PE in the transferred assemblies was confirmed by fluorescence spectroscopy which exhibited an intense peak at 576 nm characteristic of the native PE protein (Fig. 4).

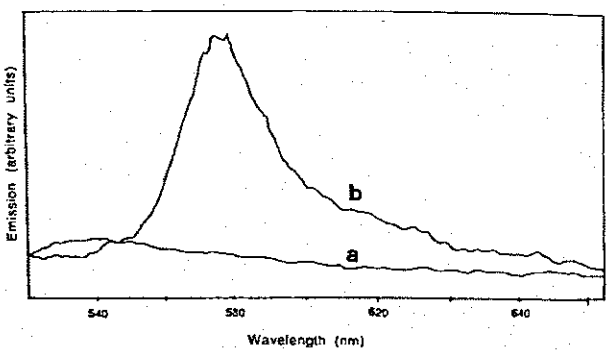

Fig. 4. Fluorescence spectra of transferred films: spectrum a, B-PUMT/ST; spectrum b, B-PUMT/ST/B-PE.

4. Conclusions

It is demonstrated that a novel biotinylated polythiophene copolymer system may be used for integrating photoactive proteins into an electroactive matrix. Although the organization, distribution and orientation of the proteins are not well defined at this stage we believe that we have been able to show that a biotinylated optically active protein may be bound to a biotinylated copolymer using streptavidin as a bridging protein. Ongoing experiments include ellipsometry, atomic force microscopy, surface plasmon resonance and freeze fracture transmission electron microscopy to elucidate the detailed structure of these systems and electro-optic studies to determine signal transduction properties. New synthetic routes are also being pursued which will directly control the ratio of the biotin and alkylated functional groups and vary the length of the spacer arm (distance the biotin group will hang below the monolayer) to maximize protein interaction and organization. It is believed that this is a versatile methodology which may be applied to any biotin-derivatized system (such as bacteriorhodopsin, antibodies and enzymes) for a host of biotechnological applications.

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