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The Monomolecular Organization of a Photodynamic Protein System Through Specific Surface Recognition of Streptavidin to Biotinylated LB Films

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**The Monomolecular Organization of a Photodynamic Protein
System Through Specific Surface Recognition of Streptavidin to
Biotinylated LB Films**

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Abstract

This paper focuses on a novel methodology for the two-dimensional ordering of a photodynamic protein system using the Langmuir-Blodgett (LB) technique. The specific versus non-specific surface recognition of biotin on biotinylated LB monolayers by streptavidin and avidin conjugated phycoerythrin was investigated. Both avidin and streptavidin conjugates, when injected under the biotinylated monolayer, were found to preferentially adsorb to the biotin while at the air-water interface. Pressure-area isotherms displayed a biotin-streptavidin/avidin complex dependent increase in surface pressure at expanded areas indicating protein adsorption. Fluorescence measurements of transferred films confirmed the binding of phycoerythrin to the monolayers and provided evidence that the avidin conjugated system may bind by both specific and non-specific mechanisms, while the streptavidin systems bind through only a specific mechanism. The extension of this methodology to any biotin or avidin/streptavidin derivatized protein system is expected to lead to the fabrication of ultrathin, ordered, protein molecular assemblies with potential bioelectronic, optical and protein structure research applications.

Introduction

Two-dimensional ordering of highly pigmented protein complexes would be extremely valuable for a wide range of potential bioelectronic, optical, biomedical and protein research applications that take advantage of the evolved, intelligent materials properties of these protein systems. One such property of a dynamic colorant system could form the basis for a dynamical optical mimic strategy or conversely a dynamic contrast strategy. Such systems could be made capable of sensing a background image, translating it into a signal, which could then cause emission of a similar or contrasting image to the recorded background. These ordered protein assemblies may also serve as unique environments for TEM crystallographic structural analysis, for systems where it is difficult or impossible to obtain large three-dimensional protein crystals¹ or two-dimensional protein crystals by other TEM mounting techniques.

The incorporation of these photodynamic systems into organized two-dimensional films of electrically conducting polymers should also provide novel electronic and optical properties for the development of bioelectronic and optic sensors, spatial light modulators, and optoelectronic switching devices. Recent studies with the light transducing proton pump protein from the purple membrane of *Halobacterium holobium*, bacteriorhodopsin, have shown this chromophore containing protein to possess interesting nonlinear optical properties².

A methodology is necessary which has the capability of simultaneously orienting and coupling photodynamic, water soluble protein systems to suitable optical and electronic devices. The Langmuir-Blodgett (LB) technique has recently been employed for the

orientation and spatial organization of protein molecular assemblies^{3,4}. In particular, Blankenburg et al. have utilized the highly specific interaction between biotin lipids and the proteins avidin and streptavidin in LB monolayers to form oriented two-dimensional protein domains³. Avidin and streptavidin are tetramer proteins (four binding sites) which have a high specificity for binding biotin functionalities.

Independently, we initiated a research project which has proven to be an extension of this work, in that we have attached a highly pigmented, water soluble antennae protein, phycoerythrin, to the streptavidin and avidin tetramer proteins in an attempt to form a two-dimensional array of a photodynamic conjugated protein system⁵. Our approach has involved utilizing the highly specific recognition of biotin on the LB trough subphase surface of biotinylated LB lipid monolayers by the streptavidin conjugated phycoerythrin (Str-PE) and avidin conjugated phycoerythrin (Av-PE). The biotin-avidin or -streptavidin complex is well known^{6,7}, self-assembling, and once formed is essentially irreversible ($K_a = 10^{15}$) with a stability that is comparable to that of a covalent bond. The unusual high affinity of the biotin streptavidin complex has been rationalized in molecular terms through crystal structure determinations⁸.

Recently, avidin and streptavidin have been found to irreversibly bind to and form functionally active monolayer films spontaneously on gold and silver surfaces from aqueous solutions. These films have demonstrated biosensing by using an avidin-coated quartz crystal microbalance to detect a target strand of viral DNA⁹. Our approach offers two distinct advantages. The first being that the monolayers are functionalized, highly oriented, two-dimensional

films. Using the Langmuir-Blodgett technique direct control over the amount, orientation and thickness of the final films is possible. Thus, one has the ability to create molecular motifs that possess signal transduction mechanisms to subsequently allow direct control of the ultimate sensitivity and size of the biosensing device.

One such class of molecular motifs would be to vary the lipid matrix to which the proteins are being attached, to include electrically and optically active functionalities thereby creating novel electronic and optical membrane-protein interactions for signal transduction. The second advantage could ultimately involve the simultaneous incorporation of photodynamic proteins, such as phycoerythrin (as Str-PE), into an electroactive membrane assembly comprised of biotinylated lipid mixed with a conducting polymer. In particular, such electroactive membrane assemblies could include conducting functionalized monolayers with biosensing redox active groups^{10,11}. In this way, optically active monolayer films can be fabricated which have potential biooptoelectronic applications.

Photodynamic biological proteins have been extensively investigated for their light harvesting properties. However, only recently^{12,13} has the possibility of light transducing proteins as active components of photonic devices such as spatial light modulators and holographic recording media for optical data storage been demonstrated. Birge¹⁴ has reported spatial light modulators based on bacteriorhodopsin to have performance levels competitive with commercially available devices. Soskin and Taranenko¹³ have also reported the use of bacteriorhodopsin for optical phase conjugation and fabrication of optically bistable devices. The phycobiliproteins represent another important class of

photodynamic proteins and may support applications similar to those demonstrated in bacteriorhodopsin. However, before the phycobiliproteins are used to demonstrate optical devices it is important to investigate and understand the optical properties of thin films of these proteins.

Phycobiliproteins are a class of proteins which are known as the antennae proteins for light harvesting by microorganisms under very low light intensity conditions. These proteins are organized as arrays of stacked discs in phycobilisomes, in the order of phycoerythrin-phycoerythrin-allophycocyanin (outer-inner) which are bound to the external face of the thylakoid membranes of cyanobacteria (blue-green algae) and red algae¹⁵. In this unique organization, these proteins are able to efficiently absorb and transfer minimal light energy, via a Forster mechanism, to the chlorophyll for photosynthesis with greater than 90 % efficiency^{16,17}.

