INTERACTIONS OF *Bacillus thuringiensis* CRY4Ba TOXIN WITH LIPID LANGMUIR-BLODGETT MONOLAYERS

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ABSTRACT

Bacillus thuringiensis Cry4Ba insecticidal crystal protein insertion into lipid monolayer was investigated by Langmuir-Blodgett technique. Three types of synthetic lipid, dipalmitoylphosphatidylcholine (DPPC), dioleylethanolamine (DOPE) and cholesterol (Chol), were used as the model monolayer. The insertion study was done by compression isotherm where the adsorption kinetics of pure protein, pure lipid and mix lipid-protein were carried out at the gas/water interface. Protein molecules only partially inserted some part into the lipid monolayer. This occurred along with rearrangement of the protein structure at the interface. In lipid layer at π=0, the amount of adsorbed protein was found to depend on the induction time. There was an increased area due to protein insertion at all temperatures in all three layers. The increased area was higher in DPPC and Chol layer than DOPE layer. The inserted protein alters the elasticity property of the lipid layer as evidenced by a decreased in the compressibility modulus. The insertion kinetics can be fitted with double exponential process where the increasing surface pressure ($\Delta\pi$) from the protein insertion is fitted as a function of time. Fluorescence spectroscopy revealed that there is a structural change associated with proteins at the interface.

KEY WORDS: LIPID-PROTEIN INTERACTIONS /LIPID MONOLAYER / CRY4Ba INSERTION

71 pp.

การศึกษาปฏิกิริยาระหว่าง *Bacillus thuringiensis* Cry4Ba กับชั้นฟิล์มบางของไขมันที่ถูกเตรียมโดยวิธี Langmuir-Blodgett
(INTERACTIONS OF *Bacillus thuringiensis* CRY4Ba TOXIN WITH LIPID LANGMUIR-BLODGETT MONOLAYERS)

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บทคัดย่อ

การแทรกผ่านชั้นฟิล์มบางของไขมันสังเคราะห์สามชนิด dipalmitoylphosphatidylcholine (DPPC), dioleylethanolamine (DOPE) และ cholesterol (Chol) โดย *Bacillus thuringiensis* Cry4Ba โปรตีนได้ถูกศึกษาโดยวิธี Langmuir-Blodgett โดยสังเกตการถูกบีบอัดของชั้นโปรตีน ชั้นไขมัน และชั้นไขมันที่ถูกแทรกโดยโปรตีน บนผิวรอยต่อระหว่าง ก๊าซและของเหลว จากการศึกษาพบว่าโปรตีนนั้นได้แทรกเฉพาะบางส่วนของโครงสร้างเข้าไปในชั้นฟิล์มบางของไขมันซึ่งเกิดขึ้นควบคู่กับการเปลี่ยนแปลงโครงสร้างของโปรตีนที่ผิวรอยต่อ ถ้าชั้นไขมันนั้นอยู่ในสภาวะของที่ไม่มีแรงดันระหว่างกัน การเคลื่อนที่ของโมเลกุลโปรตีนมายังชั้นไขมันจะขึ้นกับเวลา เมื่อชั้นไขมันถูกแทรกจะพบว่าพื้นที่ต่อโมเลกุลของไขมันนั้นเพิ่มขึ้นโดย การเพิ่มขึ้นของพื้นที่ต่อโมเลกุลไขมัน ในชั้นฟิล์มบางของ DPPC และ Chol มากกว่า ชั้นฟิล์มของ DOPE นอกจากนี้ โปรตีนโมเลกุลที่แทรกในชั้นฟิล์มของไขมันยังทำให้สมบัติความยืดหยุ่นของชั้นไขมันเปลี่ยนไปสังเกตได้จากการลดลงของค่าโมดูลัสของการบีบอัด (Compressibility modulus) ในการศึกษาจลศาสน์ของการแทรกผ่านชั้นไขมันของโปรตีนนั้น ผลการเปลี่ยนแปลงของแรงดันระหว่างโมเลกุลไขมันเทียบกับเวลาสามารถนำคำนวณเปรียบเทียบได้กับ สมการเอ็กโปเนนเชียลลำดับที่สอง และการเปล่งแสงฟลูออเรสเซนส์ (Fluorescence)ของโปรตีนยังบ่งชี้ถึงการเปลี่ยนแปลงโครงสร้างของโปรตีนอีกด้วย
71 หน้า.

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CHAPTER I
INTRODUCTION

1.1 *Bacillus thuringiensis* (Bt) Toxin

Bacillus thuringiensis (Bt)[1] is the ubiquitous gram-positive soil bacterium with rod-shape that produces insecticidal protein during its sporulation. Because of this characteristic, the *Bt* is widespread use to control the important plant pests with considering safe to people and non specific species [2]. There are different strains[3,4] of B.t. which specific toxicity toward a particular type of insect ; *Bt* aizawai (against wax moth larvae in honeycombs), *Bt* kurstaki (against Lepidotera), *Bt* isaraelensis (against Diptera, primarily mosquitoes and blackflies) and tenebrionis (Against Leptinotarsa decemlineata). *Bt* strains produce two types of toxin encoded by plasmid genes. The main type is the Cry (crystal) toxin and the second is the Cyt (cytolytic) toxins.

Table 1.1 Classification of Cry toxin

Gene	Crystal shape	Protein size (kDa)	Insect activity
Cry I Subgroups : A(a), A(b), B, C, D, E, F, G	Bipyramidal	130-138	Lepidoptera larvae
Cry II Subgroups : A, B, C	cuboidal	69-71	Lepidoptera and diptera
cry III subgroups A, B, C	flat/irregular	73-74	coleoptera
cry IV subgroups A, B, C, D	bipyramidal	73-134	diptera
cry V-IX	various	35-129	various

The mechanism of the Cry protein is generally considered as the multistage process[5-7] (Figure 1.1). Firstly, the toxin is ingested in the alkaline condition and

activated performing by protease in the larvae midgut. The activated toxin binds to the specific receptors located on the apical microvillus membrane of midgut epithelium following by changing the structural conformation allowing toxin insertion into membrane to form ion pore that leads to the osmotic lysis of the cell. The Cry4Ba toxin structure which correlates to the pore-forming mechanism[8] has been investigated by x-ray crystallography [9].

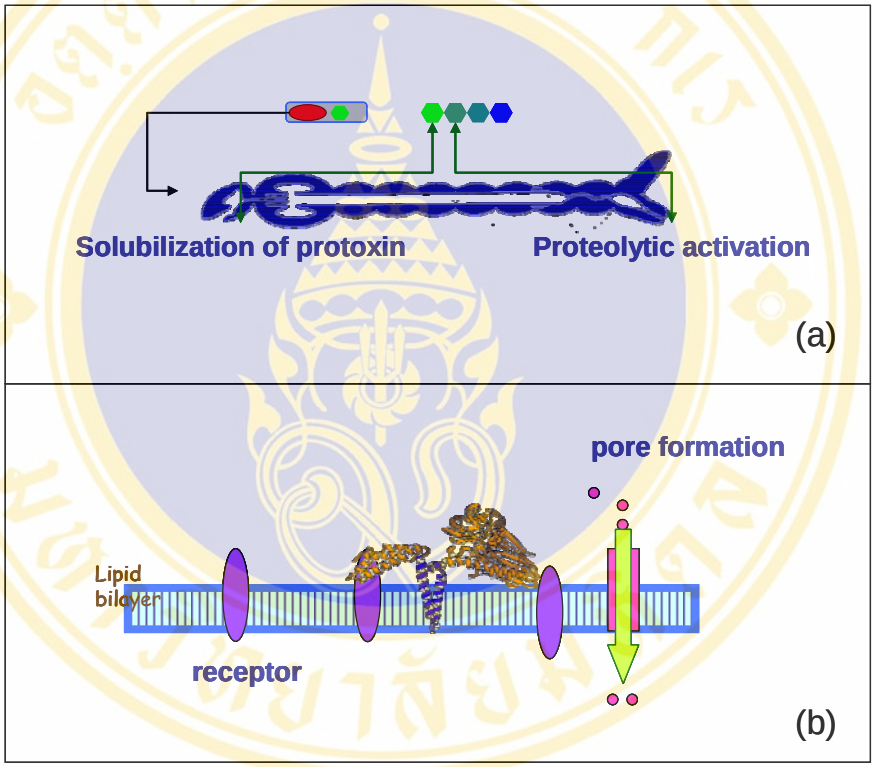

Figure 1.1 (a) Toxin activation process
(b) Toxin insertion process

Cry4Ba toxin consists of three distinct domains(Figure 1.2). Domain I is pore-forming domain consisting of four amphiphatic α-helices surrounding the hydrophobic α5 helix. Domain II , the receptor-binding domain, is a β-prism of three antiparallel β-sheet and domain III is a β-sandwich of two antiparallel β-sheets. It was proposed that helices α4-α5 in domain I are the insertion parts of protein into the membrane reconcile with the “umbrella-like model”[10]

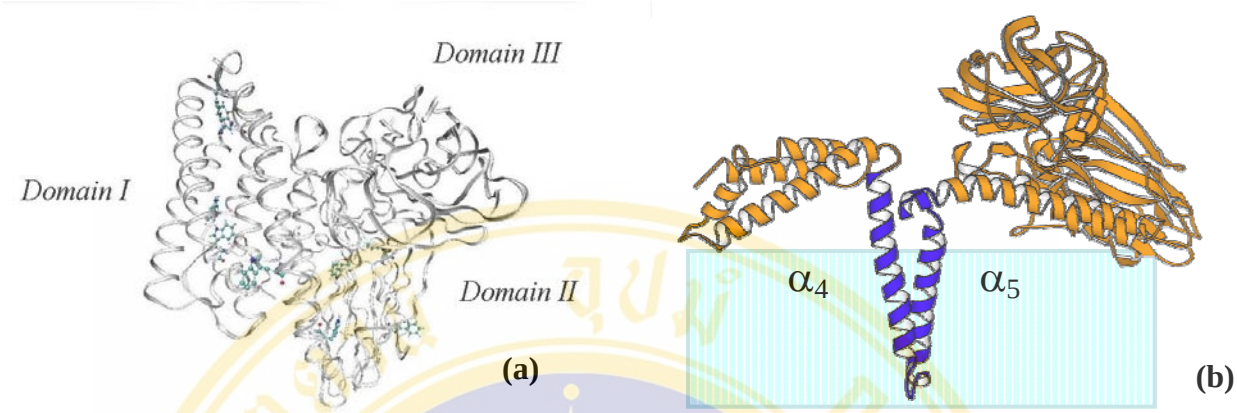

Figure 1.2 (a) Cry4Ba structure
(b) umbrella-like model

1.2 Biomimetic membrane

1.2.1 The biological membrane

Membranes [11,12] are the vital component of the cell that separate the cell from its environment (plasma membrane) or separate one region to the other region within the cell. The cell membranes are not just the boundary of the cell but the maintenance of the cellular structure by controlling the movement of materials passing in and out the cell. Furthermore; membranes are required for many mechanism of the cell such as cell-to-cell communication, cell recognition both itself and another and signal transduction. Function and properties of the cell membranes depend on their composition or their structure. In general, the major components of membranes are membrane protein and membrane lipid. The first component, membrane protein, represents in 50 % of the membrane and mainly derived into 2 basic types ; i) integral membrane proteins ii) peripheral membrane proteins. The second is membrane lipids which organized in two-dimensional fluid; lipid bilayers [13] (Figure 1.3) due to their amphipathic characteristic that the hydrophilic (polar) portion facing out to the aqueous and the hydrophobic (non-polar) portion forming the bilayers interior.

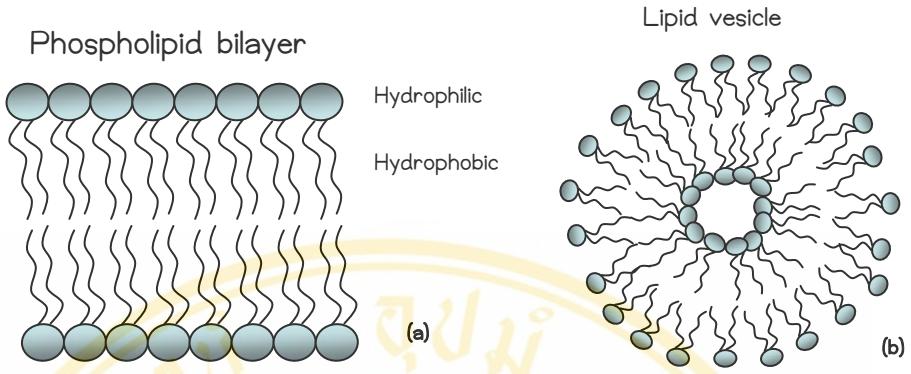

Figure 1.3 Model membrane
(a) Phospholipid bilayers
(b) Lipid vesicle

Consequently; the principal membrane lipids are phospholipids and cholesterol (Figure 1.4). Phospholipids which represent about 50% of membrane lipid compose of the polar head group linked to two fatty acid tail by phosphate group and glycerol backbone. These two moieties act the phospholipids as amphiphatic molecule. The most abundant phospholipids found in the membranes are phosphatidylcholine (lecithin), phosphatidylserine and phosphatidylethanolamine (cephalin). Another major membrane lipid is cholesterols which show steroid structure; rigid ring system connecting to a short branched hydrocarbon tail. This amphiphatic cholesterols insert the hydrophobic aromatic ring system into lipid bilayers with the same orientation of phospholipids and align the hydrophilic hydroxyl group with polar head of phospholipid toward the aqueous. Cholesterols are important in maintaining the membrane stability as served as a temperature-stability buffer, decreasing permeability to small molecule and reserving the membrane fluidity.

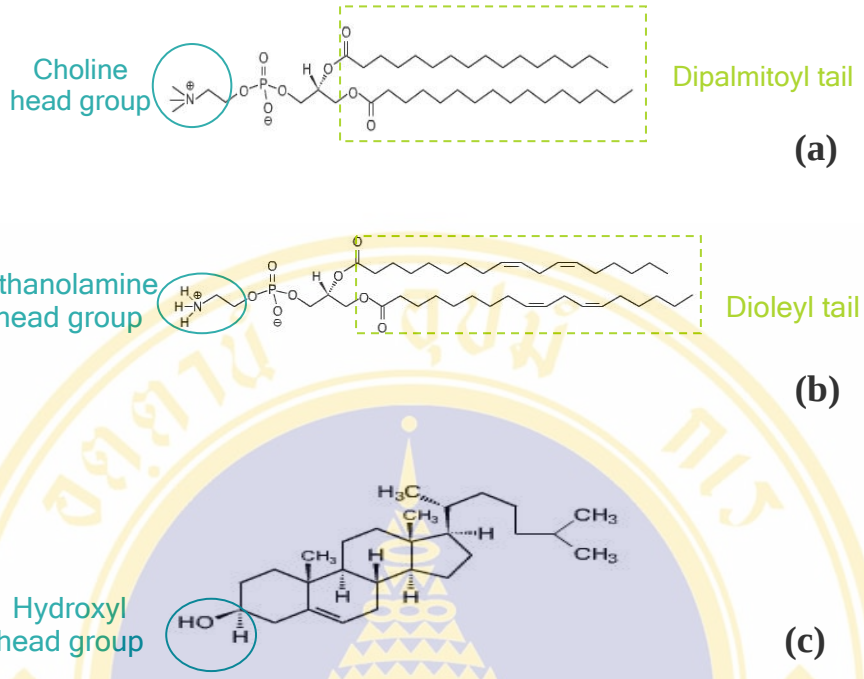

Figure 1.4 Membrane lipid structure
(a) Dipalmitoylphosphatidylcholine
(b) Dioleylphosphatidylethanolamine
(c) Cholesterol

1.2.2 Model membrane

The interaction and activity at the membrane level are the most striking phenomena which introduce to many applications. The study of the natural membranes is difficult because controlling the experimental parameters due to the complexity in membrane structure and only the global phenomenon can be observed. To simplify this problem, the model membranes are the suitable nomination. There are many approaches on mimetic membrane system such as bilayer lipid membranes (BLMs)[14], vesicle and monolayer[15]. BLMs forming, like natural membranes, are a good model membrane because it allows the variation of many parameters such as electrical properties, transport properties, fusion and molecular recognition. The spherical or ellipsoidal closed form of bilayer structure in term of both natural and synthetic phospholipids are known as liposomes. In contrast, those formed from the synthetic surfactant are termed in vesicles. In this biomembrane model is enormously used in studies of permeability due to the internal aqueous compartment. The vesicles

system show the advantage on the ease of experimental set up where the result can be observed by spectroscopic measurement. Some disadvantage occurred in the difficult on homogeneous (in size and layer number) vesicles preparation , the strong constraints at the polar head group as the small curvature radius of vesicles and the regularity of preparation. In contrast, with used of monolayer system, it has the great advantage over other membrane models in which various properties can be measured and chosen without limitation. Additionally; the monolayer homogeneity , stability and planar geometry can be easily manipulated.

1.3 Motivation and objective

Though there are many reports on insect's larvae killing by Cry protein , how this protein penetrates into membrane and makes cell die is still not clear. It is of interest to understand this biological phenomenon which involves the lipid-protein interaction at the membrane level. The biomimetic membrane is the reasonable candidate to achieve this goal. As the advantage of monolayer over the other model membrane, Langmuir-Blodgett technique [16] is used to imitate the lipid monolayer in a simplified manner offering a simple way for observing the interactions that occur in the monolayer by monitoring the force. The information about the interaction of lipid-protein in vitro, yet; will provide the picture on how the protein penetrates into the cell membrane.

Cry4Ba insertion into lipid Langmuir-Blodgett monolayer has previously been reported [17]. This protein spreading isotherm shows the smaller interfacial area per molecule than the whole crystal structure surface suggesting that the only the hydrophobic part of protein involve in the insertion into lipid monolayer. It was found that the protein inserted only a certain part into lipid layer corresponding with a small expansion of lipid mean molecular area by isothermal compression study. They also purposed that protein-lipid interaction with neutral head group of lipid is hydrophobic interaction which is the most favorable in cholesterol than the others; phosphatidylcholine (PC) and phosphatidylethanolamine (PE) head groups and cholesterol. In addition, cholesterol also increased the fluidity of mixing lipid monolayer lead to more protein insertion. They concluded that the insertion of protein

into lipid layer largely depended on the nature of lipid, the initial surface pressure and the packing density of the lipid film.

In this study we used the monomolecular approach to observe the penetration of the crystal protein, Cry4Ba, into the mimectic membrane. Three distinct synthetic lipid types; 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) , 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and cholesterol (Chol) considered as the major component of the insect membrane were used to perform the model biomembrane. The environmental parameter,temperature and the state of packing of monolayer which perturbed the protein configuration in aqueous is examined coincide with the effect of monolayer properties and protein-lipid interactions.

CHAPTER II
INTERFACIAL ACTIVITY

Interface [18] represents the boundary between the different homogeneous phase region that forms between three common state of matter such as solid-liquid, solid-gas, gas-liquid, two immiscible liquids and two solids. Thermodynamics is the important discipline to define the interfacial properties by state function and thermodynamics variables. Of the interfacial system, the system behavior occurs in equilibrium in some certain aspects despite unstable in others, so the interfacial involving system is extensively referred to metastable system [18]. To form the interface between materials the free energy is involved; positive, negative or zero energy. The dynamics of interface are of interested in the mechanism of reaction, the rate process and other parameters. Here, we concerned in the phenomenon at the gas-liquid interfaces which encounters in physical and chemical process occurring in the bulk system. The monolayer method which can directly measure the force at the interface is applied.

