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Ultra-thin Film Assembly of a Novel Biomaterial Containing Protein and Functionalized Polymer for Sensor Application

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Abstract

A novel biomaterial capable of incorporating biotinylated biomolecule has been synthesized. Our strategy is to biotinylate one-dimensional electroactive polymers and use a bridging streptavidin protein on Langmuir-Blodgett (LB) organized films. These copolymers are derivatized with long alkyl chains and biotin moieties to bind, respectively, to the hydrophobic surface and the biotinylated species, through the biotin and streptavidin complexation. We utilize the polymer assembly approach to attach a signal transducing biomolecule biotinylated phycoerythrin (B-PE) into this novel biomaterial by binding the unoccupied biotin binding sites on the bound streptavidin (4 sites total). The pressure-area isotherm of the protein injected monolayer showed area expansion. A characteristic fluorescent emission peak at 576nm was detected from the monolayer transferred onto a solid substrate. These observations demonstrated the promise of the organized thin polymer assemblies for their application to the sensor system.

Introduction

Our aim is the incorporation of biological molecules, which possess inherent intelligent properties, into well-defined, oriented assemblies of electroactive polymer matrix. The resulting structures should possess novel electronic and optical properties for potential bioelectronic, biomedical and biotechnological applications. Our methodology to assemble such complex molecules into hierarchical systems involves the LB technique, utilization of the biotin-streptavidin interaction and design of a new electroactive polymer. The LB technique and the specific recognition of biotin for streptavidin or avdin have been employed for the orientation and spatial organization of protein assemblies[1-4].

Here we have developed a biotinylated copolymer system which could: improve LB film formation, enhance stability and mechanical strength of monolayers, immobilize proteins and potentially function in a signal transduction role as well. Copolymers of 3-undecylthiophene and 3-methanolthiophene have been chosen with regard to modification of the hydroxyl group of methanol by substitution with biotin. 3-alkyl substituted thiophene polymer is one of the most important heteroaromatic electrically conducting polymers. It possesses good thermal and environmental stability and its ease in processibility makes it amenable to manipulation on the LB through, being capable of forming organized monolayers. These properties make it attractive for possible applications in electronics, sensors and nonlinear optics[5-8].

Recently the synthesis of the biotinylated copolymer of 3-hexylthiophene and 3-methanolthiophene

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which formed ordered monolayers subsequent to interaction with streptavidin was reported[9]. In the present paper, this system has been extended by employing a longer alkyl chain and demonstrating the LB through cassette approach to fabricate ordered systems using the newly developed polymer and protein.

Our approach to assemble hierarchical systems containing proteins involves the high specificity and stability of the biotin/streptavidin interaction which is used in a wide range of biomedical research applications. The binding affinity of biotin to the tetrametric protein streptavidin is strong ($K_a=10^{15}mol^{-1}$) and once formed the complex is essentially irreversible [10-11]. This streptavidin can subsequently bind biotinylated phycoerythrin, highly fluorescent protein possessing multiple chromophores[12,13]. Our goal is to combine the unusual optical and electronic properties of the copolymer with the inherent flexibility of the biotin/streptavidin binding system.

Experimental Section

Synthesis of Poly(methanolthiophene-co-undecylthiophene) (PMUT). Synthetic grade anhydrous ferric chloride (Aldrich), 0.99mol was dried under vacuum at 100℃ prior to reaction. Then nitrogen was introduced along with 100 ml of dry chloroform (Aldrich). 0.02 mol of 3-undecylthiophene (TCI America) and 0.01 mol of 3-methanolthiophene (Aldrich) in 10 ml of chloroform was added dropwise under vigorous stirring. The reaction mixture was allowed to stir for two days till the reaction was complete. The reactant solution was precipitated into 500 ml methanol (Aldrich). The product was then purified with methanol in a Soxhlet extractor for two days.