The individual phycobiliproteins possess very interesting optical properties and have already found commercial uses as fluorescent markers in biomedical research applications. These proteins are highly fluorescent (20x more so than fluorescein) and have quantum yields as high as 0.9. Phycoerythrin is the outer most phycobiliprotein, with an unusually large Stoke's shift of 81 nm (495 nm excitation and 576 nm emission), which is 2.7 times that of fluorescein¹⁸. The chromophores responsible for this optical behavior consist of almost identical linear tetrapyrrole groups which are covalently bound to specific protein side chain residues through thioether linkages^{16,17}. In addition, the time resolved fluorescence properties and molecular environments of the chromophores in these proteins are reasonably well understood^{19,20}.

It is anticipated that these chromophores, in the native proteins, if oriented and embedded in a conducting LB matrix will afford some novel electronic and optical interactions, as well as serve to enhance the overall stability of the films. In addition, modification in the native protein assemblies or linker peptides are expected to modulate the wavelength of reception.

We describe here research directed towards the two-dimensional ordering and incorporation of the highly pigmented phycoerythrin protein using the LB technique. Biotinylated lipid monolayer films were first prepared at the air-water interface. Streptavidin or avidin conjugated phycoerythrin was then injected under the monolayers and incubated to allow for adsorption of the protein complex to the monolayer. Protein binding to the monolayer was observed through pressure-area isotherms and fluorescence spectroscopy. Optical microscopy showed uniform coverage of the highly fluorescent protein system over the monolayer surface. The role of specific versus non-specific binding mechanisms in the conjugated protein systems was investigated. Using various controls, we have demonstrated that a specific biotin-streptavidin interaction is responsible for the protein binding, while both non-specific and specific binding mechanisms occur with the biotin-avidin complex.

Experimental Section

The biotinylated phospholipid, N-(biotinoyl)dipalmitoyl-L- α -phosphatidylethanolamine, triethylammonium salt, (B-DPPE), was

purchased from Molecular Probes (Eugene, Oregon) and used as received. L- α -dipalmitoyl phosphatidylethanolamine, (DPPE), was used as a control lipid and was purchased from Avanti Polar Lipids (Pelham, Alabama). The proteins, unconjugated phycoerythrin (PE), and avidin (Av-PE) and streptavidin conjugated R-phycoerythrin (Str-PE) were purchased from Biomedica Corporation (Foster City, California). These proteins were received as 1 mg/ml stabilized suspensions and were used without further purification.

All monolayer work was carried out on Lauda MGW Filmwaag troughs with a surface area of approximately 930 cm². Highly purified Milli-Q water (Millipore Co. Ltd.) was used for all subphase preparations. The subphase was composed of an aqueous solution of 0.1 mM sodium phosphate, 0.1 M NaCl, at pH 6.8. Pressure-area isotherms were carried out by spreading a 0.5 mM chloroform solution of the lipid onto the subphase surface. In the case of protein studies, 0.1 mg of the protein in 5 ml of the subphase was injected under the lipid monolayer and allowed to incubate for 2 hours at 30° C prior to compression. Compression was then carried out at a speed of approximately 2 mm²/min until collapse of the film was observed. For transfer studies, the monolayer lipid was prepared and annealed in the expanded state, followed by injection of the protein and incubation for 2 hours. The film was then compressed to a constant surface pressure of 15 mN/m for vertical deposition. Monolayer films were then transferred onto hydrophilic glass substrates with a sample lift speed of 5 mm/min.

Fluorescence spectra were obtained using an Argon Ion laser (coherent, Innova 90E), ranging from 25 to 50 mW power, at 496.5 nm which is close to the absorption peak of the protein as

shown schematically in figure 1. The laser beam was collimated using a 100 mm focal length cylindrical lens. The excitation laser beam was oriented perpendicular to the axis of the monochromator. The fluorescence was collected by a spherical lens of dimensions, $F = 60$ mm, $D = 45$ mm. The illuminated area of the sample was imaged, with a 1 x 1 ratio, on the entrance slit of the monochromator such that the numerical aperture of the collection system is $N.A. = 0.18$.

A color filter was placed in front of the entrance slit of the monochromator which had a cutoff wavelength of 515 nm. The monochromator, a Jarrell-Ash model 78466 SP, of 2 m focal length was used with a 300 μ m slit size which offered a spectral resolution of 1.2 Å. The effective aperture of the monochromator was $N.A. = 0.05$. On the exit slit of the monochromator joints, a photomultiplier tube (EMI 9658-RF) in a housing thermoelectrically cooled down to -20° C was attached. The signal from the photomultiplier tube was processed by an amplifier-discriminator (SSR-1120) and photocounter (EG&G 1109). For the monolayer samples, an integration time of 3 s was used which limited the spectral resolution to 6.25 Å. Since the sample was degrading under intense laser illumination, the laser power was kept under 60 mW and the cylindrical lens was slightly defocused such that the laser spot was a 0.5×2 mm² rectangular shape on the sample.

Results and Discussion

Pressure-Area Isotherms. To establish the adsorption of protein to the monolayer films while at the air-water interface, a series of

pressure-area isotherm measurements was performed. The structure of the B-DPPE and a direct comparison of isotherms of the pure B-DPPE to protein (PE, Av-PE and Str-PE) injected B-DPPE monolayers is given in figure 2. In all cases, the isotherms displayed a relatively steep slope after a pressure of 15 mN/m which corresponds to an area per molecule of approximately 100 Å². However, the biotin binding Str-PE and Av-PE protein monolayers exhibited much different behavior in the expanded state. A significant increase in surface pressure was observed with these monolayers at larger areas per molecule. This behavior suggests that the conjugated protein systems are in some way incorporating themselves into the biotinylated monolayer in the expanded state. Then, as compression is continued the protein injected monolayers reach a stage where they actually overlap the pure B-DPPE isotherm. This behavior is much different than that observed by Blankenburg et al. where fluorescein labelled streptavidin was injected under the biotinylated monolayer using similar preparative conditions. The isotherms in this case displayed a continuous expansion in area throughout the compression cycle without any observed overlap with the pure lipid system³.