2.1 Monolayer

The term "*monolayer/monomolecular layer*" usually represents to a layer of the slightly soluble amphiphile molecules floating on liquid surface. Since amphiphile molecules consist of hydrophilic head group (polar) and hydrophobic tail (non-polar) which tend to locate at the interface of water by dissolving the head group in water region and exerting the tail in to an air region. In general, water surface have surface tension as the present of cohesive energy between water molecules or termed in the surface excess free energy at the interface. By forming of monolayer, reduction of water surface tension is occurred. The difference of water surface tension in absent and present of molecules at interface is normally termed in *surface pressure* or mean of each molecules exert force to each others.

Interfacial tension

Langmuir-Blodgett is one method which is performing systematic study of monolayer force at air/water (generally gas/water) interface. In this performing method, after the amphiphile molecules are dropped on water subphase, the solution spreads rapidly to cover the available area then a monolayer is formed. In this condition the area available for molecule quite large so that the interaction of each adjacent molecule is weak producing slightly change of water surface tension .If the area available for monolayer is reduced by a barrier system (Figure 2.1) the molecules start to exert a repulsive effect on each other then the surface pressure (π) is presented ,and given by

$$\pi = \gamma - \gamma_0 \qquad (2.1)$$

where γ is the surface tension in absence of a monolayer

γ_0 is the surface tension with the monolayer present

The surface pressure is measured by the Wilhelmy plate-method[19] in which a measurement is made by determining the force due to surface tension on a thin plate suspended so that it is partially immersed in the subphase (Figure 2.2). This force is then converted into surface tension (mN/m or dynes/cm)

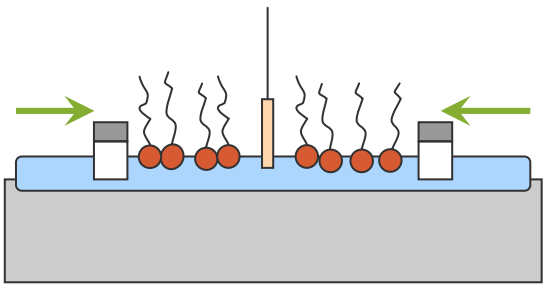

Figure 2.1 Monolayer compression system

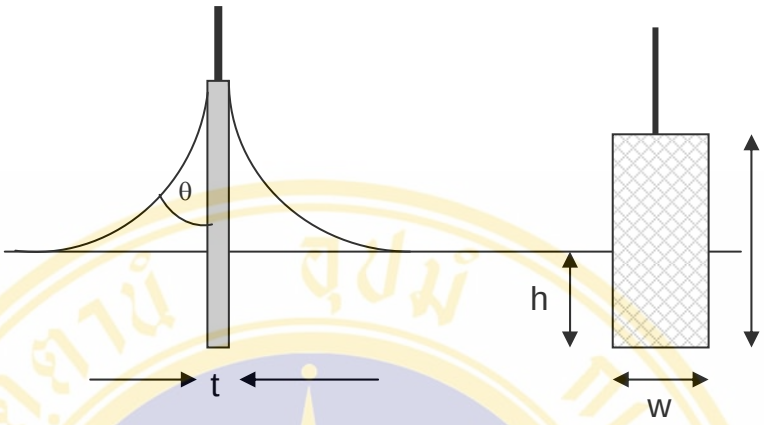

Figure 2.2 Schematic of surface tension on Wilhelmy plate

The plate is often very thin and made of platinum due to its wetting properties influence in the θ of 60° , but even plates made of glass, quartz, mica and filter paper can be used. The forces acting on the plate consist of the gravity and surface tension downward, and buoyancy due to displaced water upward. For a rectangular plate of dimensions l_p, w_p and t_p, of material density ρ_p, immersed to a depth h_l in a liquid of density ρ_l, the net downward force is given by the following equation

$$F = \rho_p g l_p w_p t_p + 2\gamma(t_p w_p)(\cos\theta) - \rho_l g t_l w_l h_l \qquad (2.2)$$

where γ is the liquid surface tension,

θ is the contact angle of the liquid on the solid plate and

g is the gravitational constant

The surface pressure is then determined by measuring the change in F for a stationary plate between a clean surface and the same surface with a monolayer present. If the plate is completely wet by the liquid (i.e. $\cos\theta = 1$) the surface pressure is then obtained from the following equation

$$\pi = -\Delta\gamma = -[\Delta F / 2(t_p + w_p)] \quad (2.3)$$

$$= -\Delta F / 2w_p \text{ if } \quad w_p >> t_p \quad (2.4)$$

The important property of amphiphile monolayer is given by measuring the surface pressure as a function of the area of water surface available to each molecule ,this measurement is carry out at constant temperature and is known as a surface pressure - area isotherm (simply isotherm). Usually an isotherm is recorded by compressing the film (reducing the area with the barriers) at a constant rate while continuously monitoring the surface pressure, a schematic π-A-isotherm is shown in Figure 2.3.

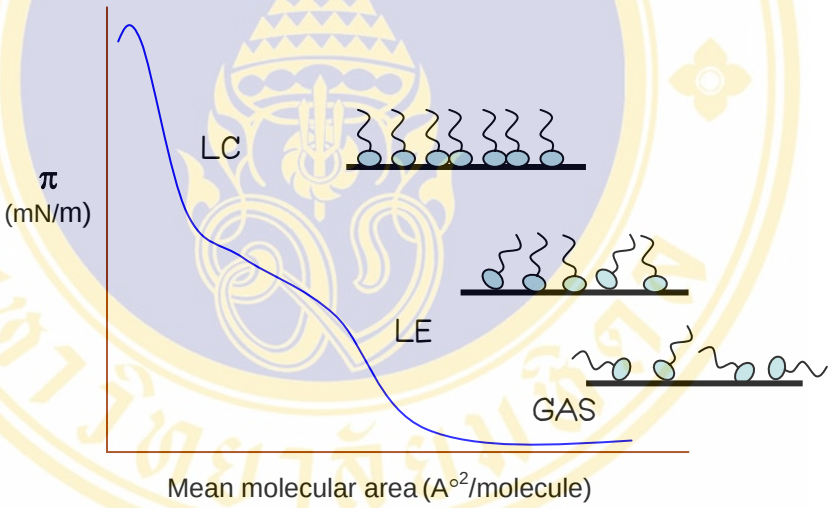

Figure 2.3 Three different phase change in π-A-isotherm

A number of distinct regions is immediately apparent on examining the isotherm. These regions are called phases. At large the monolayers exist in the gaseous state (G) and can on compression undergo a phase transition to the liquid-expanded state (L1). Upon further compression, the L1 phase undergoes a transition to the liquid-condensed state (L2), and at even higher densities the monolayer finally reaches the solid state (S). If the monolayer is further compressed after reaching the S state the monolayer will collapse into three-dimensional structures. The collapse is

generally seen as a rapid decrease in the surface pressure or as a horizontal break in the isotherm if the monolayer is in a liquid state.

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Monolayer state

This monolayer occurs in the different states which can changes from one to another state in the so-called phase transition which already discussed. These phase transition may happen from these states; gaseous, expanded and condense. All these phase can be described by the equation of state in thermodynamics aspect.

In gaseous state; molecules are far from each other and the area per molecule is large compare to the molecule size. With freely move like an ideal gas leads to the ideal gas state equation is applied in equation 2.1

$$\prod A = kT \qquad (2.5)$$

Where A is the area available to each molecule (mean molecular area)

Upon the area reduction; compression, the monolayer changes its state from gaseous to liquid expanded and liquid condense phase, respectively. In liquid expanded (LE) phase, the monolayers are fluid and coherent (liquid-like) which exist between the transition from gaseous (molecule lie flat to the surface) to solid state (molecule perpendicular orientation to surface).And liquid (LC) state, the molecules come closely pack and reduce in degree of freedom with the order arrangement. The equation of these two states is

$$(\prod - \prod_0)(A - A_0) = kT \qquad (2.6)$$

Compressibility modulus

The important parameter used to characterized a monolayer phase and monolayer elasticity is the compressibility modulus shown in eq. 2.3

$$K = -A\left(\frac{\partial \pi}{\partial A}\right)_{T,P,N} \quad (2.7)$$

Where A is the area

π is the surface pressure

With this parameter; the elasticity deduced from compression isotherm, exhibits the higher K value the lower interfacial elasticity.

2.2 Physical process at interfaces

Since the amphiphile molecules have the hydrophobic and hydrophilic moiety in which the hydrophilic group adsorbed to water molecules so they show the oriented adsorption at interface. Thai in case of the clean surface but in case of the surface spaces are already occupied the hydrophobic part tend to avoid facing to water molecule (polar) by forming aggregation. This is the two characteristic of surfactant at interfaces; aggregation and adsorption as showed in Figure 2.4. Adsorption is the passive process in which the adsorption at gas/water interface exhibited in the multistage process. The adsorption in a simple case (adsorption of gas onto solid surface) occurs due to the attraction between gas molecules and molecules on surface (adsorbate-adsorbent) but the adsorption of molecule in liquid into gas/liquid surface no such the attraction between adsorbate and air is presented.

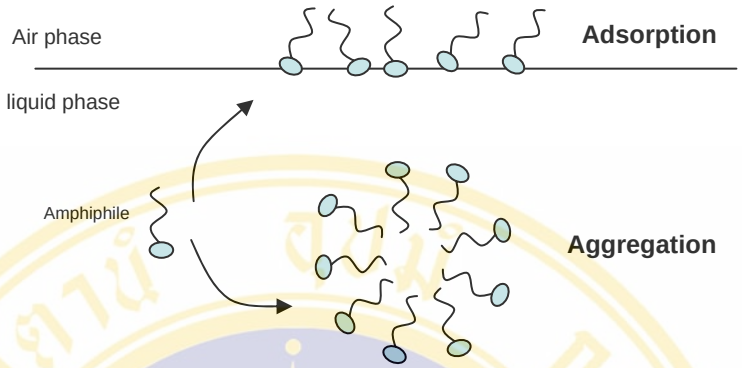


Figure 2.4 Schematic of surfactant activity at gas/liquid interface

Adsorption

Firstly, molecules in bulk solution arrive the interface with random motion known as diffusion. If the adsorption of molecules lowering the surface free energy, these molecules will rest at the interface for a long time, referred to the preference adsorption. There are two stages of adsorption [20] adsorption with the absent and present of energy barrier. In the prior case is the stage of the solute molecules adsorbed to the clean interface and form the layer. After this stage, some molecules adsorbed at the interface and the adsorption is not diffusion control since the present of energy barrier. This energy barrier exposure permits only the sufficient energy molecule adsorbed and the back-diffusion into the bulk solution is occurred. The adsorption rate can be measured by the surface pressure increasing in constant area or the area increasing in constant surface pressure assay. There are the main three types of energy barrier; i) availability of site, ii) interfacial pressure barrier and iii) electrical potential barrier.

Penetration

Penetration (Figure 2.5) is occurred when underneath the insoluble monolayer the other molecule was injected and leads to change in both surface pressure and surface potential. In some case, the penetration molecules contribution arose in the mix film of two components at the interface. The energy terms that must be considered when the penetration occurred are [21]:

i) the energy of removal of hydrophobic part of the molecule from water,

ii) the energy to make a space in monolayer (result in the barrier compression-like),

iii) the energy of interaction of the penetration molecules with molecules of monolayer,

iv) the dipole interaction between the head group.

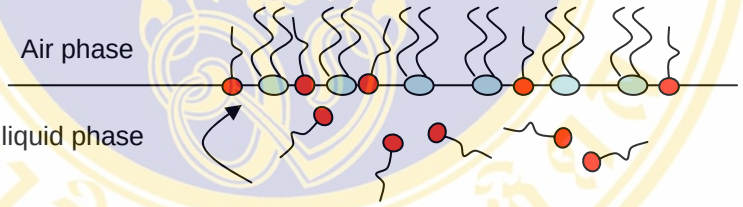


Figure 2.5 Schematic of penetration into monolayer

The interaction between adsorbate-adsorbent molecules is occurred during the adsorption process. Various types of interaction may be involved in this process [22].

i) Van der waals interaction: occurs between both polar and polarizable groups.

ii) Hydrogen bonds: occurs when the hydroxyl, amino, electron-donating and electron-accepting is presented.

iii) Coulomb interaction: occurs between charge-charge groups.

iv) Hydrophobic interaction : occurs between non-polar parts.

CHAPTER III
EXPERIMENTAL MATERIALS AND METHOD

3.1 Materials

Chemicals

The 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC ; $C_{40}H_{80}NO_8P$) of molecular weight 734.5 g/mol and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE ; $C_{41}H_{78}NO_8P$) of molecular weight 744.05 g/mol were purchased form Avanti Polar Lipids, Inc. Cholesterol of molecular weight 386.65 g/mol was obtained from Sigma Inc. Sodium hydrogen carbonate ($NaHCO_3$) of molecular weight 84.01 g/mol and Sodium carbonate (Na_2CO_3) of molecular weight 105.99 g/mol were purchased from Merck.

The analytical grade solvent (purity > 99%); Chloroform ($CHCl_3$) and Ethyl alcohol (EtOH) were obtained from Lab scan and Mallinckrodt, respectively. De-ionized and non organics water (3A) with the resistant of ≈18.0 MΩ/cm produced by Millipore Milli-Q filtering system was obtained from the Central Instrument Facility (CIF), Faculty of science, Mahidol University.

Cry4Ba protein

Activated Cry4Ba toxin of 73 kDa molecular mass was obtained from *Institute of Molecular Biology and Genetics, Mahidol University*.

Buffer solution

Carbonate buffer solution with concentration of 50 mM and pH≈9 was prepared using Carbonate(Na_2CO_3)-Bicarbonate($NaHCO_3$) buffer system.[23]

Sodium carbonate(50mM) solution of 900 ml volume mix with 100 ml of bicarbonate solution(50 mM) then stirring with magnetic stirrer for 20 minutes.

Lipid solution

All lipid solution was prepared by dissolving 20 mg lipid in 1 ml of chloroform solution and use as the stock solution. The solution 0.2 mg/ml was prepared by diluting the stock solution with chloroform. All lipid solutions were blew with nitrogen gas and tightly stored at the temperature of -20 °C. The working solution should not be used longer than 5 days due to the lipid oxidation.

3.2 Monolayer technique

3.2.2 Instrumental set up

Monolayer compression isotherm measurements were performed using KSV 2000 (KSV, Finland) system. The LB set up consists of the i) the square Teflon trough with the total volume of 185 ml and surface area of 242 cm^2, ii) the symmetry compression Delrin barriers, iii) platinum plate hangs with the microbalance sensor. This set up are placed on the Granite table in air recirculation laminar flow hood [Dwyer instruments,Inc USA]. The subphase temperature was controlled by water circulating Lauda RM 6 [Lauda instruments,Inc. USA] which flows the recirculating water into Aluminium plate under the trough. Temperature of the subphase is measured by thermocouple [model SR92 Shimaden, Taiwan).This setup system is showed in Figure 3.1

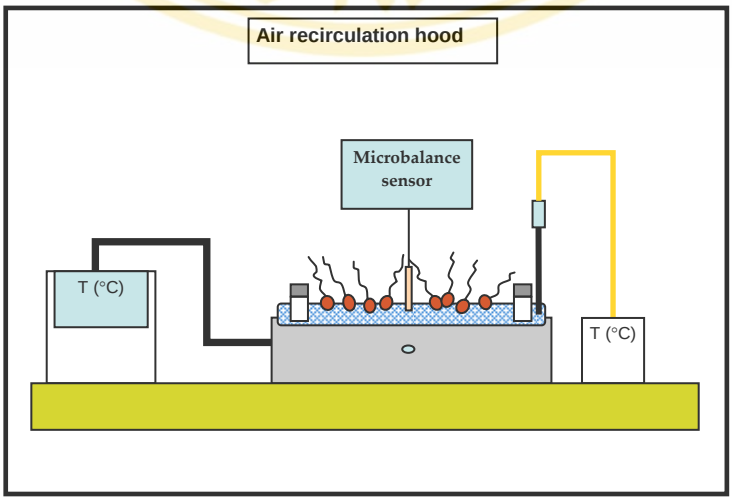


Figure 3.1 Schematic of monolayer technique setup

3.2.3 Method

The Teflon trough was cleaned with chloroform and then rinsed twice with Milli-Q water (pH≈18.0). In protein adsorption study, the protein must be equilibrated at the designate temperature for ten minutes before injection to ensure the proper experimental condition. For the compression isotherm, the surface impurities or contaminant were removed by the fine capillary tube suction. The lipid solution with concentration of 0.2 mg/ml, 100 µl was spread on carbonate buffer subphase by micropipette. Then system is approximately kept about 10 minutes before start to compress with compression rate of 10 mm/min. In the second part of this work, the adsorption of protein in solution was injected into buffer subphase then the experiment was performed.

For the insertion measurement, lipid solution was spread onto the buffer subphase, compressed to hold at the chosen surface area, and start to monitor the change in surface pressure as a function of time. The surface pressure is decreased at the initial stage due to the readjustment of the monolayer, creeping. After the surface reaches the constant value, 80 µl of protein (0.5 µg/µl in carbonate buffer),was slowly injected underneath the lipid layer by the glass syringe. Care must be taken not to disturb the lipid monolayer during the injection. The increase in the surface pressure was monitored as a function of time.

3.3 Fluorescence spectroscopy

3.3.1 Instrumental set up

LS 55 Model of Luminescence spectrometer [PerkinElmer, Inc. USA.] was used to study the protein optical properties. Heating and cooling bath equipped with the circulator [NTE 111 from Neslab instruments, Inc. USA] was used to control the temperature.

3.3.2 Method

Intrinsic fluorescence caused by Tryptophan residues excited at 280 nm in 1 mm quart cuvette at 0.27 µM Cry4Ba concentration was measured. Temperature was

controlled by flow the water through the flow cell. The temperature dependent of Cry4Ba protein emission intensity was varied from 7 to 75 °C. Protein solution was equilibrated at the observed temperatures for 15 minutes before the emission profiles were collected.

CHAPTER IV
SINGLE COMPONENT MONOLAYER

4.1 Lipid monolayer compression isotherm study

Compression isotherm study

The compression isotherm of lipid monomolecular layer, which is typically considered as the insoluble monolayer, was chosen to imitate the lipid molecules organization at air-water interface. Three lipid types; DPPC, DOPE and Chol , are investigated. DPPC and DOPE are phospholipid that composes of the polar headgroup linking with the hydrophobic fatty acid hydrocarbon chain by glycerol. Chol is the steroid lipid that composes of polar hydroxyl group joined with planar rigid ring and a short branched hydrocarbon tail. These different lipids with the polar headgroup and hydrophonic tail are studied at the interface.

Isotherm of DPPC is shown in Figure 4.1 where there is a coexistence of liquid expanse (LE) and liquid condense (LC) phase separated by the gradually increase slope which indicates the phase transition[24].

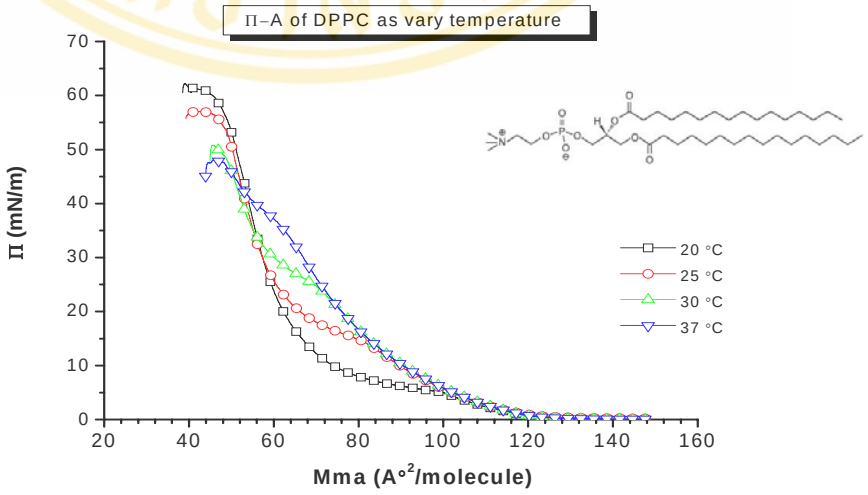


Figure 4.1 π-Area isotherm of DPPC

In case of the DOPE and Chol lipids each shows only one organization state, LE and LC, respectively (Figure.4.2). The shape of lipid molecules has the direct influence on molecular arrangement at the air-water interface. The unsaturated fatty acid moiety lipids, DOPE, shows the kink at double bond that creates disorder at hydrophobic region prevent the molecule from close packing yielding in the fluid state of the film. In case of Chol, molecules have good packing layer due to their planar structure, as a result the monolayer shows LC phase.