Synthesis of Biotinylated PMUT (B-PMUT). A solution of 0.01 mol biotin (Biomeda), 0.011 mol N,N-dicyclo-hexylcarbodiimide (Aldrich). 0.011 mol PMUT and 0.001 mol 4-pyrrolidinopyridine (Aldirch) in 50 ml dichloromethane (Aldrich) was stirred at room temperature until esterification was complete. The N,N-dicycloundecyl urea was filtered and the filterate was washed with water (3×50 ml), 5% acetic acid solution (3×50 ml) and again with water (3×50 ml), dried ($MgSO_4$) and solvent was evaporated in rotary evaporator under reduced pressure to give the B-PMUT.

LB monolayer formation. All monolayer studies were carried out on a Lauda MGW Filmwaag trough with a surface area of approximately 930 cm^2. In the case of pressure-area isotherms of BMUT and B-PMUT, 0.5 mM chloroform solution was spread onto the purified MilliQ water subphase. For the measurement of pressure-area isotherms following streptavidin injection under the B-PMUT monolayer, the subphase was composed of an aqueous solution of 0.1 mM NaH_2PO_4 and 0.1 M NaCl, at pH 6.8. Streptavidin (0.1 mg in 5 ml of the buffered subphase) was injected under the spread film and left to incubate for two hours at 30℃, and subsequently biotinylated phycoerythrin was introduced under the polymer and streptavidin layer. The streptavidin and biotinylated phycoerythrin were purchased from Biomeda Co. and used as received. Compression was then carried out at a speed of $2mm^2$/min until collapse of the film was observed. For transfer studies, the polymer was spread, followed by streptavidin introduction and incubation in the expanded state for two hours, subsequently followed by B-PE introduction and incubation for two hours and then compressed to an annealing surface pressure of approximately 15 mN/m for deposition. Monolayer films were then transferred onto glass solid supports for fluorescence spectroscopy and ellipsometry measurement.

Ellipsometry measurements. Ellipsometry can be used to give direct measurement of the thickness of very thin films, including monolayers[14]. A thin film ellipsometer (43603-200E), Rudolph Research, Flanders, NJ) was used for the measurements of thickness of a monolayer transferred to a frosted glass substrate.

Results and Discussion

Materials Synthesis and Characterization. The synthesis of biotinylated copolymer involves two steps, the synthesis of a copolymer of 3-undecylthiophene and 3-methanolthiophene and attachment of biotin in the second step as shown in Figure 1.

The number average molecular weight of B-PMUT measured by Gel Permation Chromatography was 2500 g/mol. Infrared measurements of PMUT and B-PMUT were carried out on KBr discs and are in Figure 2.

Both PMUT and B-PMUT showed a principal absorption at 780 cm^{-1} due to the C-H out-of-plane vibration of the 2,5-disubstituted thiophene and a distinct peak around 810 cm^{-1} due to the C-H out -of-plane vibration of the 2,3,5-trisubstituted thiophene [15]. B-PMUT exhibited new characteristic peaks at 1678 cm^{-1} due to an ester linkage and a sharp peak at 3400 cm^{-1} from N-H stretching. As expected, the broad O-H absorption peak at 3400 cm^{-1} shown in PMUT disappeared in B-PMUT.

The polymers were dissolved in chloroform and coated on glass substrates. Their UV-Vis spectra are shown in Figure 3. Both the copolymers showed a broad λ max around 400-450 nm with absorption increasing from 600 nm indicating the presence of an extended π -conjugation along the polymer backbone. B-PMUT showed a blue shift due to the interruption of π -conjugation by the introduction of biotin.