The overlapping of the phycoerythrin injected monolayers with the pure B-DPPE in our system may be explained by the bulkiness of the phycoerythrin protein which is attached to the tetramer proteins. Phycoerythrin is known to be disc shaped with dimensions of approximately 60 Å by 120 Å and a molecular weight of 240,000 daltons¹⁸. This additional size and weight may be sufficient to cause a "swinging" down of the conjugated proteins into the aqueous subphase during monolayer compression. This is shown schematically as the a to b transition in figure 3. If this were to

occur, one would expect to see an increase in surface pressure in the expanded state and an overlapping of the isotherm with the pure lipid upon further compression as the protein reorients down into the subphase. This behavior is evidence that the conjugated proteins are adsorbing by binding biotin moieties on the B-DPPE. This possible two-dimensional ordering of the conjugated proteins onto the biotinylated lipid LB film during compression is illustrated in figure 3.

In contrast to the Str-PE and Av-PE conjugates, the unconjugated PE injected monolayer, displayed very little change in the figure 2 isotherm in comparison to the pure B-DPPE. Since the PE does not have any tetramer protein bound to it, this further supports that the biotin binding sites of the avidin and streptavidin tetramer proteins are responsible for adsorption. However, the question is raised as to whether the protein adsorption proceeds through an entirely specific (biotin-avidin or -streptavidin) mechanism or both specific and non-specific binding mechanisms. Fluorescence spectroscopy was used to address this issue and confirm the presence of bound protein to the monolayer films.

Fluorescence Spectra. Monolayer films were transferred onto hydrophilic glass solid supports for fluorescence studies. In each case, the monolayers gave transfer ratios ranging from 100 - 150 %. The phycobiliprotein, phycoerythrin is well known for its intense and characteristic fluorescence. Therefore, fluorescence spectroscopy provides a unique probe with which to monitor and compare the presence of adsorbed protein to the biotinylated monolayer films. The measurements were carried out by exciting

the samples with 496 nm light from an Argon Ion laser and scanning the emission from 515 to 670 nm.

Figure 4 shows the fluorescence spectra of a Str-PE injected B-DPPE monolayer and two controls. The controls are glass slides which were vertically passed into the protein containing subphase after the monolayer was cleaned off. This was to demonstrate that the conjugated proteins did not spontaneously adsorb onto the bare clean glass surface. As shown, the Str-PE injected B-DPPE monolayer gives a strong emission at approximately 576 nm which corresponds to the emission of the native aqueous phycoerythrin conjugated protein. The two controls show no emission in this region. This is direct evidence that the Str-PE has adsorbed to the biotinylated monolayer film.

This raised the question whether the biotin-streptavidin complexation was responsible for the protein adsorption. To address this issue, two additional controls were investigated. The first included injecting unconjugated phycoerythrin (PE) into the subphase of the B-DPPE monolayer. The second control involved preparation of an unbiotinylated phospholipid monolayer (DPPE) and then subsequent injection of the Str-PE. Therefore, each control was missing one component of the biotin-streptavidin complex. Figure 5 gives the fluorescence spectra of the Str-PE injected B-DPPE monolayer and the two controls described previously. As shown, the Str-PE exposed biotinylated monolayer exhibits the characteristic native phycoerythrin emission at 576 nm. In comparison, the two controls show no fluorescence signal, strongly suggesting that both the streptavidin tetramer protein and biotin functionalities are necessary for binding of the phycoerythrin to the monolayer by complex formation.

Similar studies were carried out with the Av-PE conjugated protein system. As shown in figure 6, the Av-PE injected monolayer film gives a strong emission at 576 nm which again indicates biotin-avidin based adsorption of the native phycoerythrin protein to the monolayer in comparison to the two controls which gave no corresponding emission. Direct comparison of the fluorescence emission of B-DPPE monolayers with the three injected phycobiliprotein systems, Av-PE, Str-PE and PE may be made from figures 5 and 6. The emission of Av-PE is observed to be considerably stronger in comparison with that of Str-PE. This difference in intensity would be expected if both specific and non-specific binding of the avidin conjugated protein system to the monolayer was occurring. These results are consistent with what is known regarding the structures of the avidin and streptavidin tetramer proteins.

There are two possible mechanisms for non-specific avidin adsorption. First, avidin is post translationally modified by surface carbohydrate residues and will complex to the biotin lipid monolayer by both the specific binding sites and non-specific (electrostatic) binding mechanisms⁶. By contrast, streptavidin, which is not chemically modified by carbohydrate, binds by what appears to be only a specific (biotin-streptavidin) mechanism. These ideas were also supported by Blankenburg et al.³ when an inactivated form of streptavidin was found to have neither specific nor non-specific interaction with the biotinylated monolayer.

The second mechanism of non-specific avidin adsorption is electrostatic. Evidence to support the non-specific binding of the Av-PE electrostatic based complex was obtained when NaCl was added to the aqueous subphase. Figure 7 shows a comparison of the

fluorescence spectra of Av-PE injected B-DPPE monolayer films with and without NaCl present in the subphase. The decrease in emission intensity at 576 nm from the NaCl subphase is evidence that charge-charge interactions to the non-specific adsorption of avidin is occurring and may be decreased with the addition of NaCl³.

Fluorescence microscopy measurements were performed and in the Av-PE and St-PE injected films continuous coverage of the fluorescent proteins over the surface of the films was observed. These results were confirmed by irradiating different areas of the same films and measuring the fluorescence spectra. In each case, very similar emission intensities were observed over the entire film surface.