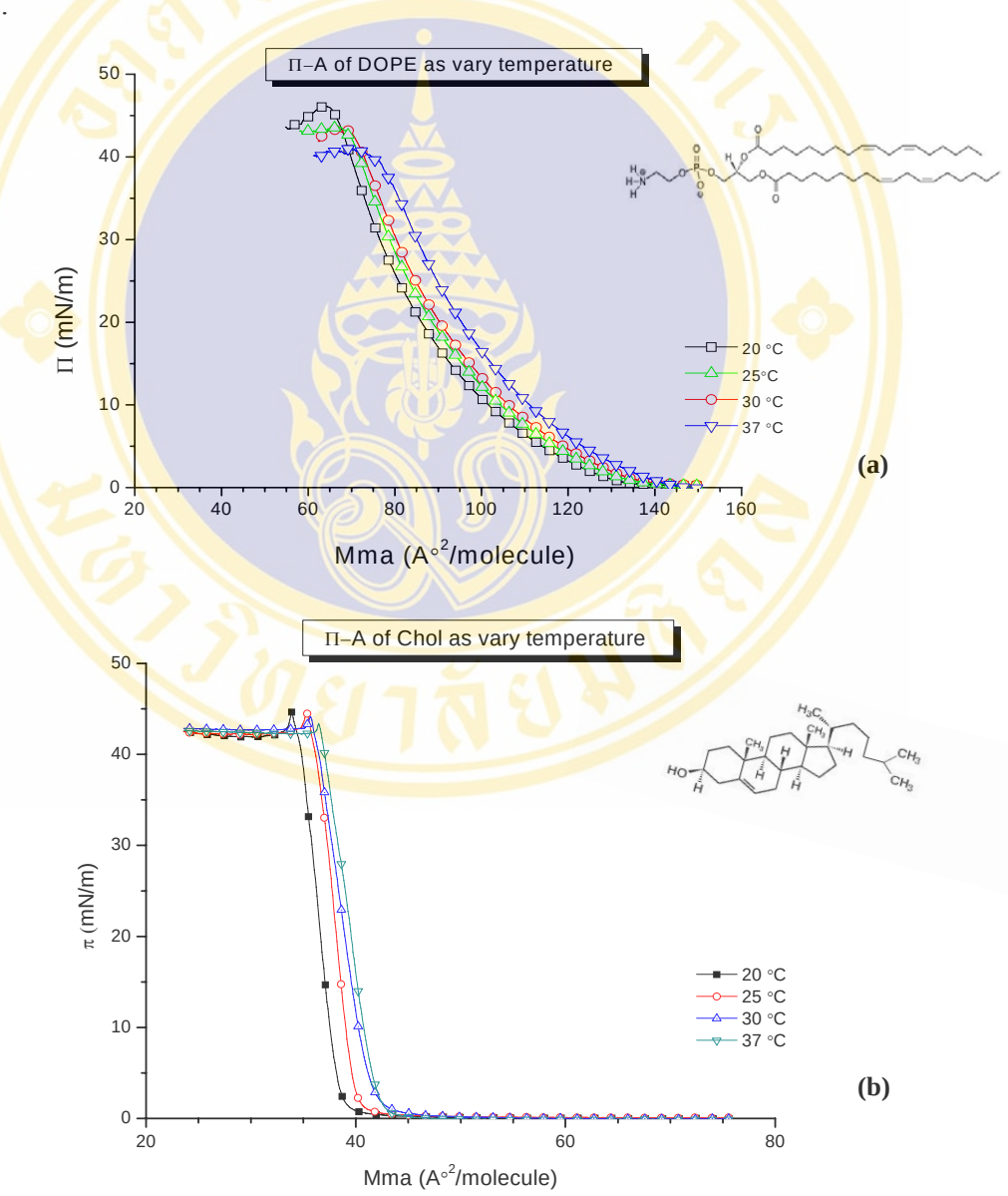


Figure 4.2 Π-Mma isotherm of single component lipid
(a) DOPE (b) Chol

Increasing temperature shows little effect on the mean molecular area for both DOPE and Chol as evidenced from their isotherm. On the contrary, in a system of saturated lipid, DPPC, an increase in the temperature renders the hydrocarbon chain melting and getting more disorder results in larger liquid expand phase region. This take places along with the rising in transition surface pressure and reducing in the collapse surface pressure (Figure 4.3). This is in agreement with the literature [24] that the phase transition of lipid monolayer is considered to be the first order transition. The major driving for of the phase transition is the disordering of aliphatic tails which require a large entropy change.

The observed mean molecular area (Table 4.1) of the Chol is around 40 $A^{\circ 2}$/molecule and 110 $A^{\circ 2}$/molecule for DOPE. The area occupied per molecule is influenced by temperature and the subphase condition where higher temperature gives rise to a larger mean molecular area. The mean molecular area of DPPC changes from 67 $A^{\circ 2}$/molecule in LC phase to 92 $A^{\circ 2}$/molecule in LE phase as the temperature is increased.

Table 4.1 Mean molecular area (Mma) of lipids monolayer

T (°C)	DOPE ($A^{\circ 2}$/molecule)	Chol ($A^{\circ 2}$/molecule)	DPPC ($A^{\circ 2}$/molecule)	
	LE	LC	LE	LC
20	106	38	138	67
25	108	40	122	69
30	109	41	111	73
37	118	42	99	92

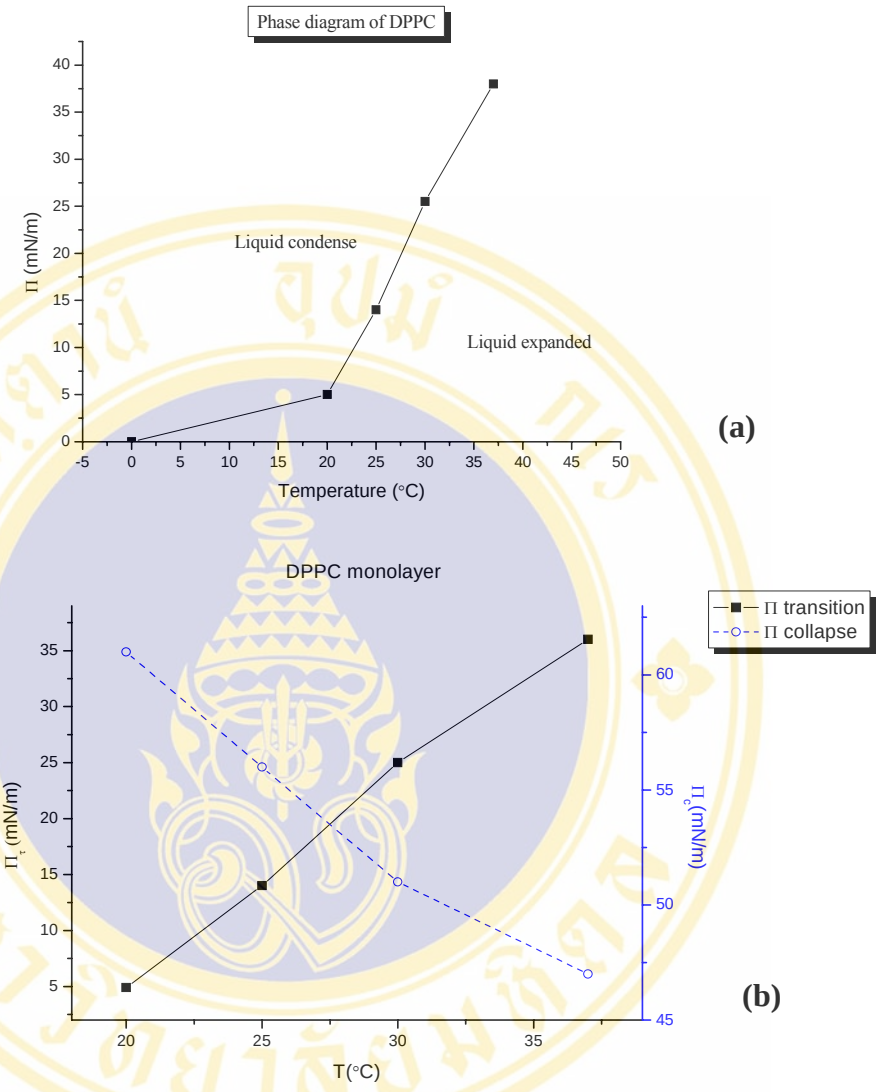


Figure 4.3 Packing state of DPPC monolayer

(a) Phase diagram of DPPC

(b) Surface pressure at phase transition and collapse point of DPPC

The morphology of lipid monolayer has been investigated on various lipid monolayers. Marité Cárdenas et al [25] showed the Brewster angle microscopy of distearoylphosphatidylcholine (DSPC) monolayer at the different state of packing (Figure 4.4). In gaseous state, the lipid monolayer shows the island like domain and upon compression the domain grows into the condense state.

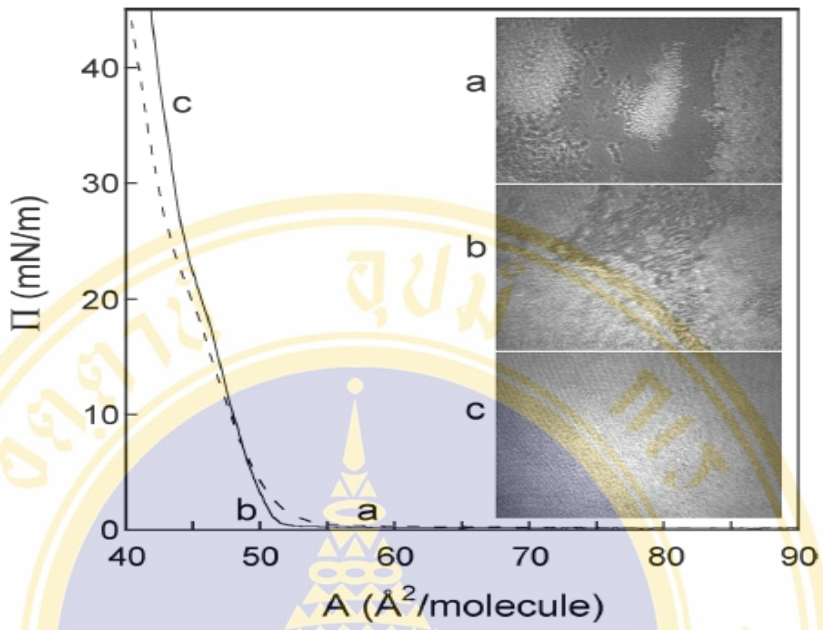


Figure 4.4. Surface pressure–area (*Π–A*) isotherm for DSPC monolayers on 2 mM NaBr aqueous solution (a) gaseous state (b) LE state (c) LC state

Kazuki Hodaa et al [26] showed the fluorescence micrographs of Chol, DPPC and dimyristoylphosphoethanolamin(DMPE) monolayer on the 0.15 M NaCl subphase. Chol (Figure 4.5 (a)) showed large domains at zero surface pressure, where the dark regions represent the gaseous phase. The bright and dark contrasts were observed for pure DPPC (Figure 4.5 (b)) which correspond to coexist between the LE and LC domains. The proportion of dark area, LC phase, increases at the expense of the bright area, LE phase, as the surface pressure is increased. For pure DMPE (Figure 4.5 (c)), their domains were observed at the coexistence state of both LE phase and LC phases. With increasing surface pressure from 15 to 18 mNm^{-1}, the percentage of LC phase in each image increased and complete LC domain.

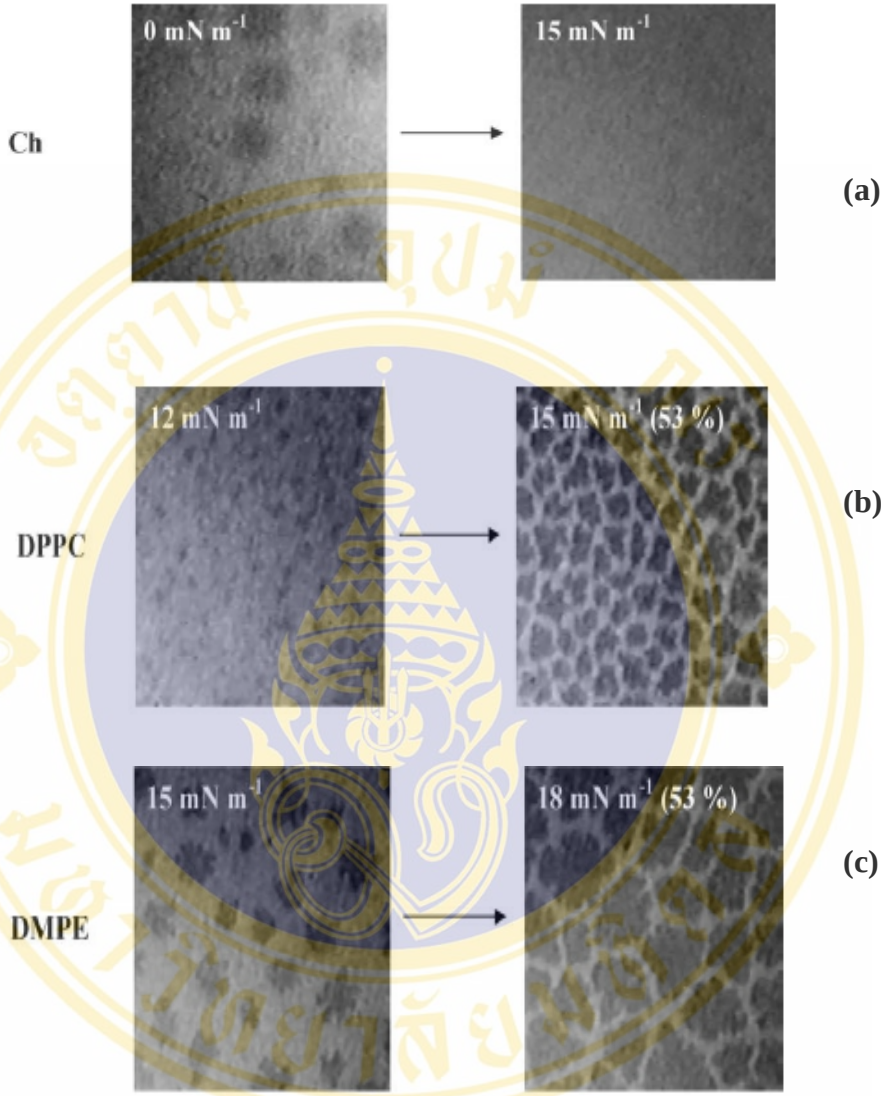


Figure 4.5 Fluorescence micrograph of
(a) Cholesterol (b) DPPC (c) DMPE

Micheala Ross et al[27] was observed DPPC layer on water interface at 20 °C by fluorescence microscopy results in Figure 4.6. No domain formation is observed at the beginning. After the surface pressure is increased to 4 mN/m where the coexistence of liquid expanded and liquid condense the micrograph appears as a small domain. This indicates the LC phase is forming.

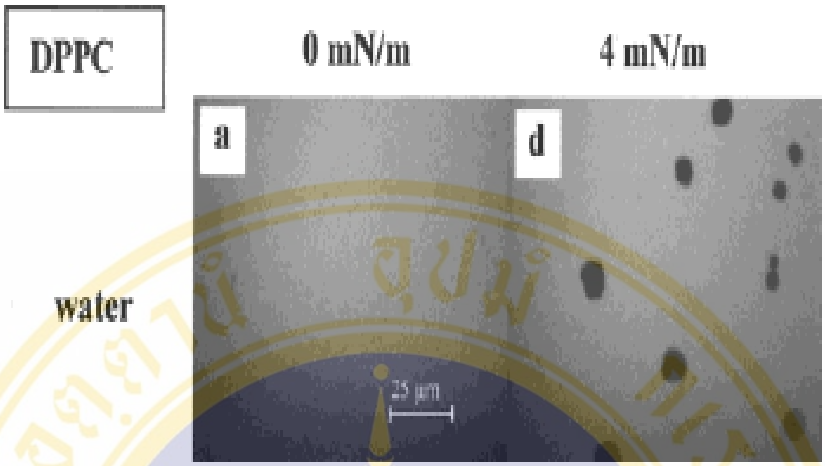


Figure 4.6 Brewster angle micrograph of DPPC at 0 mN/m (Left) and 4 mN/m(right) on water surface

Compressibility modulus

Compressibility modulus[1] was calculated from the compression isotherm of monolayers. For DOPE and Chol, the *K*-Mma curves (Figure 4.7 (a) and (b)) show no phase transition in both lipids. The elasticity is ranging from 5-110 mN/m and 5-450 mN/m for DOPE and Chol, respectively. DOPE in LE state is more elastic than Chol judging from the *K* value. The lower *K* value means the higher elasticity. When the temperature is increased, no change in *K* value is observed in DOPE film.

A lower in rigidity is observed in case of Chol monolayer if the temperature is increased.