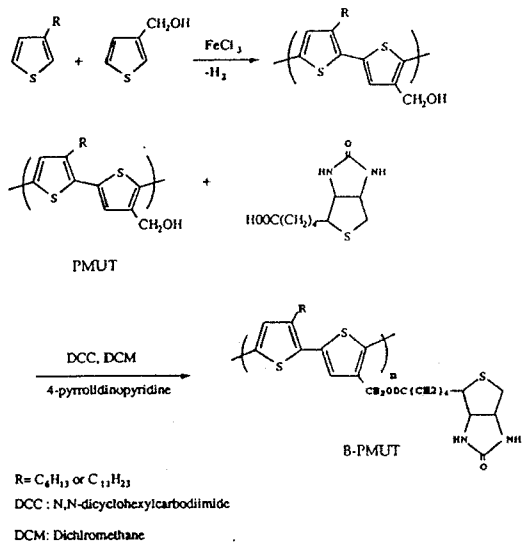

Fig. 1. The synthesis schematic of PMUT and B-PMUT

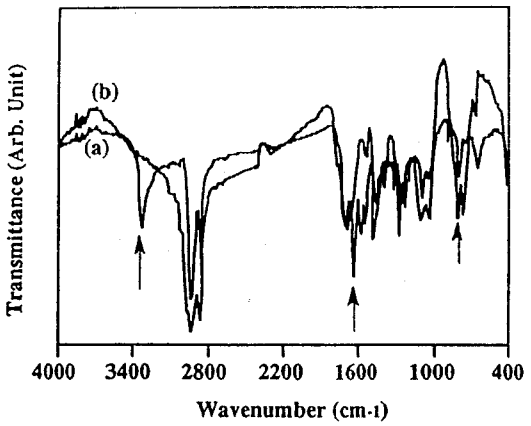

Fig. 2. FT-IR spectra of (a)PMUT and (b)B-PMUT

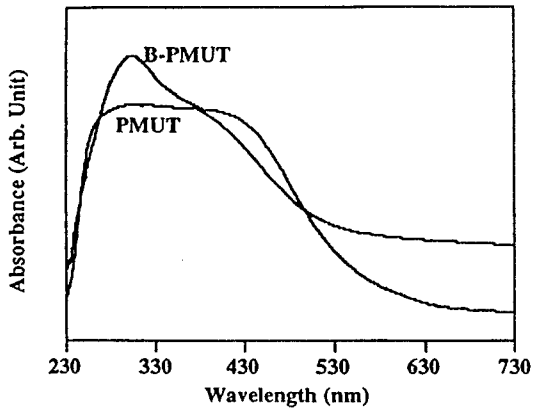

Fig. 3. UV-Visible spectra of (a)PMUT (b)B-PMUT

Pressure-Area Isotherms. In order to evaluate the effectiveness of biotinylation of PMUT on LB film formation and subsequent bindings with streptavidin and B-PE, a series of pressure-area isotherm mesurements were performed. Schematic of the experimental sequence and hierarchical ordering of the protein in the cassette aproach is shown in Figure 4. The isotherms of the PMUT, B-PMUT monolayers are given in Figure 5.

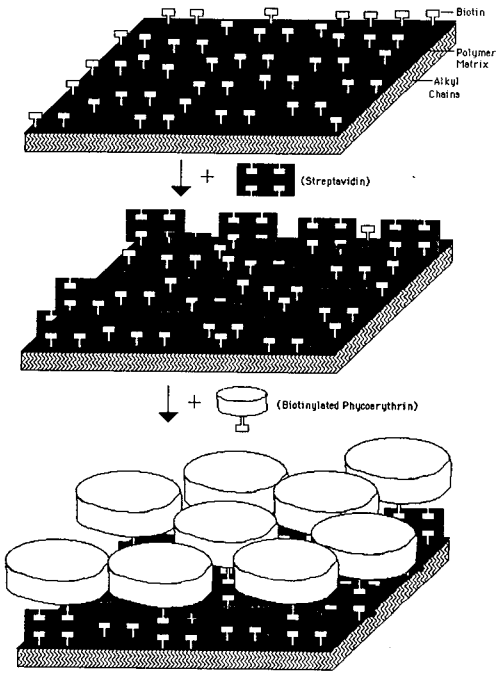

Fig. 4. Schematic of the experimental suquence and hierarchical ordering of the protein in the cassette approach