Conclusions

It has been shown that the photodynamic "antennae" protein, phycoerythrin when conjugated to either of the tetramer proteins, streptavidin or avidin, will bind to a biotinylated phospholipid monolayer (B-DPPE) at the air-water interface. The adsorption of these conjugated protein systems to the monolayer was confirmed by pressure-area isotherms which indicated a "swinging" down of the bulky protein upon compression. Fluorescence measurements gave direct evidence of the presence of the Str-PE and Av-PE on the monolayers. Comparison of various control films proved that both components of the biotin- streptavidin or avidin complex are necessary to bind the protein by complex formation, and that non-specific, spontaneous adsorption of protein to the glass surface was not occurring.

A comparison of the intensity of the fluorescence signals and the addition of NaCl to the subphase, however suggests that the avidin conjugated protein may bind by both a specific and a non-specific mechanism. By contrast, streptavidin binds by what appears to be only the specific biotin-streptavidin mechanism.

These results demonstrate, for the first time, a technique for the monomolecular organization of the photodynamic phycobiliprotein, phycoerythrin. It is important to realize, that this work also establishes a potential, general technique for the two-dimensional ordering, in a monolayer form, of any biomolecular system which may be derivatized with biotin or avidin/streptavidin. In addition, the versatility of the LB technique and the multiple binding sites of the tetramer proteins should allow for the simultaneous incorporation of various electro- and optically active surfactants and biomolecules such that one may design and fabricate biological supramolecular assemblies of tremendous diversity.

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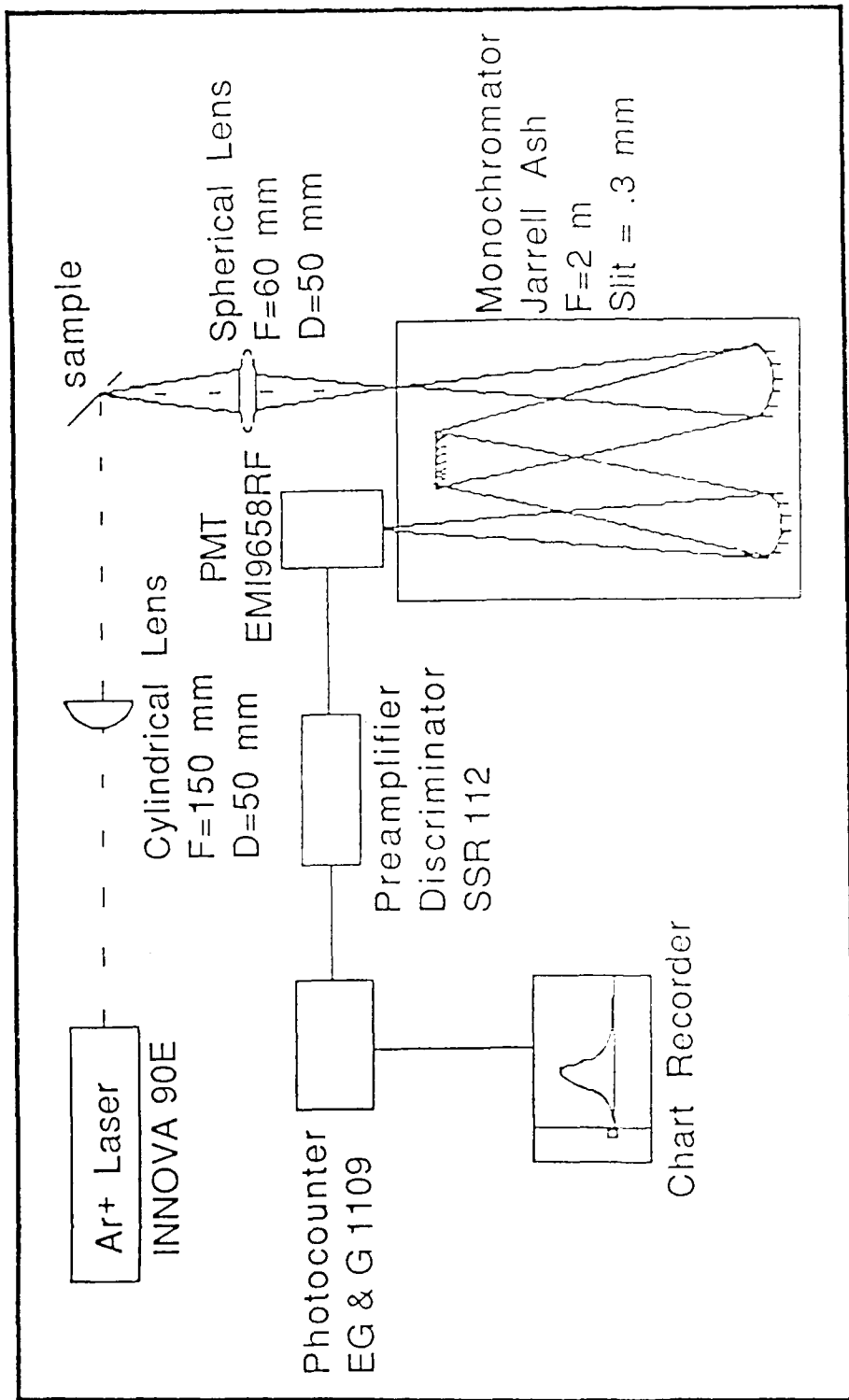


Figure 1. Schematic diagram of fluorescence set-up.