[1] Detailed in chapter II

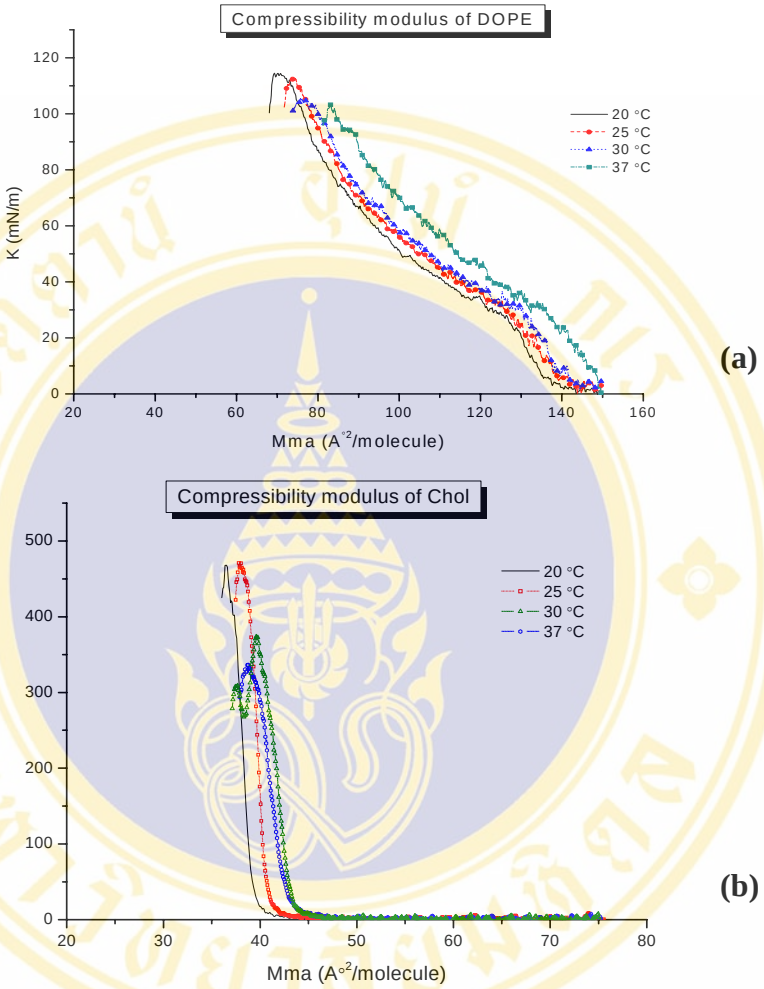


Figure 4.7 K-Mma plot of lipid monolayers at various temperature
(a) DOPE (b) Chol

In case of DPPC, phase transition causes in the dramatically decreasing in *K* value (Figure 4.7 (c)) around the phase transition and starts increasing after turning into LC phase. The *K* value of DPPC in LE state is comparable to DOPE with *K* value ranging from 5-100 mN/m. For a LC, film becomes more elastic and possesses a lower rigidity as the temperature is increased. At 37 °C, no LC is observed which indicates from the lower in *K* value. This *K* value is in agreement with the compressibility modulus of aliphatic fatty acid from literature.[18]

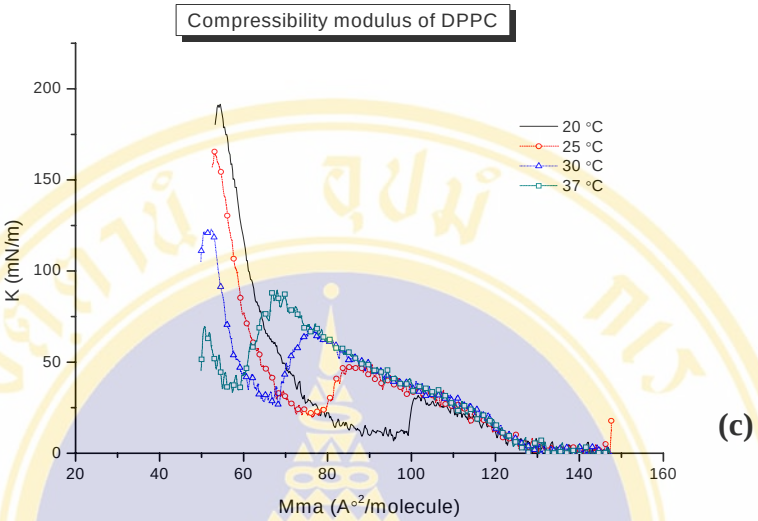


Figure 4.8 K-Mma plot (cont.) (c) DPPC

Table 4.2 Compressibility modulus of lipid film[2]

T (°C)	K (mN/m) π = 0-25		
	DPPC	DOPE	Chol
20	124.7	84.7	468.8
25	72.7	86.9	446.5
30	44.4	85.6	326.2
37	73.4	89.5	326.2

4.2 Lipid monolayer stability

The stability of the lipid monolayer is investigated in this part. The monolayer of all lipids is kept at constant surface pressure in order to observe the area reduction as a function of time. The stability tests of DPPC and DOPE at 20 °C are shown in

[2] See graph in Appendix A1

Figure 4.9. In general, when the surface pressure is kept constant at the desired value, the molecules undergo a slight adjustment in the packing, known as the relaxation. The rate of area reduction is increased by increasing the surface pressure. The results are shown in Figure 4.9 (a) for DPPC and (b) for DOPE. The percent of area reduction increase from 8, 19 and 25% in DPPC and 2.5, 2.2 and 5.7% in DOPE at surface pressure 10, 15 and 20 mN/m, respectively.

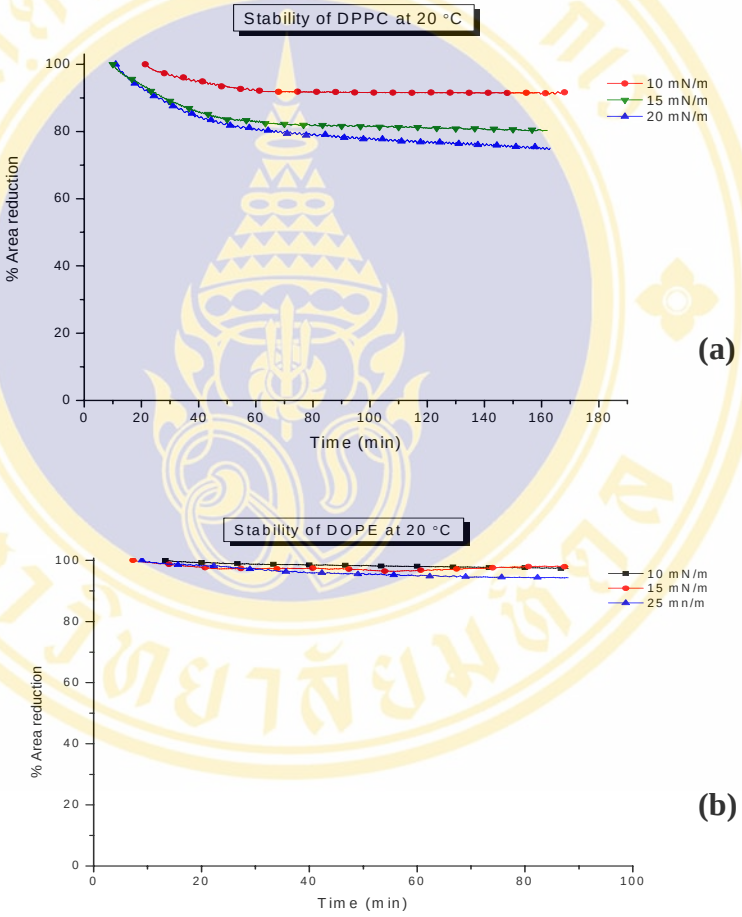


Figure 4.9 Monolayer stability at 20 °C
(a) DPPC (b) DOPE

4.2 Protein adsorption at the clean air-buffer interface

π-t observation

Adsorption of protein at the interface as function of time is carried out at two different temperatures; 20 and 30 °C. Protein is injected into buffer subphase then monitoring the surface pressure changed due to protein adsorption**.** The result is shown in Figure 3. The surface pressure started increasing after the protein is injected about 80 s and 600 s at 20 and 30 °C, respectively. Macritchie [28] proposed that the induction period represent in protein adsorption ending when saturated adsorbed at the interface and the surface pressure that continuous increased over prolong period was due to the changed in proteins conformation. Ybert and di Megio hypothesized [29] that through the induction time, the protein is adopted in a surface gaseous state, and hence the surface pressure change only slightly with surface coverage. Many reports supported this hypothesis [30, 31]. At lower temperature the protein changes its conformation with less induction time than at higher temperature and during this kinetics composes of protein adsorption along with protein rearrangement which may be due to the change in protein free energy. In solution, protein has one free energy after penetrated into interface the protein tend to change its free energy due to the change in the interaction with the surrounding. At the air/water interface, hydrophobic interactions are believed to be a dominate factor for the adsorption and this is clearly reflected in the strong correlation with the hydrophobic surface character of the protein. [21]

This adsorption kinetics can be interpreted by fitting with double exponential, equation (1). The presented in two *k* parameters possess the mix process in Cry4Ba protein interfacial mechanism.

$$\Delta\pi = k_1 + k_2 \exp(-k_3 t) + k_4 \exp(-k_5 t) \quad (1)$$

Where	$\Delta\pi$	is	the increasing surface pressure
	k_1, k_2, k_3, k_4 *and* k_5	is	the model coefficients

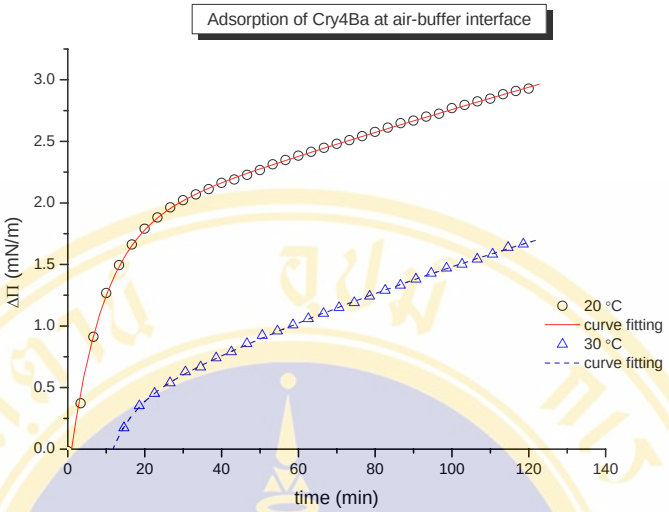


Figure 4.10 Adsorption of Cry4Ba at air-buffer interface

Table 4.3 Kinetics parameter

T (°C)	K_3 (min^{-1})	K_5(min^{-1})
20	0.00157	0.11511
30	0.0047	0.16907

The rate of adsorption process of protein molecules can be analyzed comparing to by the first-order equation

$$\ln\left(\frac{\pi_{ss}-\pi_t}{\pi_{ss}-\pi_0}\right)=-\frac{t}{\tau} \qquad (2)$$

Where π_{ss} is the surface pressure at steady state condition

π_0 is the surface pressure at time t=0

π_t is the surface pressure at any time t

τ is the relaxation time (= k^{-1})

k is the rate constant

From the previous work [32, 33], two relaxation time τ_1 and τ_2 were observed in adsorption of a model proteins; lysozyme, β-casein and bovine serum albumin (BSA), at the air-water interface. τ_1 is related with the penetration of the protein molecules at the interface while τ_2 are related to the rearrangement to steady-state condition of the protein molecule after the adsorption ceased. This can be assumed that in Cry4Ba adsorption the presence of two *k* parameters represent the two relaxation process. Both processes can be fitted with the first-order equation. The rate constants, derived from the fitting parameter in table 4.3, indicates that both adsorption and rearrangement of protein fastening at lower temperature. Proteins are more active at the interface at lower temperature due to the different in the structure of protein. The effect of the protein rearrangement at air/water interface cannot be neglected.

Compression isotherm study

The compression isotherm of proteins was study as vary the temperature. Protein solution is injected into buffer subphase and left for 10 minutes before start the compression at the barrier speed of 10 mm/min. This is done in order to allow the penetration of the protein into the least condensed phase. For the compression isotherm with time left about 5 min, no Π-A curve can be detected. For increasing temperature of 20 °C, 25 °C and 30 °C the molecular area of protein is 4361, 3988 and 3468 $A^{\circ 2}$/molecule [9], respectively.

From this result, in Figure 4.11 (a), the area occupied per each protein molecule is still lower than an estimated size, estimated from its crystal structure. This indicates that only some parts of the protein is involved in the insertion into lipid monolayer at the gas/water interface [17]. Generally, protein tends to remove their hydrophobic part by from solution. The differences in molecular area of protein observed by compression isotherm at vary temperature are consisted with the hydrophobic part of Cry4Ba; 532 $A^{\circ 2}$covering α_5 and α_7, loop α_7 -β_1 and part of loop β_{11} -β_{12} and β_{12} -β_{13}. This may imply that the protein occupies the unique conformational in solution. The hysteresis of Cry4Ba was also study at 20 °C and the

result shows that this protein viscosity in compression-expansion process (Figure 4.11 (b)), this infers that protein is not changed its conformation under stress.

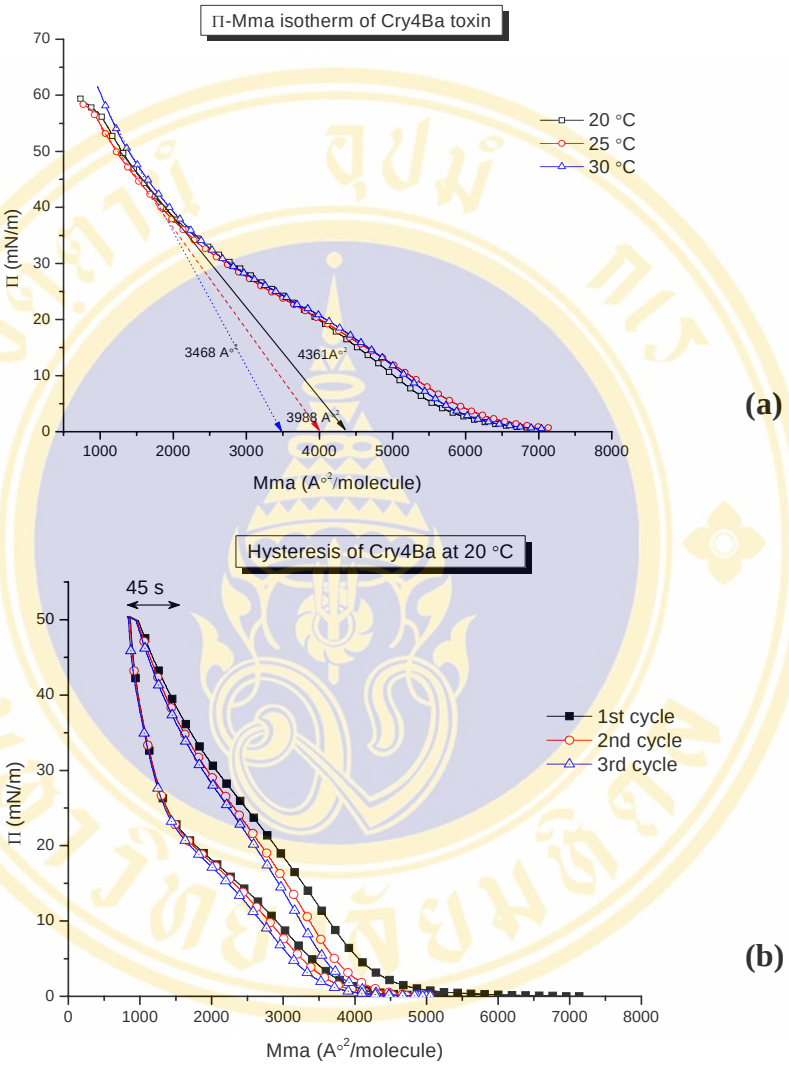
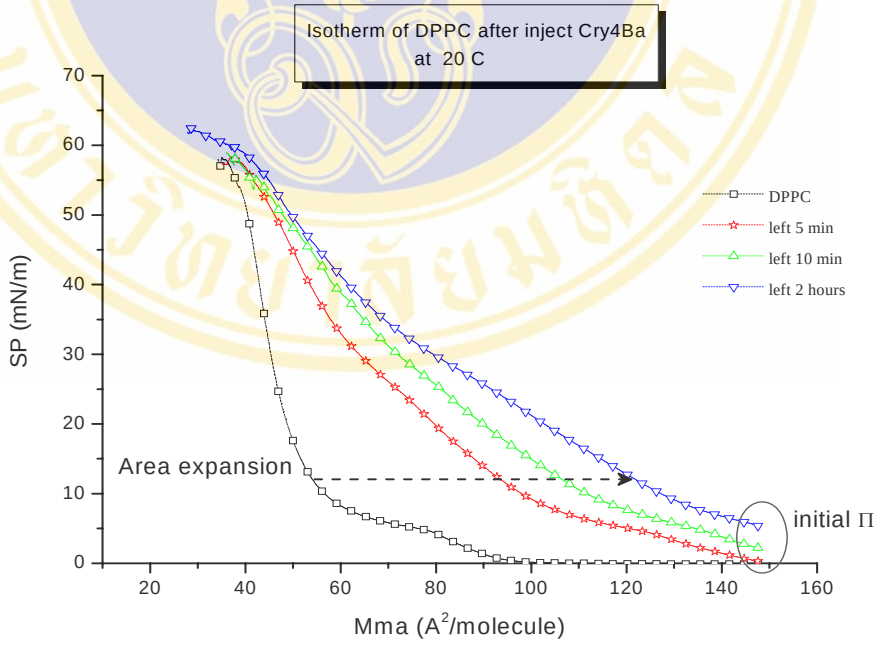


Figure 4.11 Adsorbing protein compression isotherms
(a) at various temperature (b) hysteresis at 20 °C

CHAPTER V
PROTEIN PENETRATION STUDY

5.1 Protein adsorption in the presence of lipid at ∏=0

In this part, we will consider a binary component system. The compression isotherm of three composites lipid film: DOPE, DPPC and Chol, with proteins are studied at the air-water interface. Lipid solution is first spread on the water subphase, carbonate buffer, and followed by injection of 4.35 nM proteins underneath lipid layer. The film was left at various elapse times before the compression in order to observe the effect of protein insertion at zero surface pressure. Figure 5.1 shows Π-Mma isotherm* of mixed DPPC/Cry4Ba at 20 and 30 °C and Chol/Cry4Ba at 20 °C.



(a)

Figure 5.1 Π-Mma isotherm of mix component
(a) DPPC/Cry4Ba 20 °C

* Graph in Appendix C1

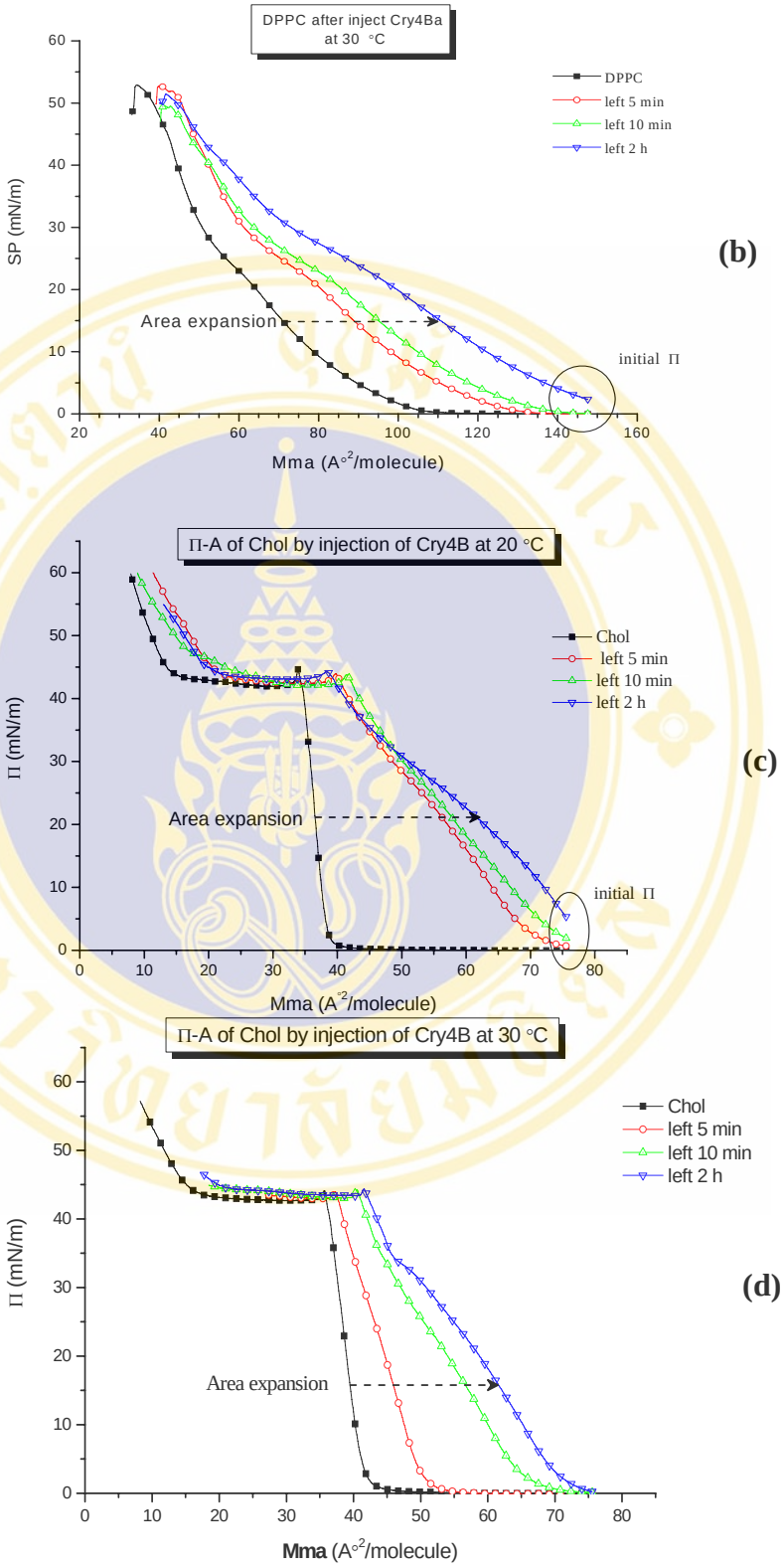
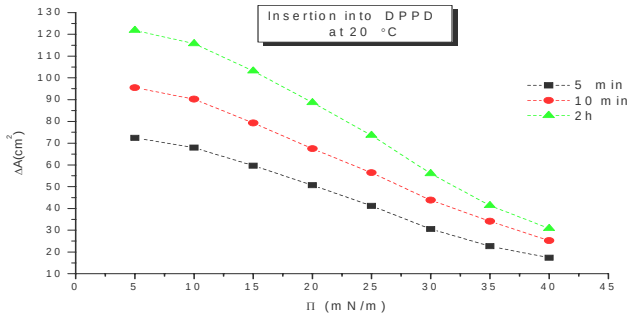


Figure 5.1 Π-Mma isotherm of mix component (cont.)
(b) DPPC/Cry4Ba 30 °C
(c) Chol/Cry4Ba 20 °C
(d) Chol/Cry4Ba 30 °C

From BAM at zero surface pressure, lipid monolayer is in the co-exist phase between the island of the LE and the disorder phase. In this mix system, Cry4Ba is injected underneath the subphase and was allowed to adsorb to the air-water interface. The protein is believed to undergo the insertion to the monolayer; however, the exact location of the insertion, whether in LE region or disorder region, is not known. It is believed to be the area which is covered with lipid layer in a gaseous state; island like layer*. The area expansion by protein penetration during the compression of lipid layer was observed when the composite films are compressed. In the short elapse time, all lipid monolayer showed only small area expansion. When the elapse time was increased, an increase in the initial surface pressure took place. This was observed only at 20 and 25 °C for DPPC and DOPE, and at all temperature for Chol. The presence of initial surface pressure means that lipid molecules are pushed closer together by the protein molecules at the air-water interface. The protein unfolding may play some role in this observed phenomenon. This suggested that the protein molecules diffuses from solution to the surface with some induction time and undergoes the structural rearrangement at the air-water interface. This is in agreement with Macritchie's hypothesis [28].