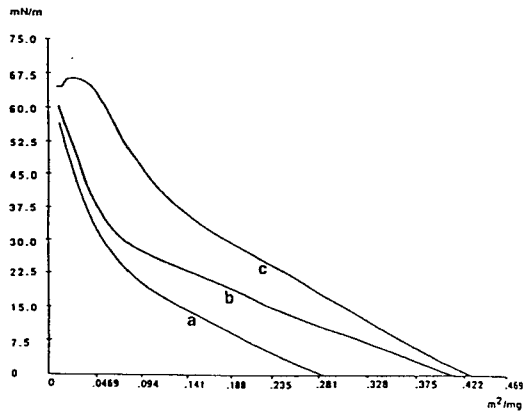

Fig. 5. The pressure-area isotherms of (a)B-PMUT (b)B-PMUT/ Streptavidin (c)B-PMUT/ Streptavidin/ B-PE

Biotinylated copolymer (B-PMUT) demonstrated a significantly better isotherm than copolymer (PMUT) implying superior packing compared to the copolymer on the air-water interface. This siginificant improvement in the formation of a B-PMUT monolayer suggests that biotinylation enhanced the LB film formation by contributing hydrophilicity to the copolymer molecule. Therefore biotinylation of PMUT not only supplied the biotin to copolymer to further bind with streptavidin but enabled the improvement of monolayer during transfer and efficient deposition onto solid supports. It was found that a constant surface pressure of 15 mN/m was maintained with a transfer ratio of approximately 60%. B-PMUT was observed to possess fairly strong mechanical properties as shown by the formation of an elastic fiber-like string when the collapsed monolayer film was drawn up using a Teflon-coated tip.

The isotherm shown in Figure 5 showed area expansions when streptavidin and B-PE were injected below the B-PMUT monolayer indicating the occurence of effective binding between the biotin and streptavidin and subsequently biotinylated PE with streptavidin. This behavior is consistent with previous observations by Blankenburg et al [3] where fluorescein labeled streptavidin injected monolayer exhibited a continuous expansion in area and pressure through the compression cycle. This pressure change supports the original goal of the biotinylation of this polymer, which was to employ the biotin-streptavidin complexation for subsequent immobilization of any biotinylated macromolecular assembly into LB films.

Fluorescence Spectra. The monolayer films were transferred to hydrophobic solid glass

supports using the horizontal dipping technique at an annealing pressure of 15 mN/m. The presence of the phycoerythrin is probed by its intense and characteristic fluorescence. Mesurements were carried out by exciting the samples with 496 nm light from an Argon ion laser and scanning from 510 to 670 nm. The fluorescence spectra of B-PMUT with streptavidin, and B-PMUT and streptavidin with B-PE, are shown in Figure 6. As shown, only the B-PMUT/ streptavidin/ B-PE monolayer gives a strong emission at 576 nm which corresponds to the fluorescence spectrum of the native phycoerthrin [12,13]. These resutls provide further evidence that the protein has adsorbed to the B-PMUT/ streptavidin monolayer via the bridging biotin/ streptavidin interaction.

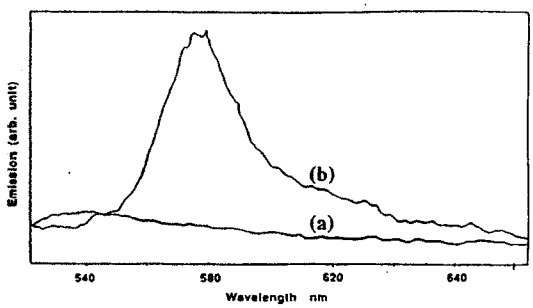

Fig. 6. Fluorescence spectra (a)B-PMUT/ Streptavidin (b)B-PMUT/ Streptavidin/B-PE

Ellipsometry mesurements. Optical ellipsometry is a way to measure the monomolecular thickness the films formed. The monolayer films were transferred to hydrophobic frosted solid glass supports using the horizontal dipping technique at an annealing pressure of 15mN/m. The thickness of B-PMUT, B-PMUT/ streptavidin and B-PMUT/ streptavidin/ B-PE were obtained as: 26Å, 54Å and 126Å in that order as shown in Table 1.