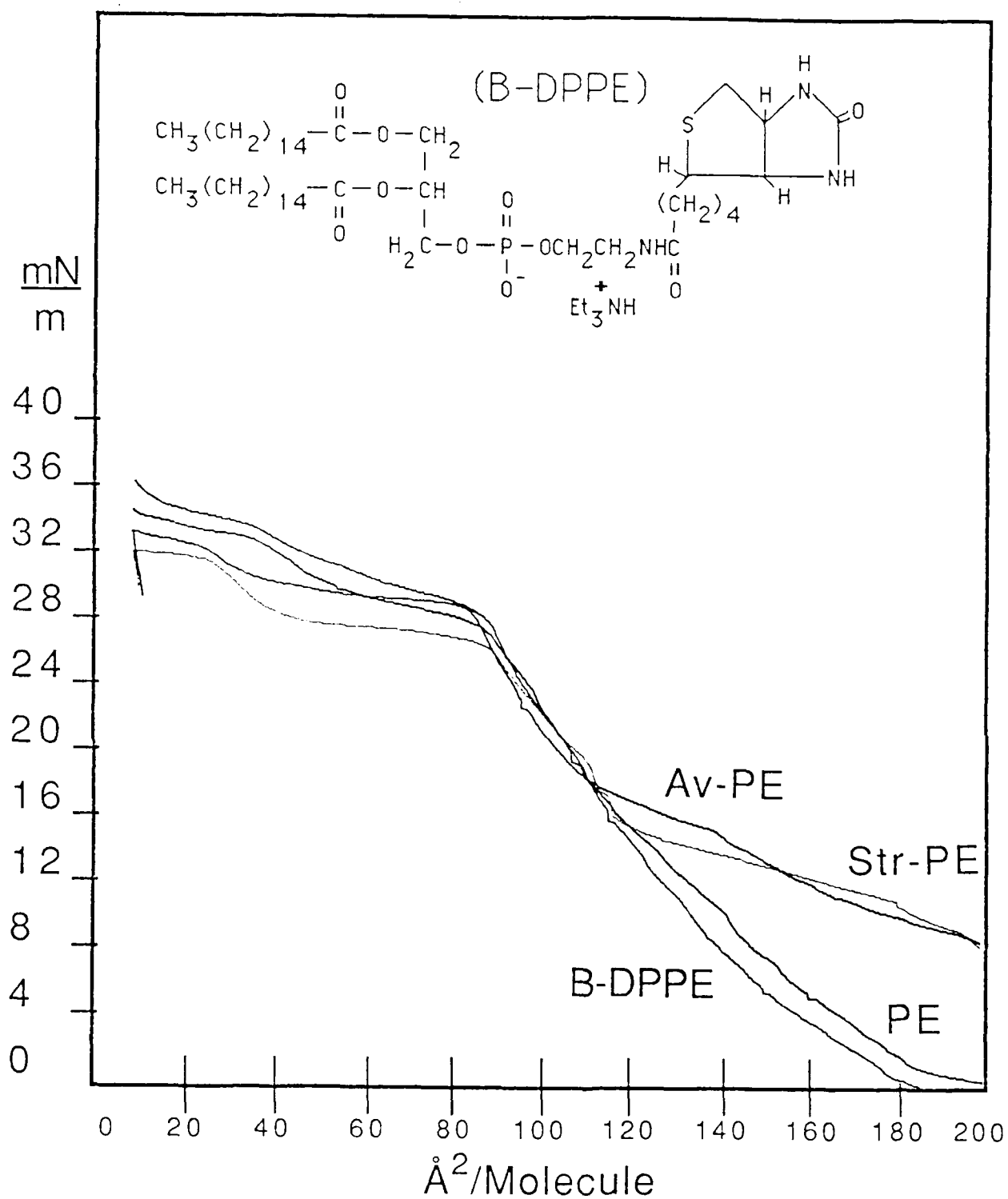


Figure 2. Structure of the biotinylated phospholipid (B-DPPE) and pressure-area isotherms of B-DPPE and protein injected B-DPPE (PE, Av-PE and Str-PE).