The protein insertion is characterized by the plot of area increased (ΔA) at various elapse time and the result is shown in Figure 5.2. When the elapse time is increased, isotherm shows higher ΔA due to an increased in the amount of protein at the interface. At lower temperature, protein takes more time to diffuse and reach the interface.



(a)

Figure 5.2 ΔA at various surface pressure

(a) DPPC 20 °C

* See picture in Chapter IV

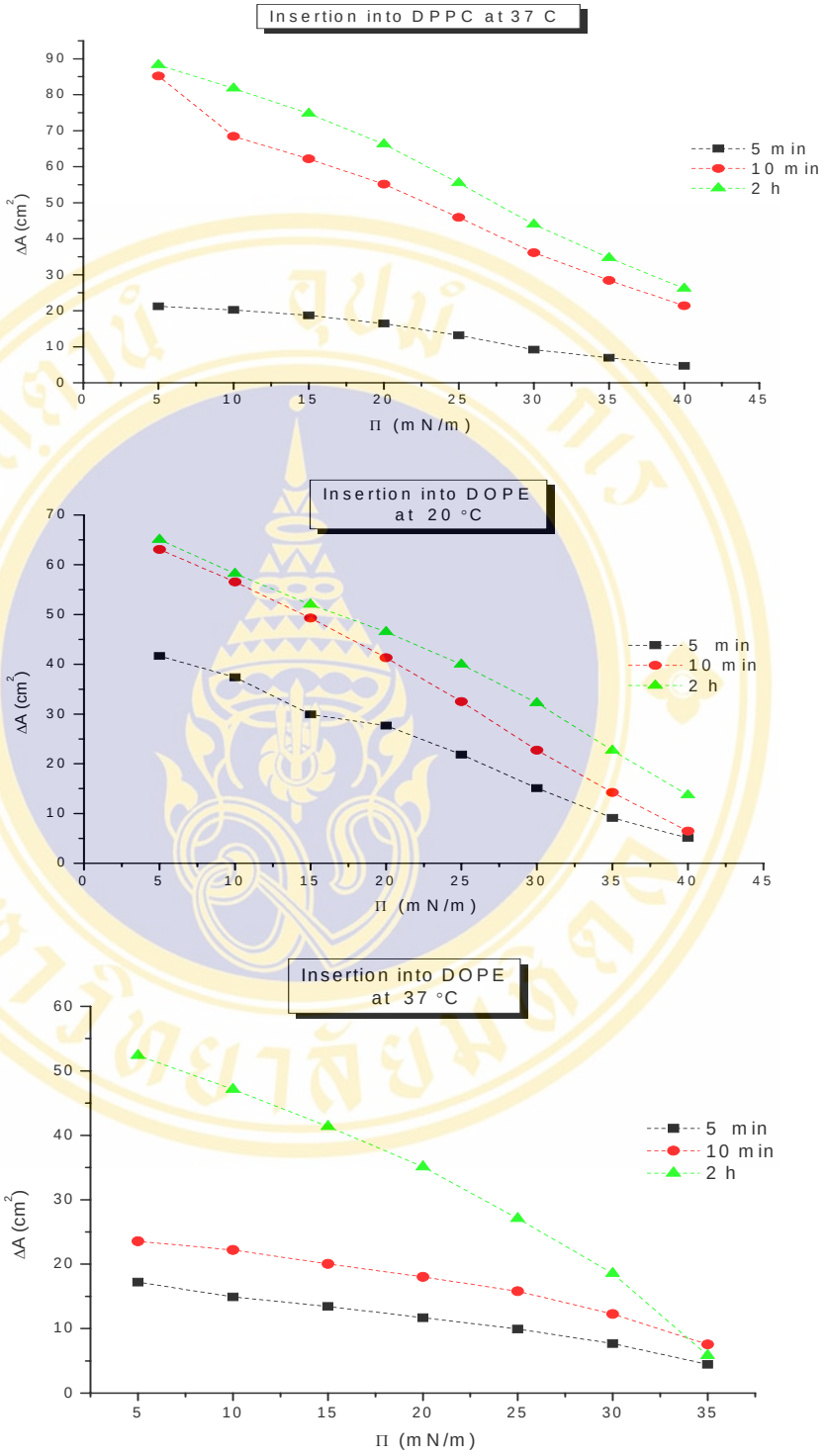


Figure 5.2 ΔA at various surface pressure

(b) DPPC 37 °C (c) DOPE 20 °C

(d) DOPE 37 °C

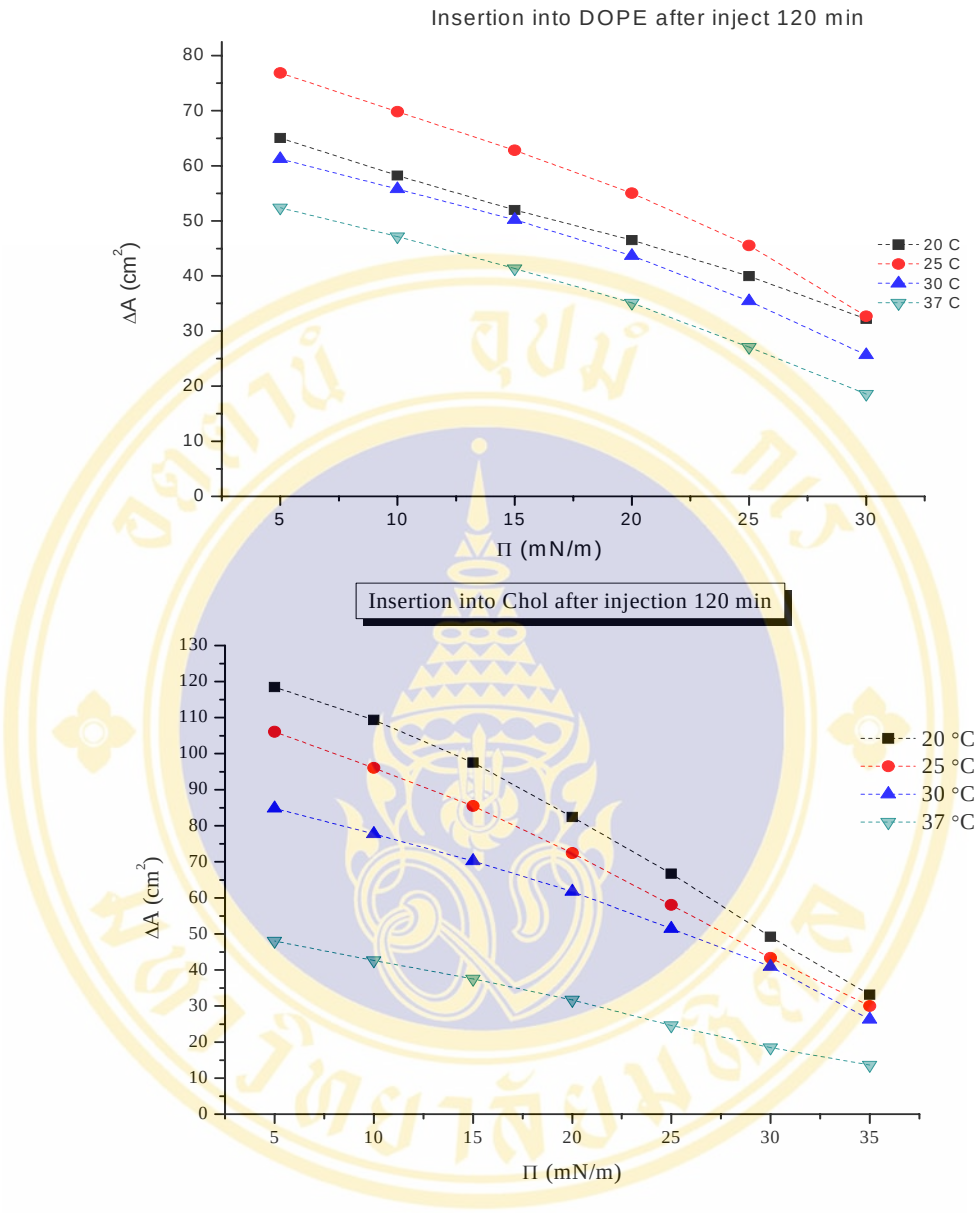


Figure 5.3 ΔA-Π as a function of temperature

(a) DOPE with 120 minutes time left

(b) Chol with 120 minutes time left

At the longest elapse time, DPPC and Chol show the highest area increase due to the insertion at a low temperature. As the temperature is increased, the insertion is decreased, except for the DOPE where the largest ΔA is observed at 25 °C (Figure 5.3).

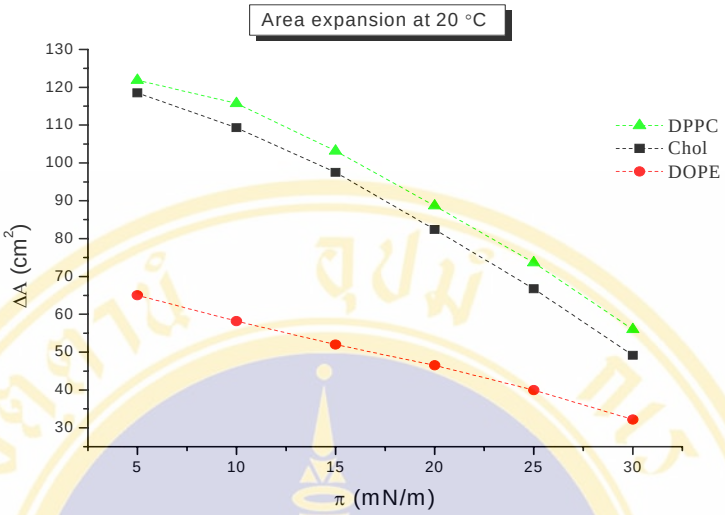


Figure 5.4 area expansion at 20 °C after inject 2 hours

At any temperature, DPPC layer has larger ΔA than Chol and DOPE. Figure 5.4 shows ΔA as a function of the surface pressure at 20 °C. It should be noted that the both DPPC and Chol are in the LC phase while DOPE is in the LE phase. By considering the compressibility modulus, the change in ΔA can be readily explained which will be mentioned later.

The compressibility modulus is calculated for lipid-protein monolayer. The value is calculated by considering the change in the surface pressure with respect to the change in molecular area as shown in equation (2). This value will be designated as *K* and it is used as the parameter to characterize the elasticity of the monolayer. The higher *K* value means the lower film elasticity. Adsorption of protein to the lipid monolayer alters the elasticity of the lipid film. In general, an incorporation of the protein into the lipid monolayer increases the elasticity of composite films for all lipids. This is due to the proteins which possesses the viscoelastic property. The lipid-protein monolayer and the pure protein monolayer show a similar respond in their modulus. The elasticity is introduced to the monolayer by incorporation of the protein which is a polymer. This property can be deduced from the compression isotherm of the adsorption at π=0. The steep slope of isotherm corresponds to the higher *K* value. A lower in compressibility modulus indicates that (a) the proteins insertion lowers the elasticity by its own interfacial properties or (b) the interactions

between proteins and lipid are the dominating factor governing the property of the mix monolayer. As shown in Figure 5.5, the compressibility modulus of mix lipid-protein monolayer is rather constant along the compression until the collapse point. This suggests that the proteins did not squeeze out during the compression. The raise in the collapsed pressure is observed in the mix lipid-protein monolayer.

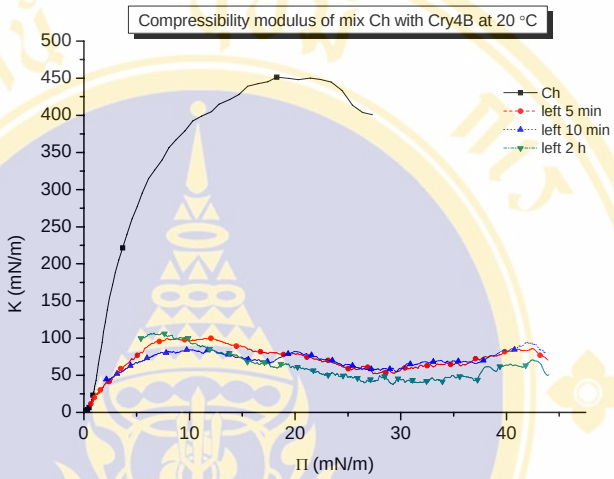


Figure 5.5 The compressibility modulus of
(a) mixed Chol-Cry4Ba at 20 °C

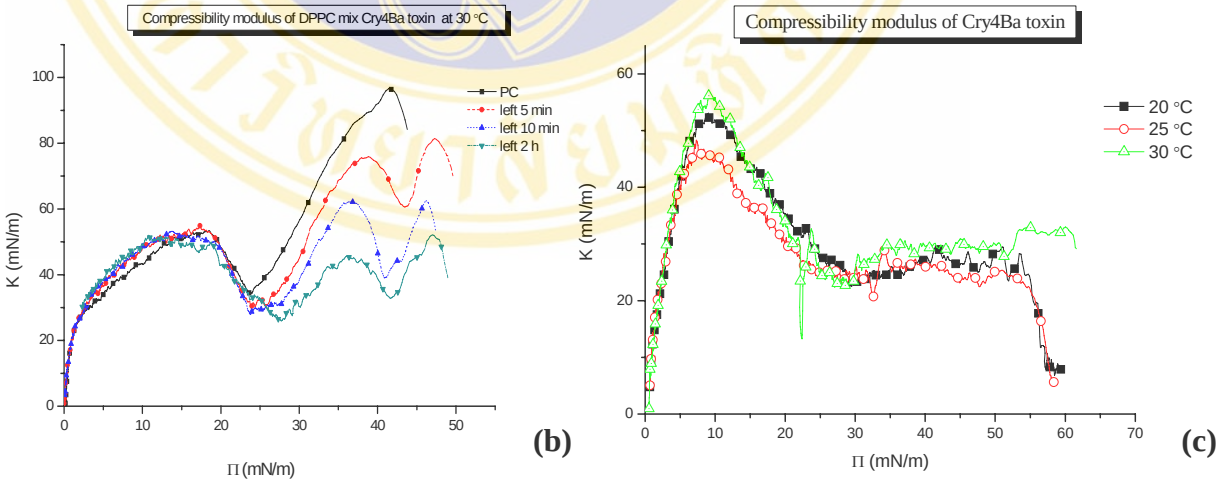


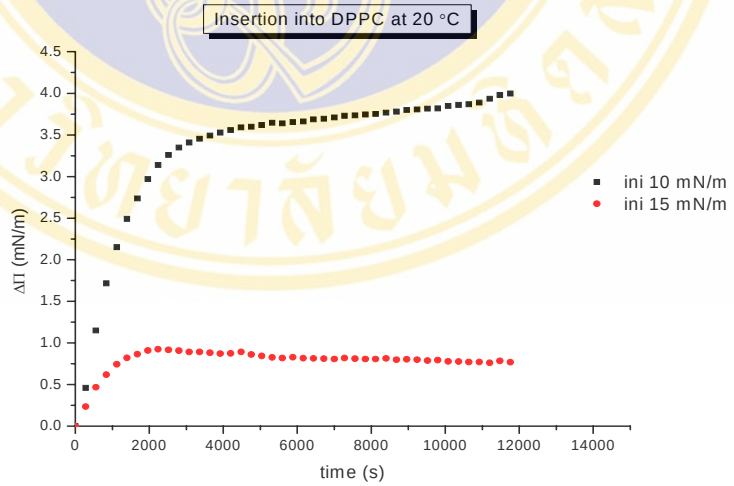
Figure 5.5 (cont.) The compressibility modulus of
(b) mixed DPPC-Cry4Ba at 30 °C
(c) Cry4Ba at 20,25 and 30 °C

The monolayer of Chol and mix Chol-protein is differ from the case of DOPE and DPPC. This may be due to the interaction between the Chol and the proteins.

In this section, the effects of the molecular organization of the lipid monolayer on the protein insertion are considered. In the previous section, the insertion of the protein into the lipid monolayer in its expand phase was considered. It was found that only some part of the protein was involved in the insertion into the lipid monolayer. The question is then arisen regarding an effect of the molecular packing, LC and LE, on the protein insertion. The comparison is made between DOPE, represents the LE phase, and DPPC, represents the LC phase. It was found that the protein can penetrate into the LC more readily than in the LE phase.

5.2 Protein adsorption in the presence of lipid with constant area assay

In this experiment, three types of lipid are use as the model membrane. Lipid monolayer is held at constant molecular area, at a given initial surface pressure (Π_i), then the surface pressure changed ($\Delta\pi$) due to protein insertion into the monolayer is monitored as a function of time. Protein is injected underneath the monolayer while the area of the trough is held constant. Any insertion of the protein into the monolayer causes an increased of the observed surface pressure due to a reduction of the area occupied per molecule at the interface. Two major effects, the molecular packing and the lipid-protein interaction, will be investigated in this section. For the effect of the molecular packing on the protein insertion, DPPC will be used as the example because the molecular packing can be readily controlled. DOPE and Chol, where the lipids are in LE and LC, will be used as the example to investigate the effect of lipid-protein interaction. The effect of temperature at 20, 25, 30 °C on the protein insertions are performed at the surface pressure of 10 and 15 mN/m. The $\Delta\pi$-t curve of the insertion of Cry4Ba into DPPC, DOPE and Chol at 20 °C is shown in Figure 5.7.