The observation of gradual increases in the thickness when each protein was bound to B-PMUT monolayer supports our previous evidence for binding. The increase of about 70 Å when phycoerythrin was bound to the B-PMUT/ streptavidin monolayer appears to agree resonably with the phycoerythrin geometry of approximately 60Å by 120Å.

Table 1. Thickness of LB Film

system	refractive index	thickness(Å)
B-PMUT	1.518	31 ± 3
B-PMUT/Str	1.49	55 ± 2
B-PMUT/Str/B-PE	1.49	121 ± 4

Based on these investigations, we plan to utilize these novel assembly approach for sensor application. As an example, here creation of a biosensor is based on optical fiber mesurement technology where the fiber surface has been treated to make it hydrophobic. To this biotin derivatized polyalkyl thiophene copolymer, shown in Figure 7, is bound by dispersion and Van der waals interactions. Enzymatically biotinylated single stranded probe DNA can be bound to one of the unoccupied streptavidin sites. Only a complementary single stranded DNA or RNA analyte will be detected by hybridization and subsequent intercalation of the dye [16].

Critical to the functioning of this type of biosensor is the use of a dye that has little or no fluorescence when not bound to double helical DNA. When intercalated into DNA, it must intensely fluorescent when excited at a suitable wavelength through the optical fiber. The optical fiber then transmits the fluorescent signal at the emmission wavelength, indicating the presence and perhaps concentration of unknown analyte. One additional attractive feature of this molecular system is the electronically conjugated polythiophene polymer backbone, which makes this polymer contribute as well as providing it with optical properties. These properties may be

exploited in integrated signal transduction strategies involving optical fiber based measurement technology.

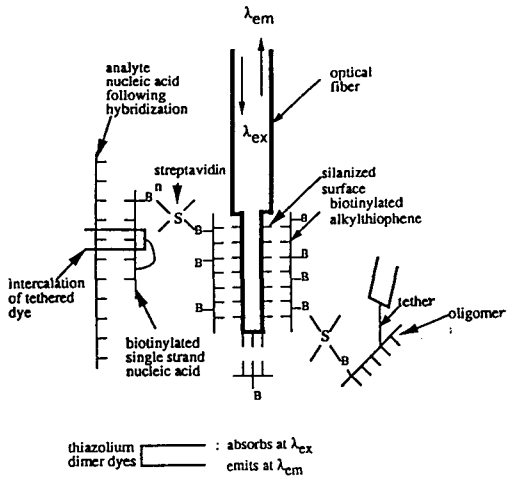

Fig. 7. Schematic of a DNA Biosensor

Conclusion

We have created a novel hierarchical structure assembly utilizing an electroactive biotinylated thiophene copolymer and the biotin/streptavidin interaction to attach biotinylated phycoerythrin. Biotinylation of thiophene copolymer was shown to promote better formation of monolayer LB films by contributing an enhanced flexibility and/or hydrophilicity to the copolymer molecule. The effective binding of biotinylated phycoerythrin to the novel LB polymer films has been demonstrated by area expansions in pressure-area isotherms, characteristic fluorescence emission from the protein and thickness increases. In addition, this copolymer demonstrated mechanical and elastic properties. These results suggest that this novel copolymer is a promising material for potential device applications in which any biotinylated macromolecule may be attached the polymer monolayer via the bridging biotin/streptavidin interacting system.

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