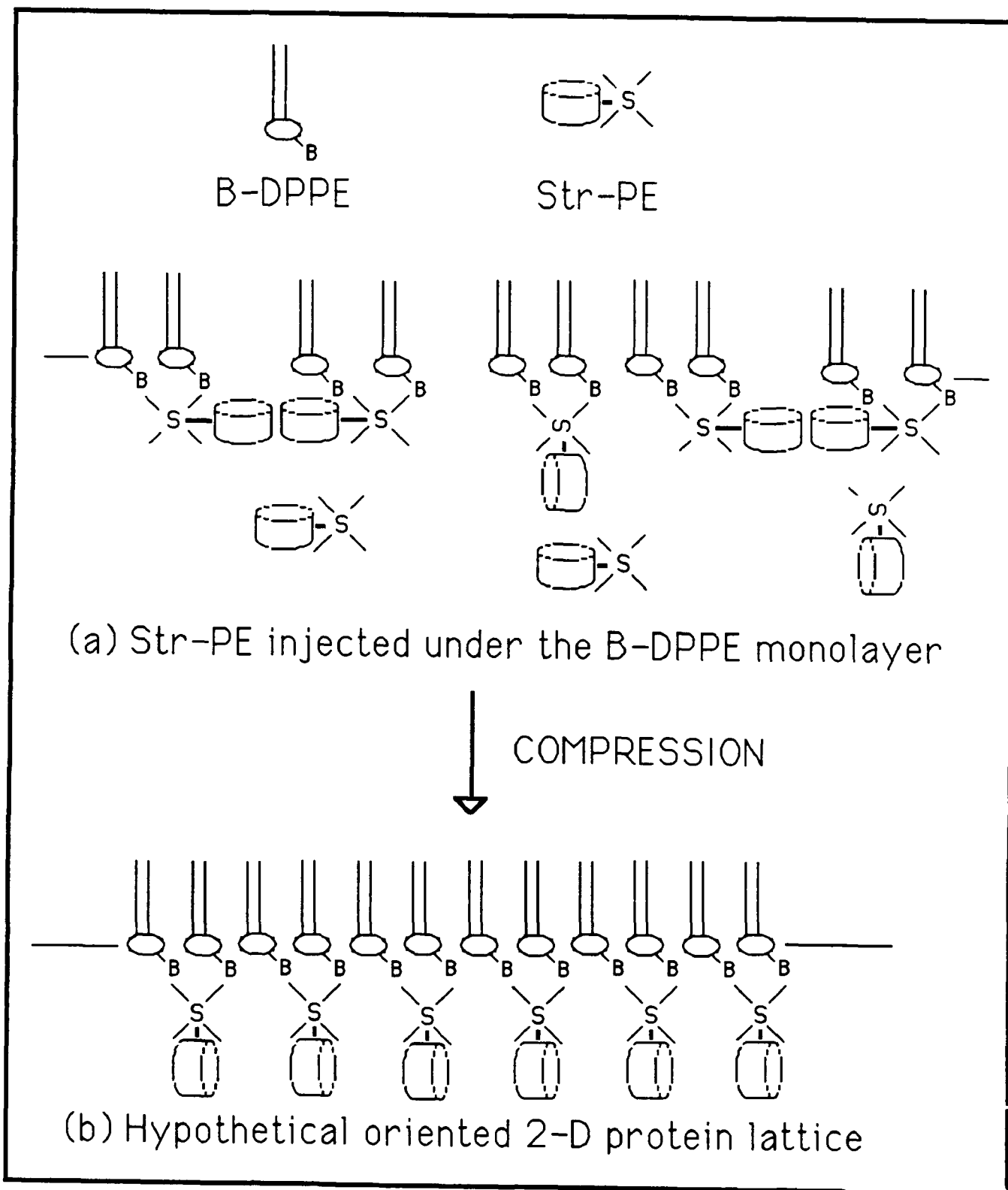
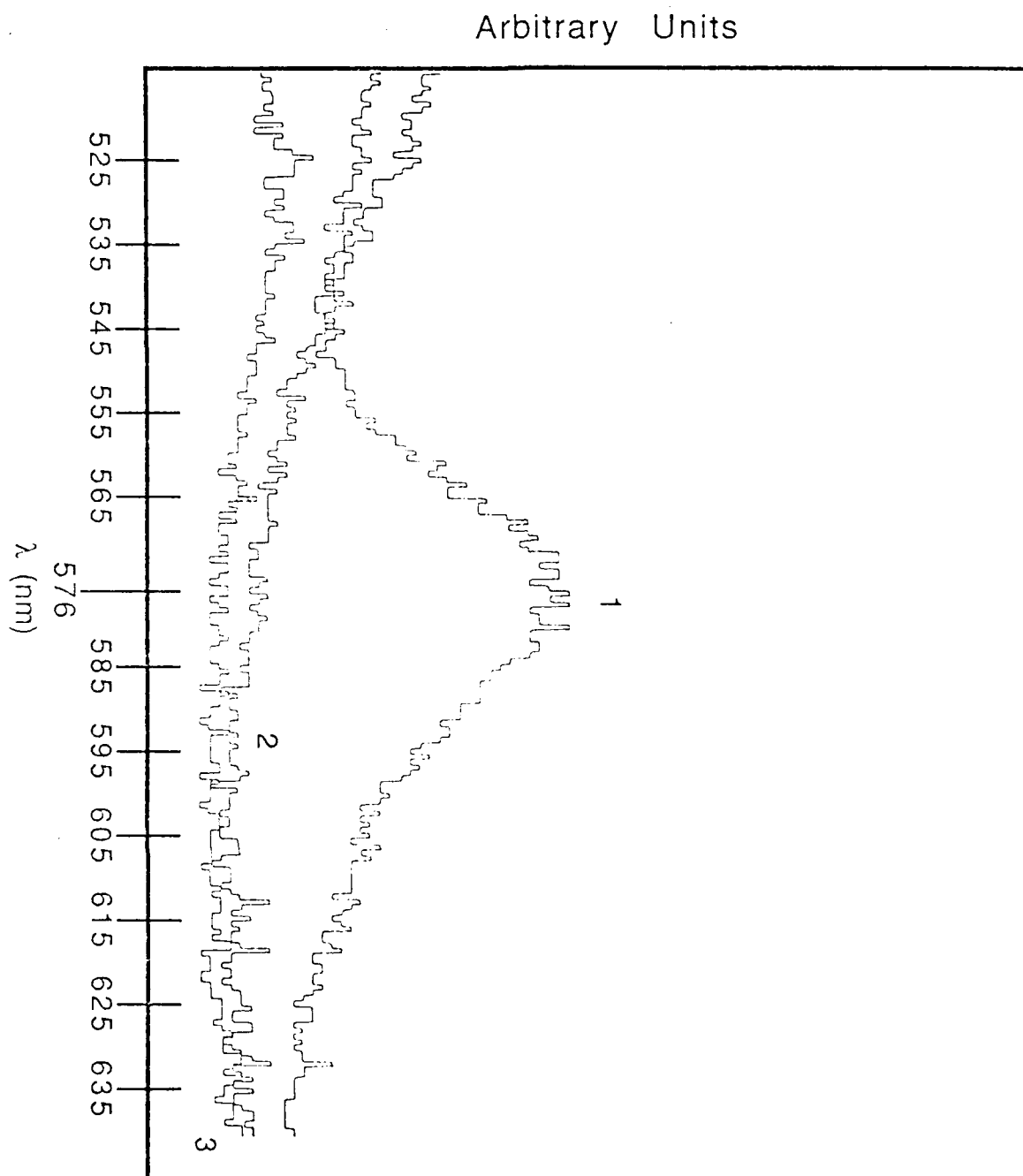