(a)

Figure 5.6 π-t curve of Cry4Ba insertion into lipid layer of (a) DPPC at 20 °C

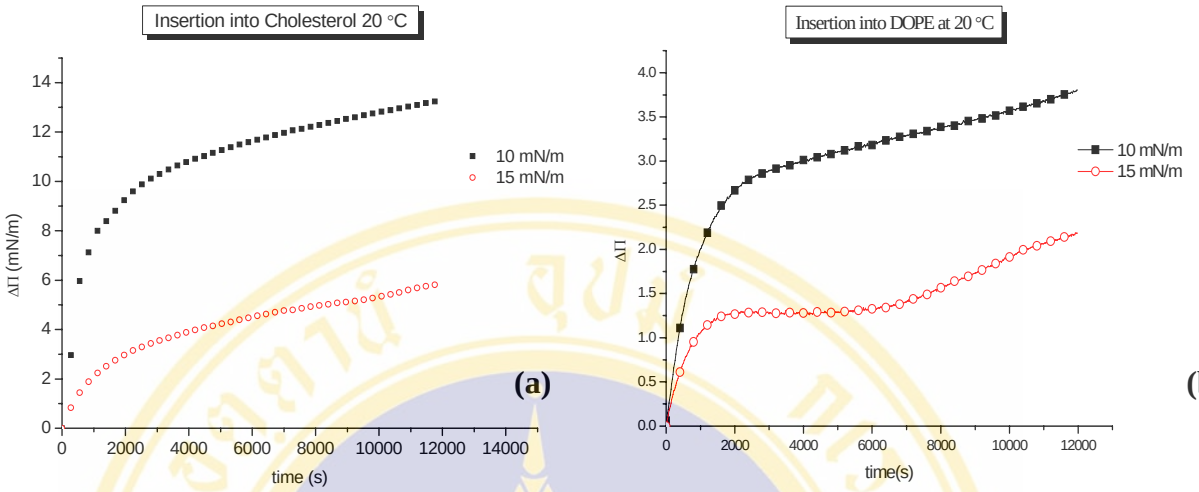


Figure 5.6(cont.) π-t curve of Cry4Ba insertion into lipid layer of (b) Chol at 20 °C (c) DOPE at 20 °C

The surface pressure is found to be increased after the protein is injected into the subphase. The amount of Δπ depend on the initial surface pressure. The changed in the surface pressure at initial surface pressure of 10 mN/m and 15 mN/m are investigated. The states of the molecular packing of DPPC molecules under each condition are tabulated in table 5.1. The results show that all lipid layers exhibit lowering of Δπ by increasing the initial surface pressure.

Table 5.1 Packing state of DPPC

DPPC	π = 10 mN/m	π = 15 mN/m
20 °C	LE-LC*	LC
25 °C	LE	*LE-LC
30 °C	LE	LE

* Approach to this state

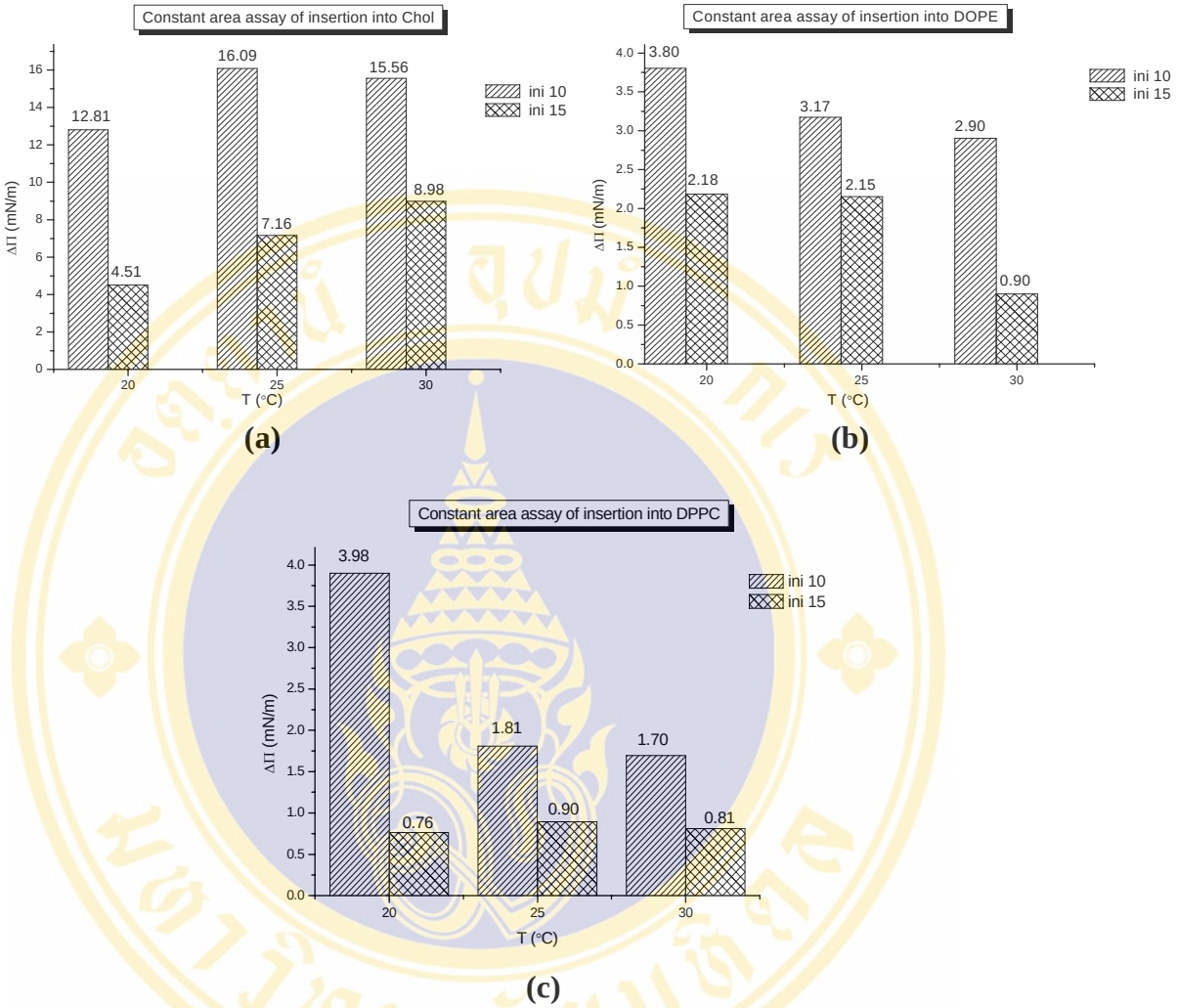


Figure 5.7 ΔA of each insertion as a function of temperature
(a) Insertion into Chol layer
(b) Insertion into DOPE layer
(c) Insertion into DPPC layer

Temperature seems to have an effect on the insertion for DOPE and DPPC where the reduction in $\Delta\pi$ is observed as a function of temperature. The effect is more pronounce at the lower surface pressure, 10 mN/m. Base on the initial thought, the observed result may be an effect of the difference in the molecular packing. DOPE only has one state of molecular packing which is LE phase. The system consistently shows a reduction in the $\Delta\pi$ as the temperature is increased. In DPPC at 20°C and surface pressure of 10 mN/m, even though the monolayer is in co-exist phase of LE-

LC or the molecule is in a denser pack than in LE phase, the protein still have a higher insertion than at the higher temperature, 25 and 30 °C. This suggests that the state of molecular packing may not have any effect on the protein insertion. It is possible that the insertion is governed by the lipid-protein interaction. The temperature does not seem to have any effect on the insertion at the surface pressure of 15 mN/m where the total amount of insertion is almost constant.

The protein insertion into Chol has a different mechanism from DOPE and DPPC. Chol only has the LC phase. The insertion shows a surface pressure dependent where the insertion is more favorable at the lower initial surface pressure than at the higher surface pressure. The increased in $\Delta\pi$ is higher in Chol than the DPPC and DOPE. It is reasonable to conclude that the protein insertion may be different from the DOPE and DPPC due to the different in molecular structure. This further strengthens the assumption that the lipid-protein interaction may control the degree insertion.

If one assume that the protein possesses the temperature dependent conformational change, this would lead to a different degree in lipid-protein interaction as a function of temperature. As a result, the DOPE and DPPC show the similar trend in the insertion because of their similarity in the molecular structure. The Chol behaves differently due to different in the chemistry of the molecule. To clarify this observation, the temperature induced protein conformational change experiment is carried out by using fluorescence spectroscopy.

The emission intensity of the tryptophan residues (Figure 5.8) in Cry4Ba toxin is showed in Figure 5.10. It is well known that the emission of tryptophan residues is sensitive to the change of the protein conformation. The fluorescence spectrum of the tryptophan residues are excited by the light at 280 nm and the fluorescence spectrum are monitored as the function of temperature from 7 °C to 75 °C. The proteins are equilibrated for 15 min at each temperature before the emission spectrum is taken to ensure the complete change in its structure. Figure 5.9 (inset) shows the emission intensity at 340 nm of the tryptophan residues of Crys4Ba as function of temperature while inset shows the full emission spectrum. The emission intensity is increased as the temperature is increased from 7 °C to 20 °C. A continuous reduction in the fluorescence intensity is observed from 25 °C to 75 °C. The change in the emission

intensity is related directly to the change in the protein conformation as a function of temperature. J. Fitter et al[34] found a change in the secondary elements of two enzymes; *Bacillus amyloliquefaciens* (BAA) and *Bacillus licheniformis* (BLA) caused by thermal induction unfolding. The Tryptophan and Tyrosine residues were excited in this fluorescence spectroscopy measurement. Yu et al [35] also reported the thermal denatural of SARS coronavirus (S protein) by observing in the decreasing of fluorescence emission intensity of Tryptophan residues. The thermal induced unfolding/denature of various proteins has been studied.[36-39]

The Crys4Ba shows a reversible conformational change as a function of temperature. This evidenced by the black line in figure 5.9 which shows the fluorescence intensity from the cooling experiment. With increasing and decreasing temperature, Crys4Ba undergoes the reversible conformational change as shown in Figure 5.9. This can be interpreted as the rearrange in its structure by folding-unfolding of the residue.

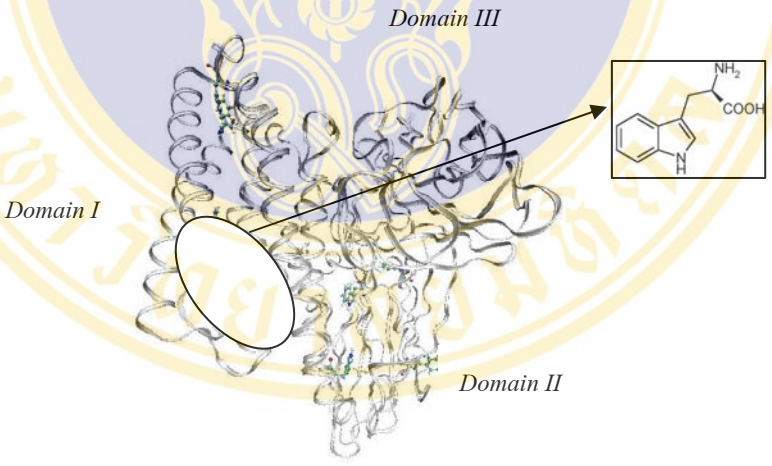


Figure 5.8 Tryptophan residues in Cry4Ba Structure

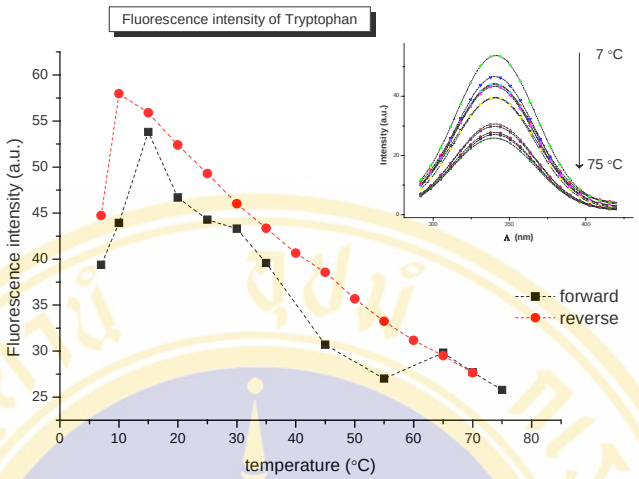


Figure 5.9 Fluorescence intensity of Tryptophan

This suggests that the Cry4Ba changes its conformation as function of temperature. By changing the conformation, the insertion behavior of the Crys4Ba may change due to the different in the interaction between inserted part of Crys4Ba and the lipid monolayer. As a result, the insertion in different lipid monolayer gives rise to a different insertion characteristic. Based on the lipid-proteins interaction, the insertion of DOPE and DPPC show a similar result but differ from the Chol.

The sigmoidal curve of insertion kinetic (Figure 5.11) is fitted with the two exponential processes related to the first-order equation (1)* involving the insertion and conformational change.

* detail in Chapter IV

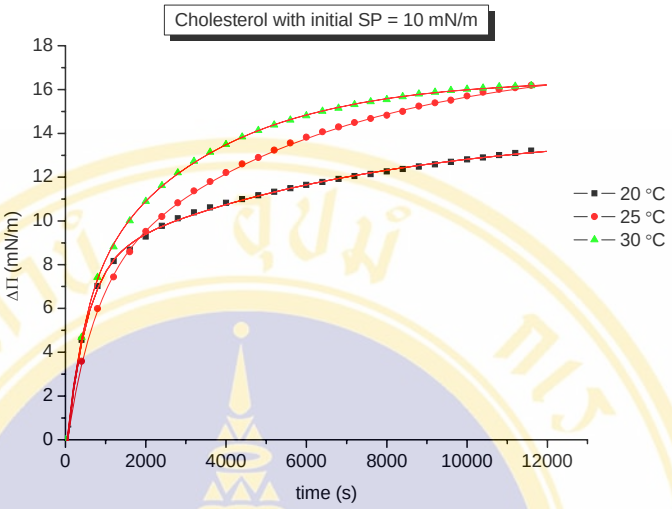


Figure 5.10 Curve fitting of insertion kinetics into Chol layer

The fitting parameter k_1 and k_2 are apparent the first-order rate constant for the individual process. The shorter time constant, *k1*, is involved with the insertion process while the slower, *k2*, is related with the structural rearrangement. [40-42] In all insertion dynamics were fit and show the parameter in table 5.1

Table 5.2.Fitting parameter of insertion kinetics curve

Type	T (°C)	k_1 (10^{-5}) (min^{-1})		k_2 (10^{-5}) (min^{-1})	
		π_i=10	π_i=15	π_i=10	π_i=15
DPPC	20	9.971	-	94.941	-
	25	12.46	5.71	243.28	5.72
	30	17.3	23.71	5.51	25.08
DOPE	20	6.51	-	143.9	-
	25	4.61	4.65	4.63	4.66
	30	4.35	16.97	4.35	17.29
Chol	20	14.8	3.21	207	93.29
	25	17.49	9.42	147.87	9.42
	30	30.17	26.92	214.68	384.15

The fitting parameters k_1 which represent the insertion rate of DPPC and DOPE of both 10 mN/m and 15 mN/m is showed to be the temperature dependence. At higher temperature, protein takes less induction time to insert into lipid layer corresponding to the higher k_1 value. Contrary to DOPE layer which posses the liquid expanded phase, at initial surface pressure of 10 mN/m shows the opposite trend that protein takes more induction time when temperature is increased. But at the 15 mN/m of DOPE occurs like in DPPC and Chol. The rate of insertion into LE phase is slower than LC phase. This indicating the lipid coverage surface is the driving force for Cry4Ba insertion which depends on the structure of lipid. From table 5.2, rate of rearrangement at 10 mN/m for all lipid show the difference of k_1 and k_2 rate constant. The high k_2 presents the faster of rearrangement at a lower molecule density or molecular packing. The closer packing of lipid molecules suppresses the movement of protein after insertion.

It was proposed that the protein does not squeeze out after incorporated into the lipid monolayer. The temperature was found to have an effect on the adsorption. The temperature hysteresis in constant area assay was performed on DPPC layer at 20 °C with $\prod_i$ =10 mN/m and shown in Figure 5.12. For a single component lipid layer, a rising in temperature leads to an increasing of the surface pressure due to the thermal induced disorder of the side chain. The adsorption of Cry4Ba protein showed the same activity as the pure lipid layer with a lower surface pressure. This may imply that the immediately changing temperature effect the molecular rearrangement on surface.

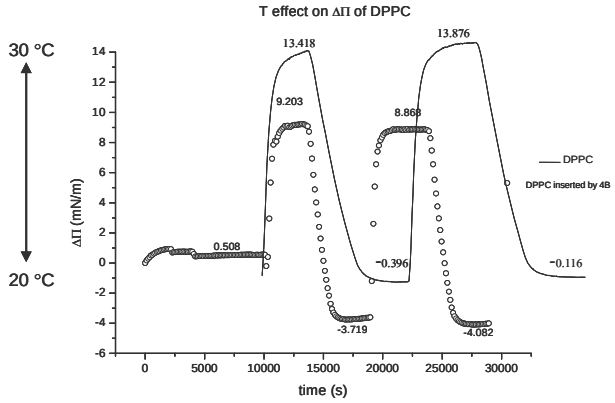


Figure 5.11 Temperature hysteresis of insertion kinetic curve

CHAPTER VI
CONCLUSION

The insertion of Cry4Ba proteins, which have the efficiency in killing the black flies and mosquitoes larvas, is reported. This work is aiming to elucidate the mechanism of this protein in destroying the larvae midgut by mimicking the insertion into the monolayer at the gas-water interface.

The study of the penetration of insecticidal Cry protein in the real membrane often involves with many experimental parameters. The extraction of the meaningful result requires extensive investigations. The fabrication of the monolayer at the gas-water interface allows a convenient way of studying the films property. Lipid monolayers of DPPC, DOPE and Chol which are the major component in insect membrane are used as the model membrane to help elucidating the effect of the molecular packing and temperature on the insertion of Cry4Ba protein. DOPE and Chol are a single phase lipid monolayer, liquid expanded (LE) and liquid condense (LC). The DPPC monolayer shows the coexistence of LE and LC phase depending on the temperature. As increasing the system temperature, DOPE and Chol film show a small area expansion while the DPPC film shows a thermal induced phase transition of the film at the gas-water interface. All of the lipid and the composite film of lipid-Cry4Ba monolayer are characterized in term of compressibility modulus. This parameter reflects the elasticity of lipid film and Chol is forming the most rigid film, lease elastic. DOPE and DPPC layer at the same phase of the molecular packing, they show a comparable elasticity modulus.

The property of the pure Cry4Ba film at the gas-water interface is investigated. The Cry4Ba is found to have a very high area occupied per molecule, around 4000 A^2/molecule. The film is subjected to the compression and expansion cycle, hysteresis.

The insertion of Cry4Ba protein shows that the adsorption into clean interface following the two stage adsorption process, i) adsorption process and ii) protein

rearrangement at the interface. At lower temperature, Cry 4Ba shows a slower rate of the adsorption at the interface than at the higher temperature. This indicates that the higher change in surface pressure will be observed at lower temperature.

The protein insertion into lipid monolayer at π=0 mN/m, shows that there is an observed induction time prior to the protein adsorption. The number of inserted Cry4Ba is increased as the induction time increased which is observed as an increased in the molecular area. This takes place along with change in the protein conformation. During the compression, the elasticity of mix protein-lipid film is higher than the pure lipid monolayer. During the compression both isotherm and compressibility modulus analysis indicates that the protein is incorporated into lipid monolayer.

The adsorption kinetics shows that the process is the multistage process. The insertion of protein leads to the change of initial surface pressure. The closer packing suppresses the protein insertion. The lipid-protein interaction is proposed to be the dominant factor controlling the insertion process. Chol shows the most favorable toward Cry4Ba insertion. DOPE and DPPC possess a similar structure and was used to demonstrate the effect of the molecular packing on the insertion. Increased in temperature is resulting in the reduction of the insertion in both DOPE and DPPC. This further strengthens the fact that the interaction between the inserted part of the protein and lipid is the major factor governing the protein insertion.

Cry4Ba shows the thermal induced conformational change. The different temperature induces the conformational change of the protein. As a result, the protein is exposed a different part toward the monolayer insertion depending upon its unfolding state. This is consistent with the fluorescence study of the Tryptophan residue in Cry4Ba where the emission intensity was found to depend on the temperature. This thermal induced unfolding is the reversible process.

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APPENDIX

APPENDIX A

COMPRESSIBILITY MODULUS

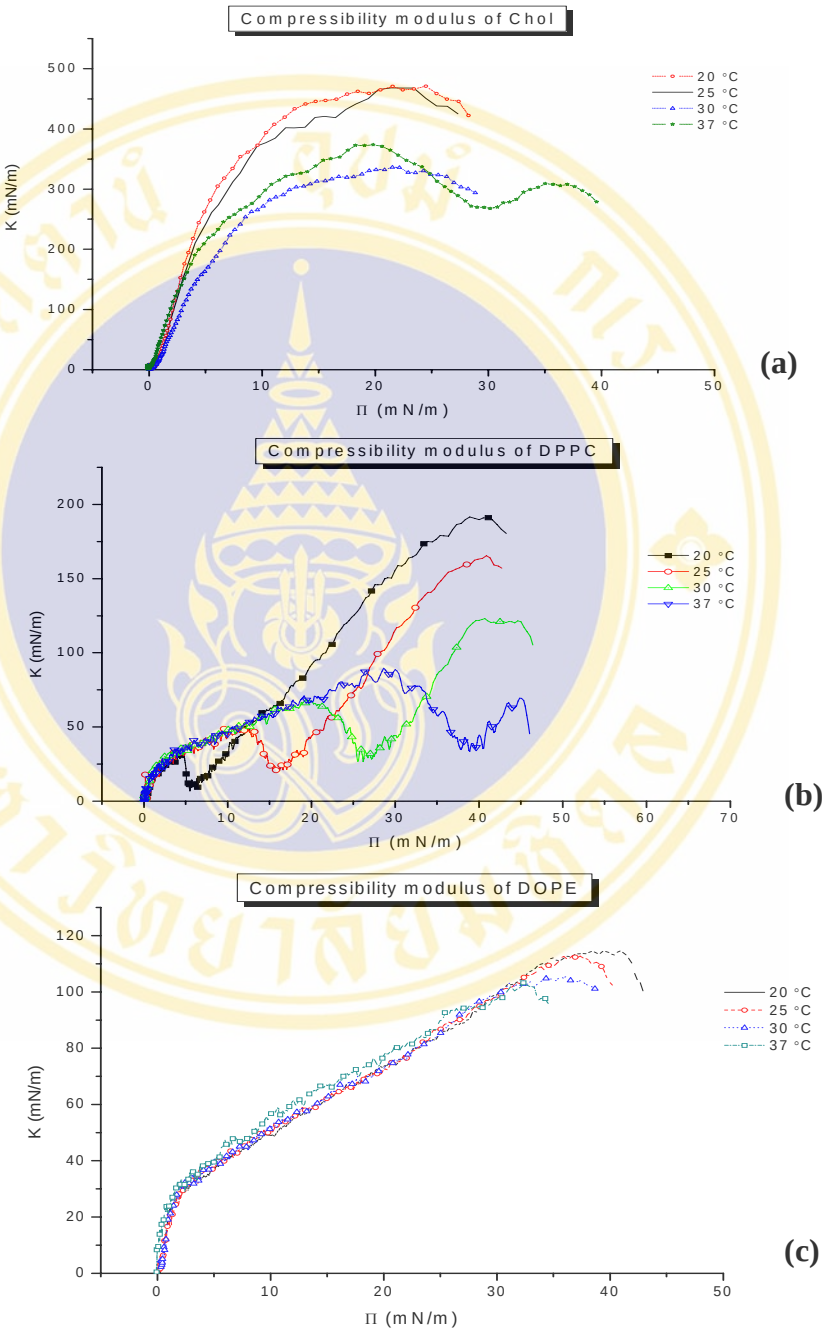

Figure A1. Compressibility modulus of single lipid component
(a) Chol (b) DPPC (c) DOPE layer

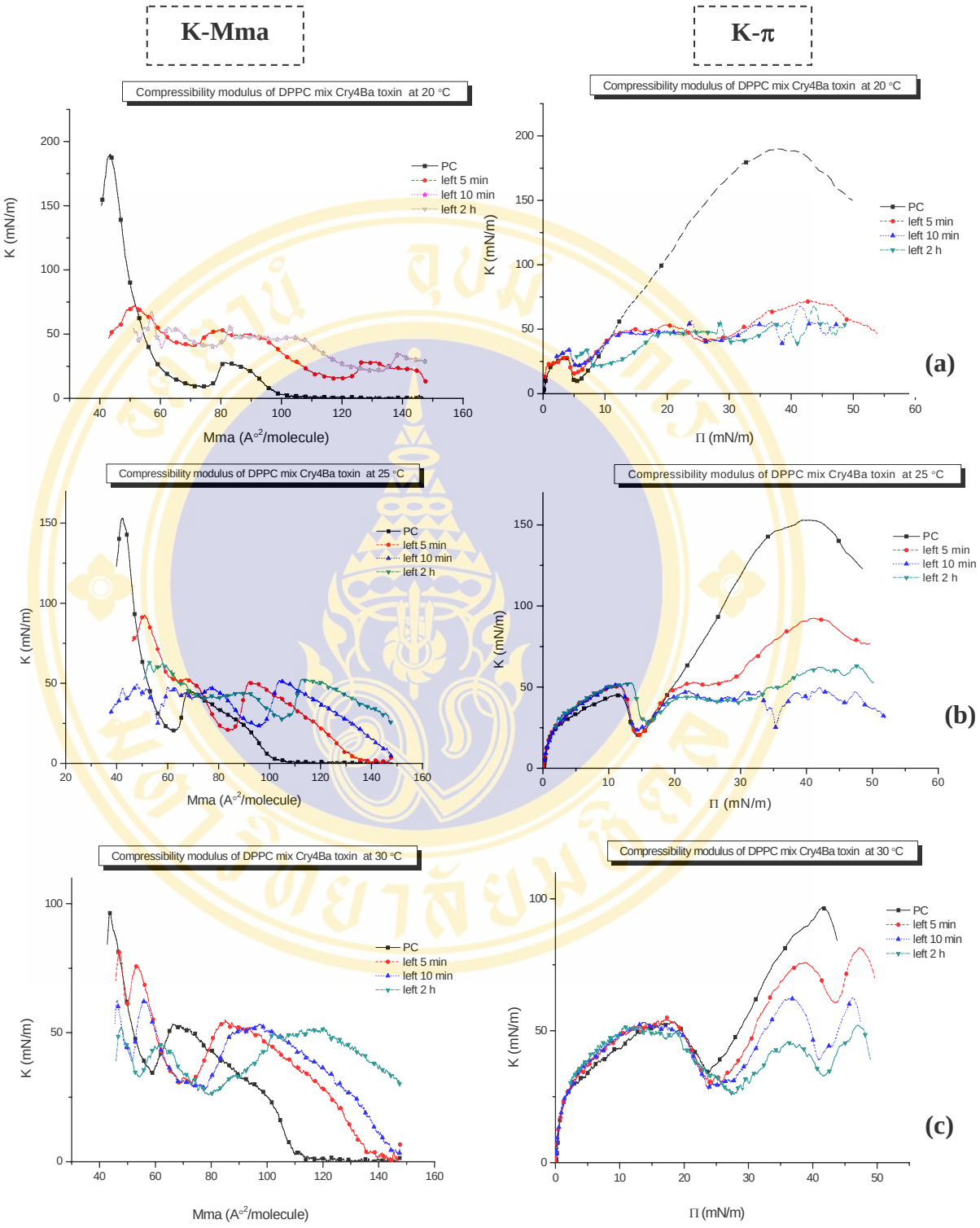

Figure A2 Compressibility of mix DPPC-Cry4Ba with various time left of temperature

(a) 20 °C (b) 25 °C (c) 30 °C

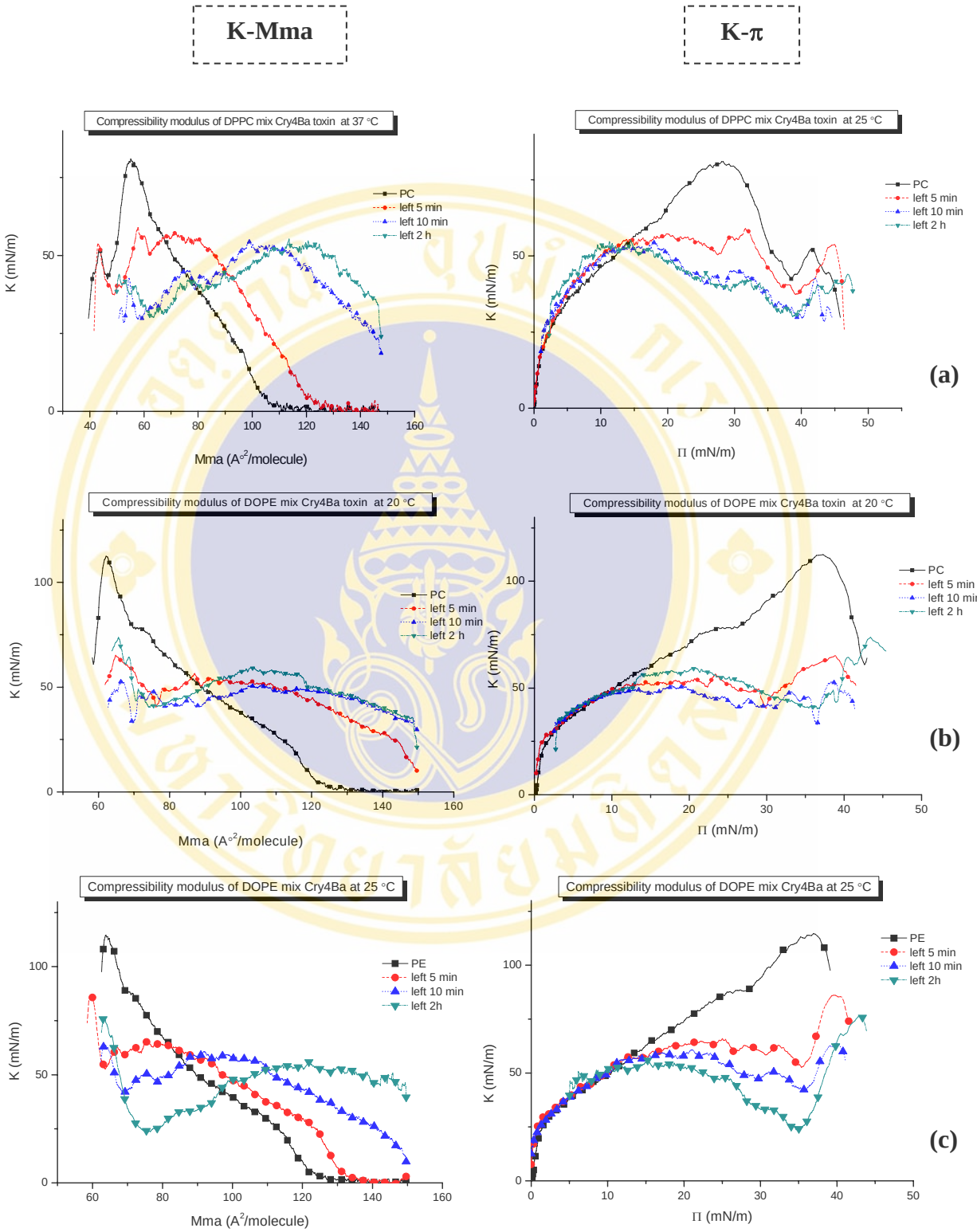

Figure A3 Compressibility modulus of mix
(a) DPPC-Cry4Ba at 37 °C (b) DOPE-Cry4Ba at 20 °C
(c) DOPE-Cry4Ba at 25 °C

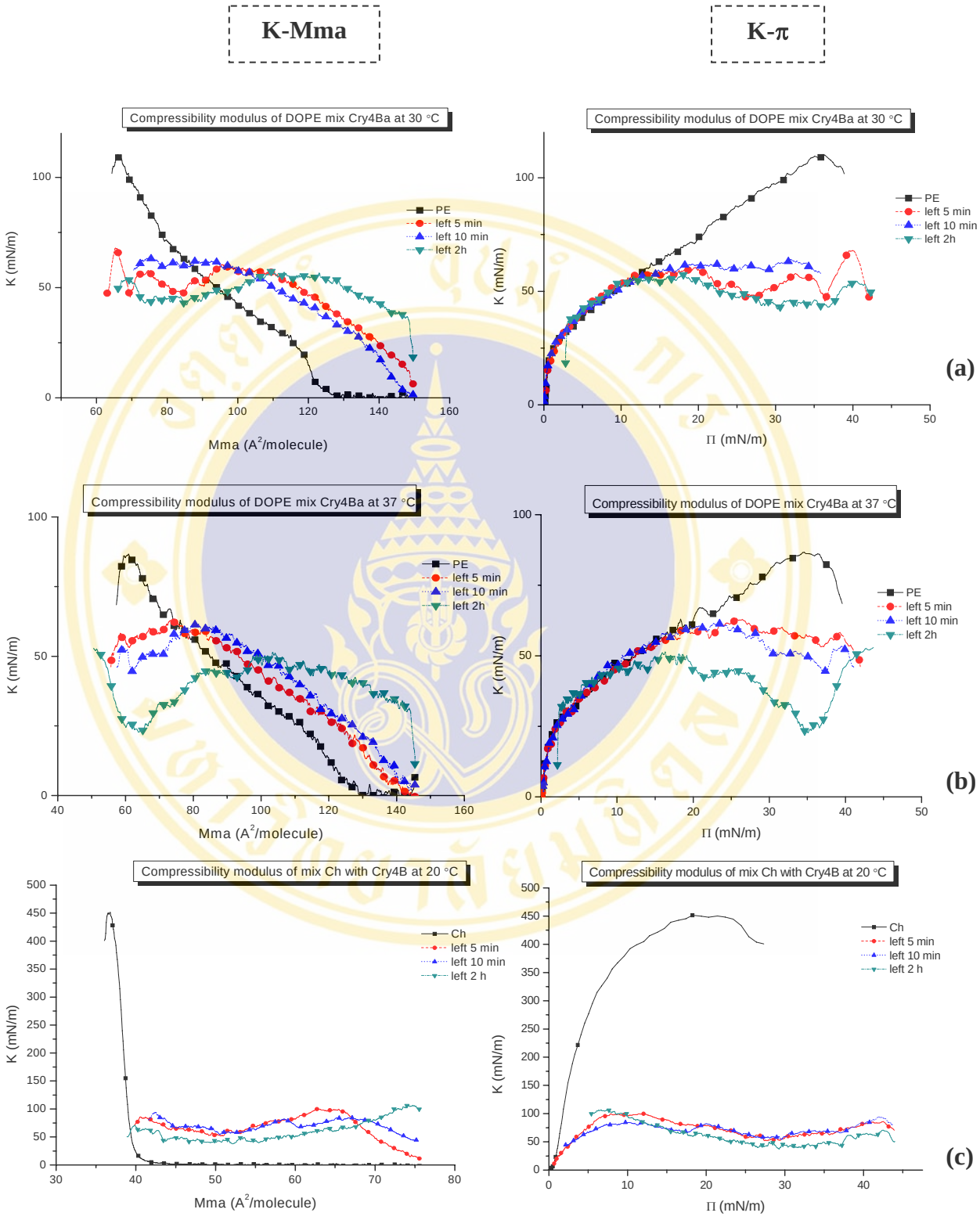

Figure A4 Compressibility modulus of mix
(a) DOPE-Cry4Ba at 30 °C (b) DOPE-Cry4Ba at 37 °C
(c) Chol-Cry4Ba at 20 °C

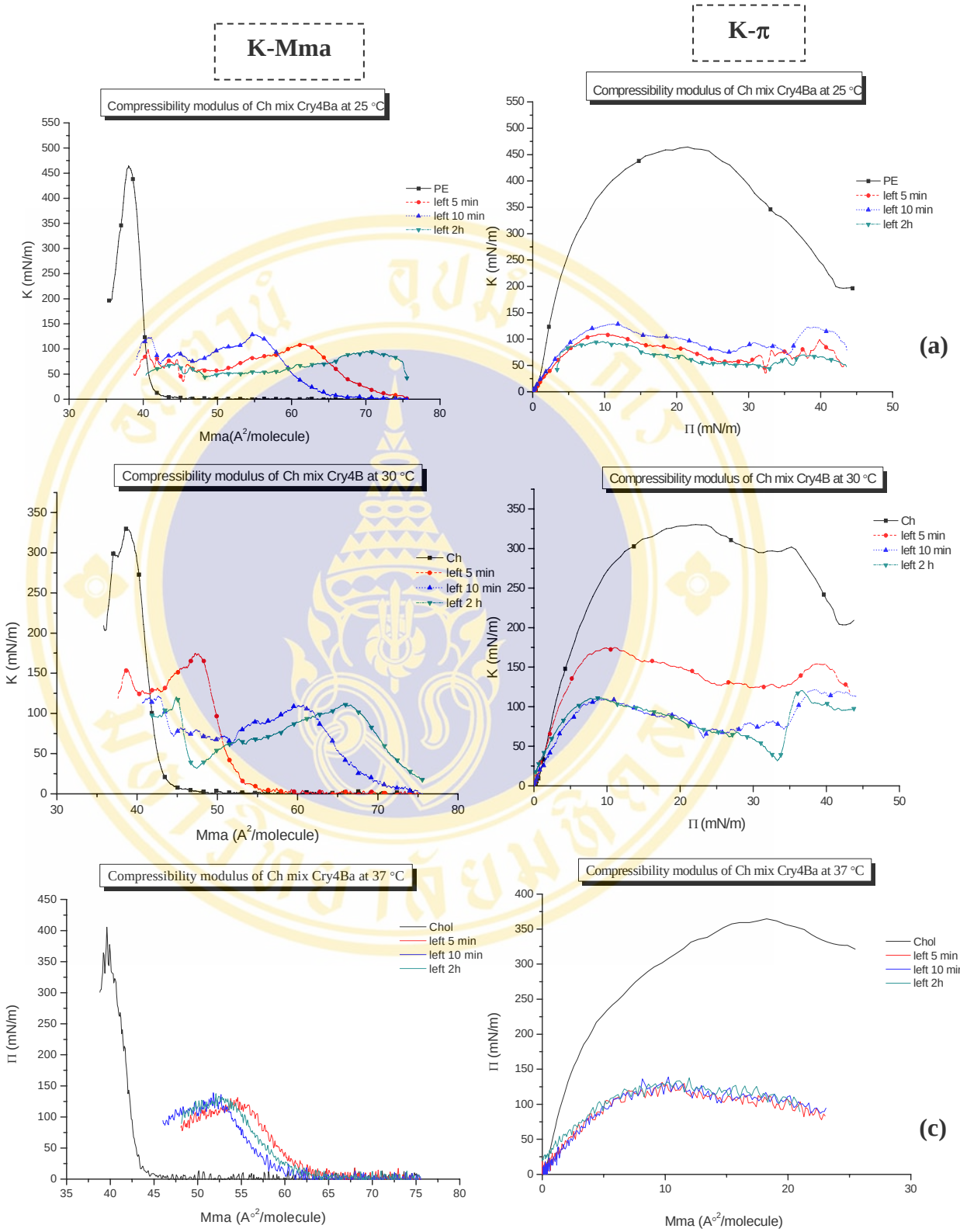

Figure A5Compressibility modulus of mix
(a) Chol-Cry4Ba at 25 °C (b) Chol-Cry4Ba at 30 °C
(c) Chol-Cry4Ba at 37 °C