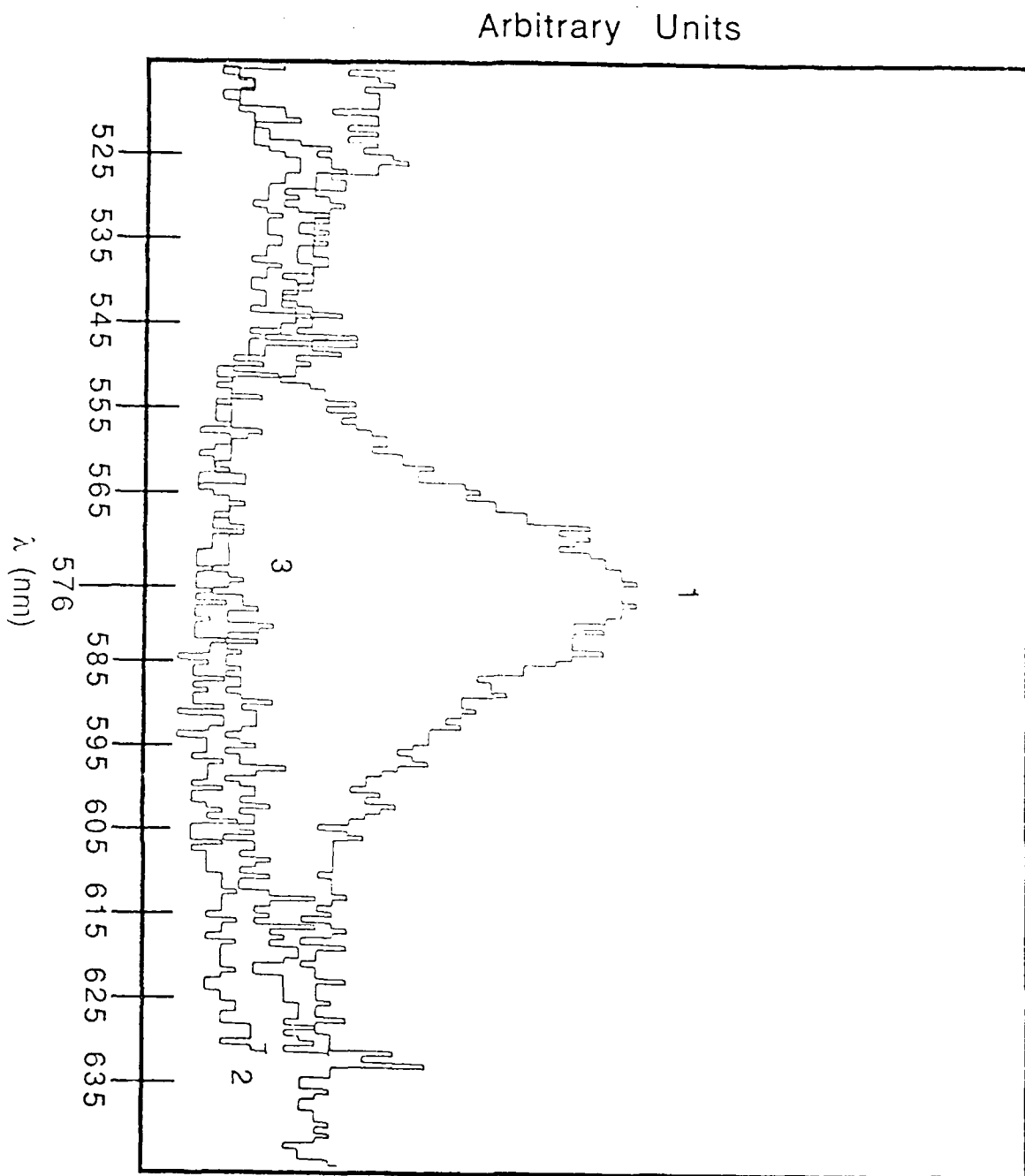
Figure 3. Idealized schematic of the two-dimensional ordering of derivatized protein monolayers onto a biotinylated lipid LB film.



1. B-DPPE + Str-PE Injected
2. Control (monolayer cleaned off the subphase)
3. Control (slide down in subphase only)

Subphase (1,2 & 3): 0.1 mM Na phosphate buffer, pH 6.8, 0.1 M NaCl

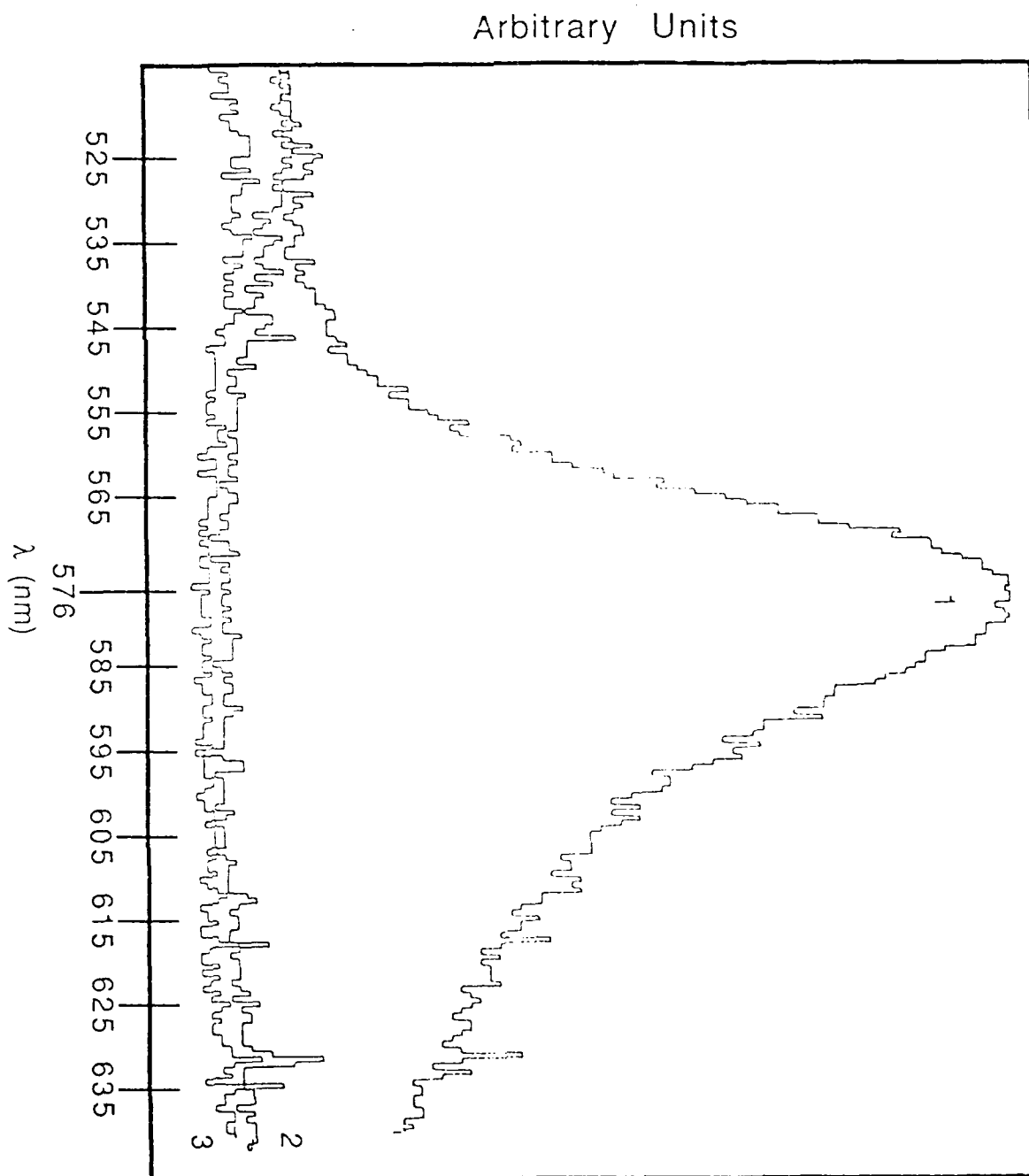
Figure 4. Fluorescence spectra of protein adsorbed LB films.



1. B-DPPE + Str-PE Injected
2. B-DPPE + PE Injected
3. DPPE + Str-PE Injected

Subphase (1,2 & 3): 0.1 mM Na phosphate buffer, pH 6.8, 0.1 M NaCl

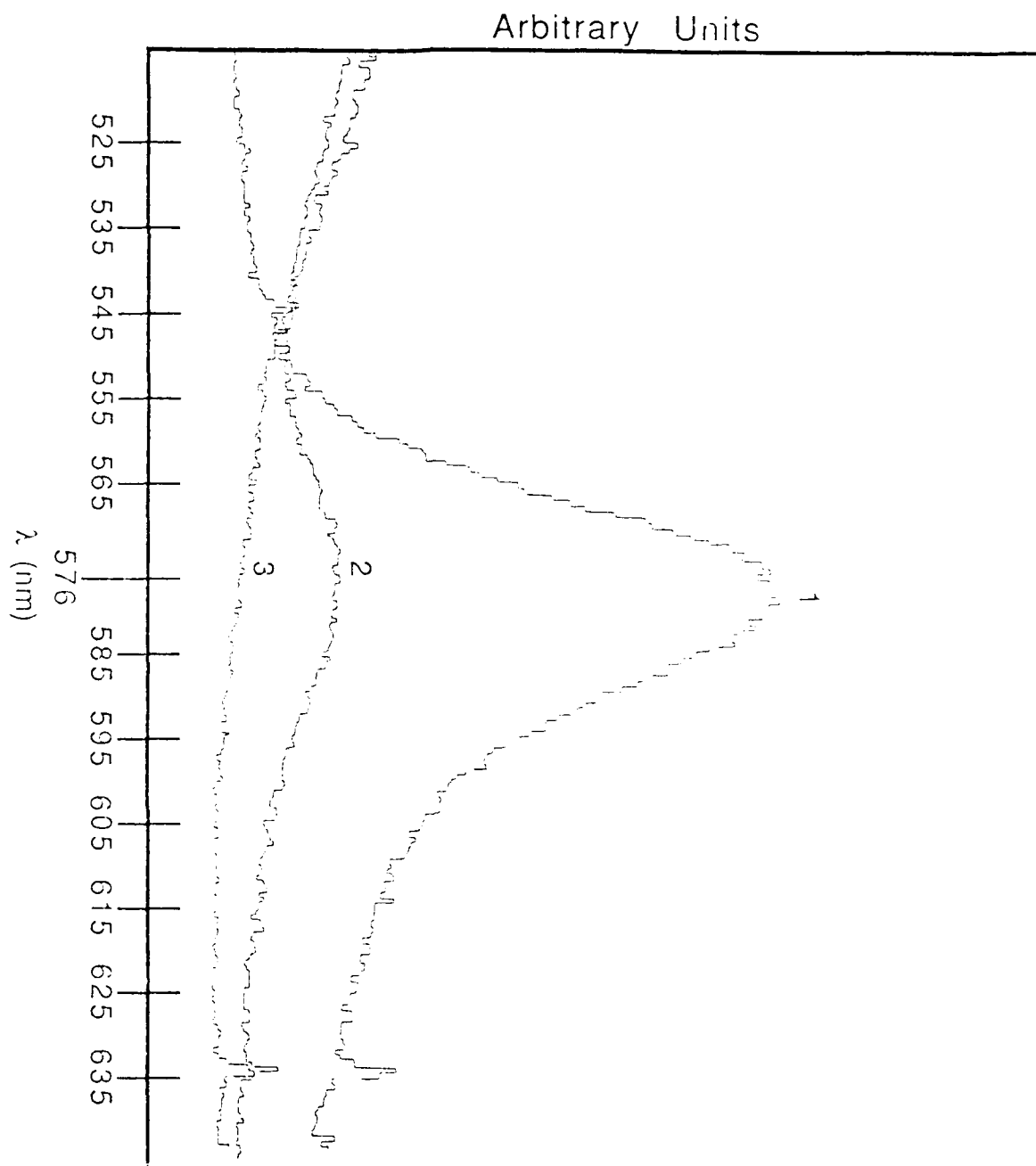
Figure 5. Fluorescence spectra of protein adsorbed LB films.



1. B-DPPE + Av-PE Injected
2. Control (monolayer cleaned off the subphase)
3. Control (slide down in subphase only)

Subphase (1,2 & 3): 0.1 mM Na phosphate buffer, pH 6.8, 0.1 M NaCl

Figure 6. Fluorescence spectra of protein adsorbed LB films.



1. B-DPPE + Av-PE Injected (without NaCl in the subphase)

2. B-DPPE + Av-PE Injected

3. Control (monolayer cleaned off the subphase)

Subphase (1): 0.1 mM Na phosphate buffer, pH 6.8

Subphase (2 & 3): 0.1 mM Na phosphate buffer, pH 6.8, 0.1 M NaCl

Figure 7. Fluorescence spectra of protein adsorbed LB films.