APPENDIX B

MONOLAYER STABILITY

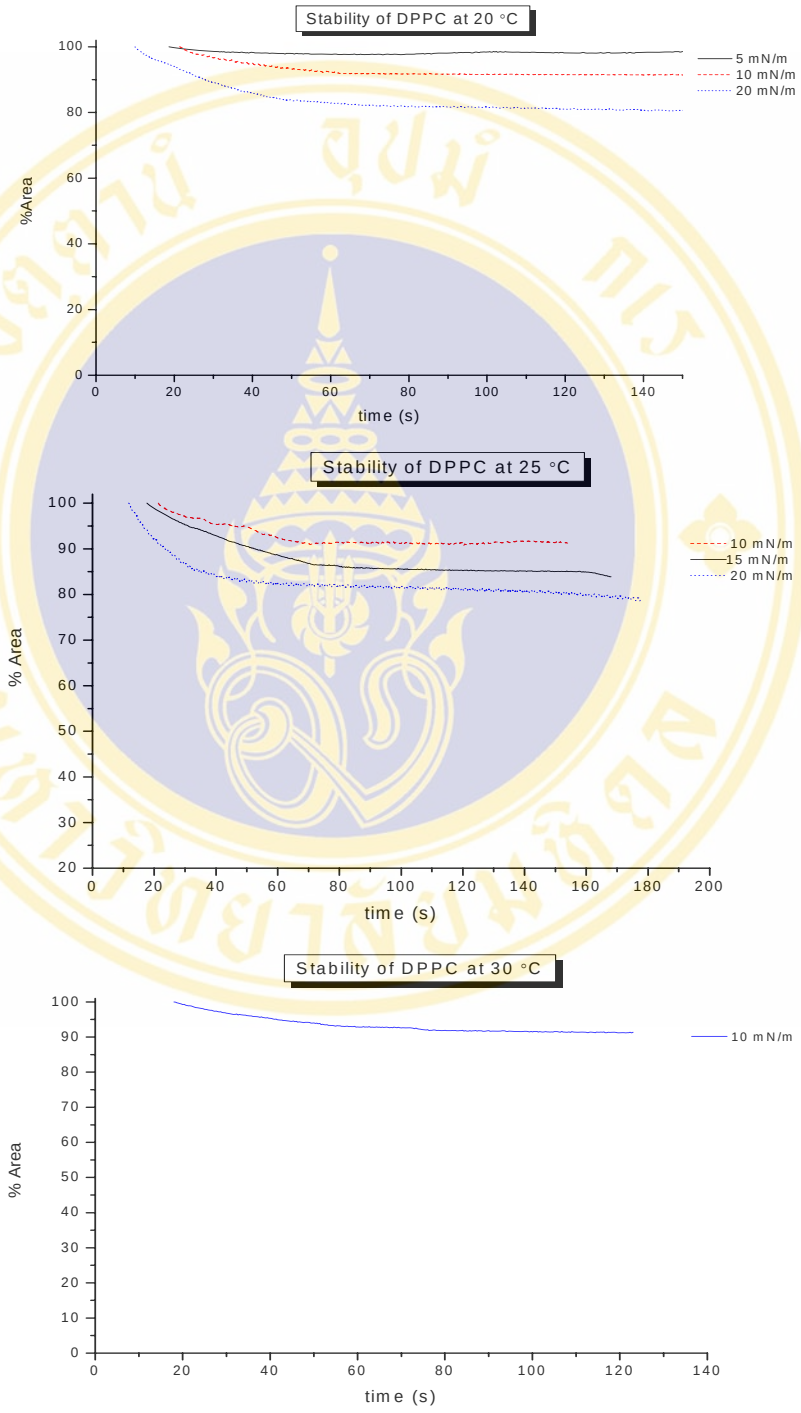

Figure B1 Stability of DPPC at
(a) 20 °C (b) 25 °C (c) 30 °C

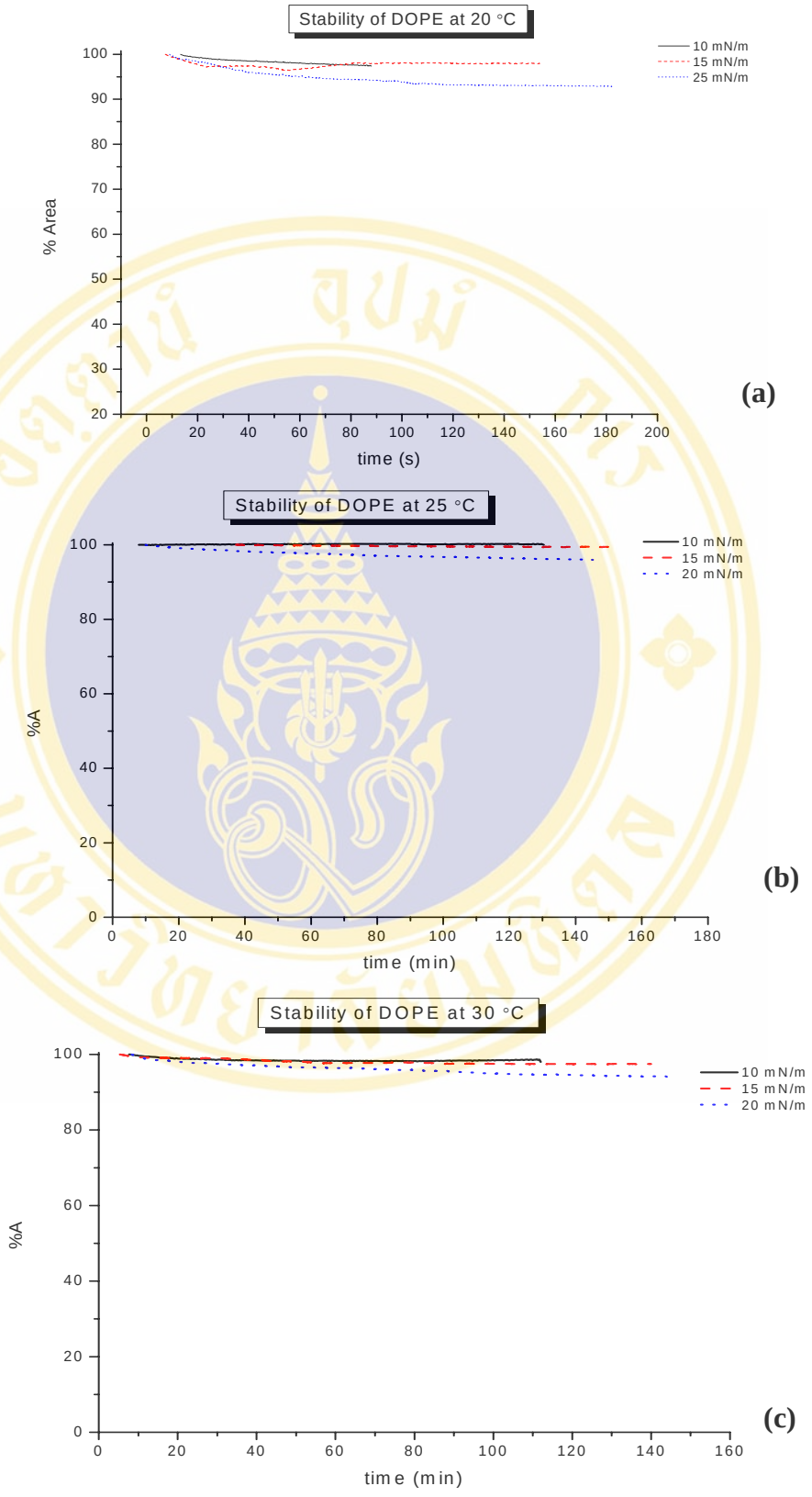

Figure B2 Stability of DOPE at
(a) 20 °C (b) 25 °C (c) 30 °C

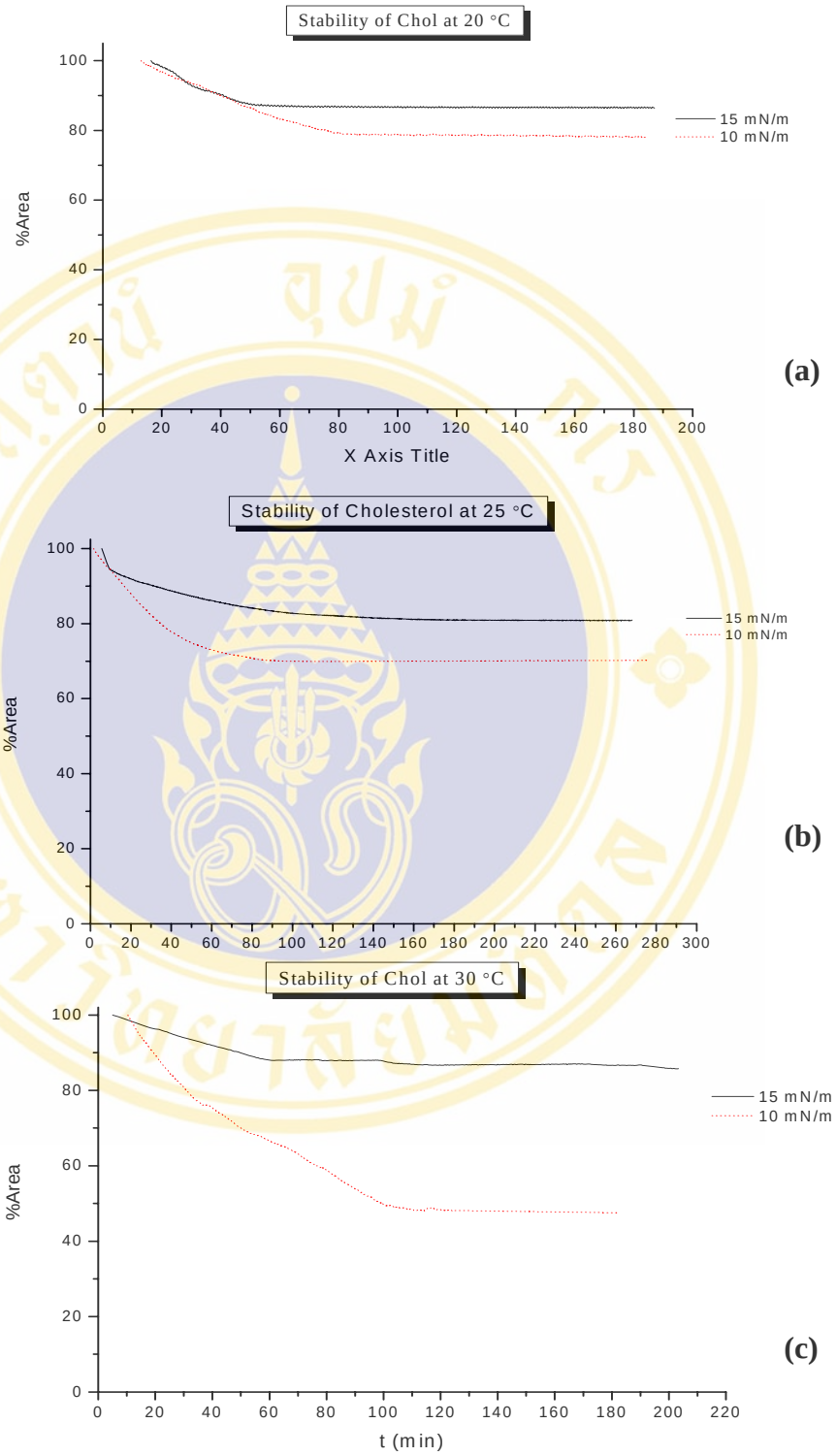

Figure B3 Stability of Chol at
(a) 20 °C (b) 25 °C (c) 30 °C

APPENDIX C

COMPRESSION ISOTHERM OF MIX LIPID-CRY4Ba

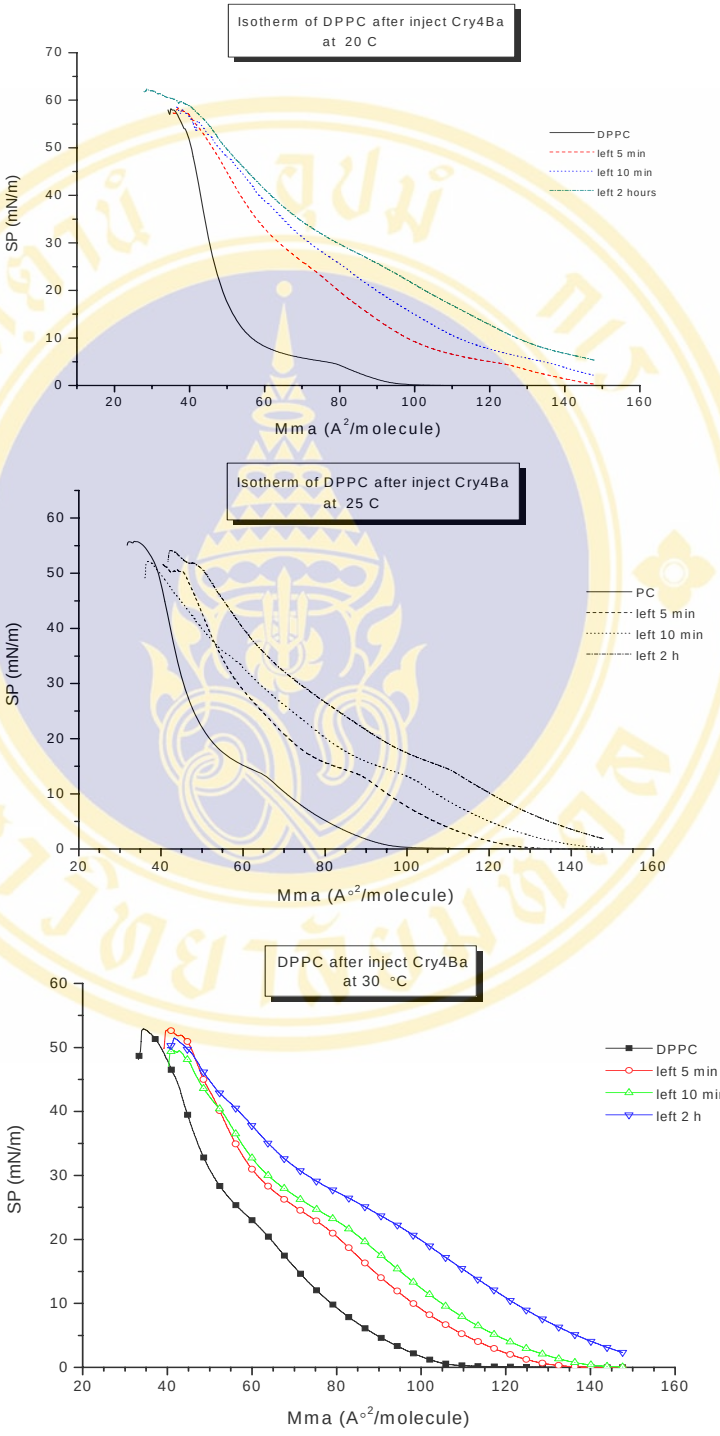

Figure C1 Π-Mma isotherm of mix
(a) DPPC-Cry4Ba at 20° C(b) DPPC-Cry4Ba at 25 °C
(c) DPPC-Cry4Ba at 30 °C

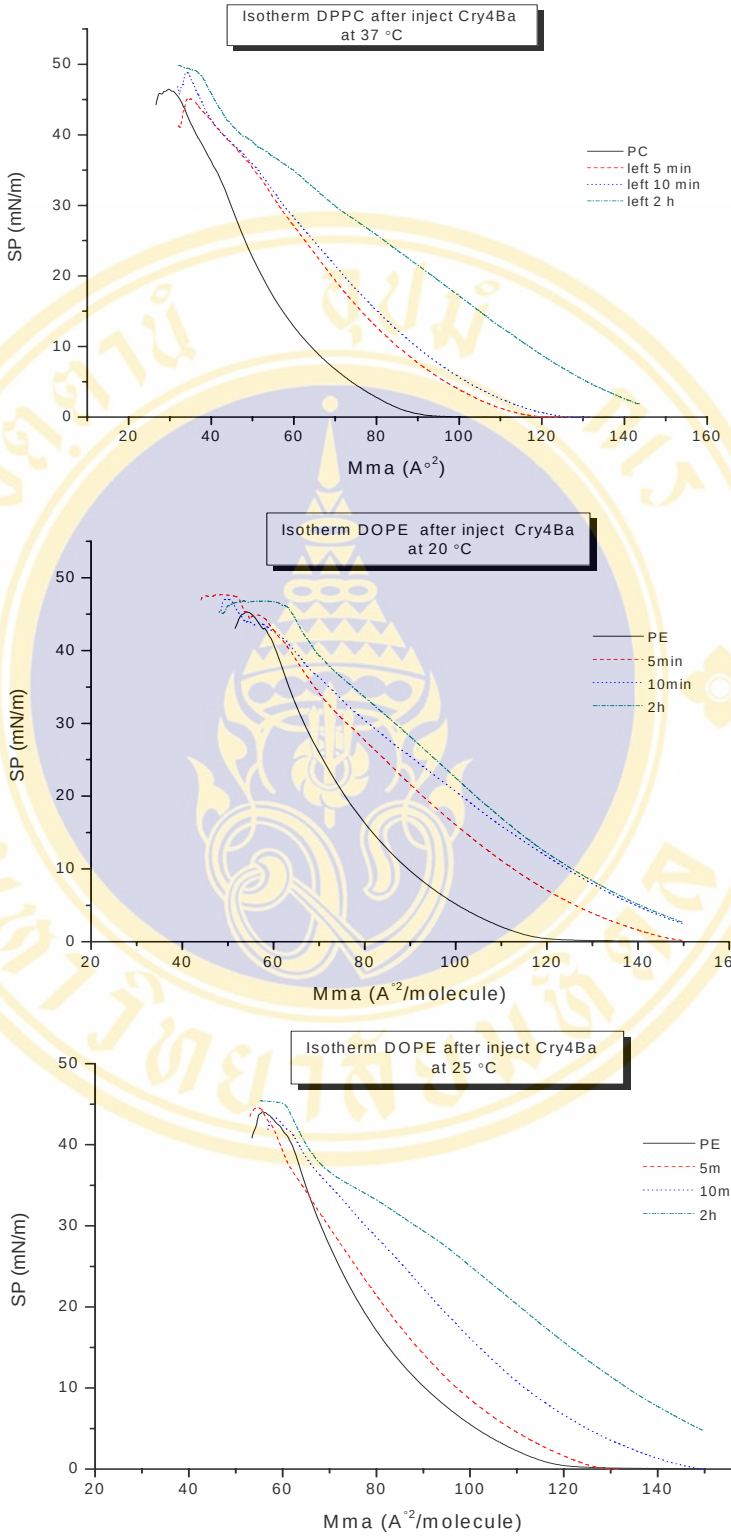

Figure C2 Π-Mma isotherm of mix
(a) DPPC-Cry4Ba at 37 °C (b) DOPE-Cry4Ba at 20 °C
c) DOPE-Cry4Ba at 25 °C

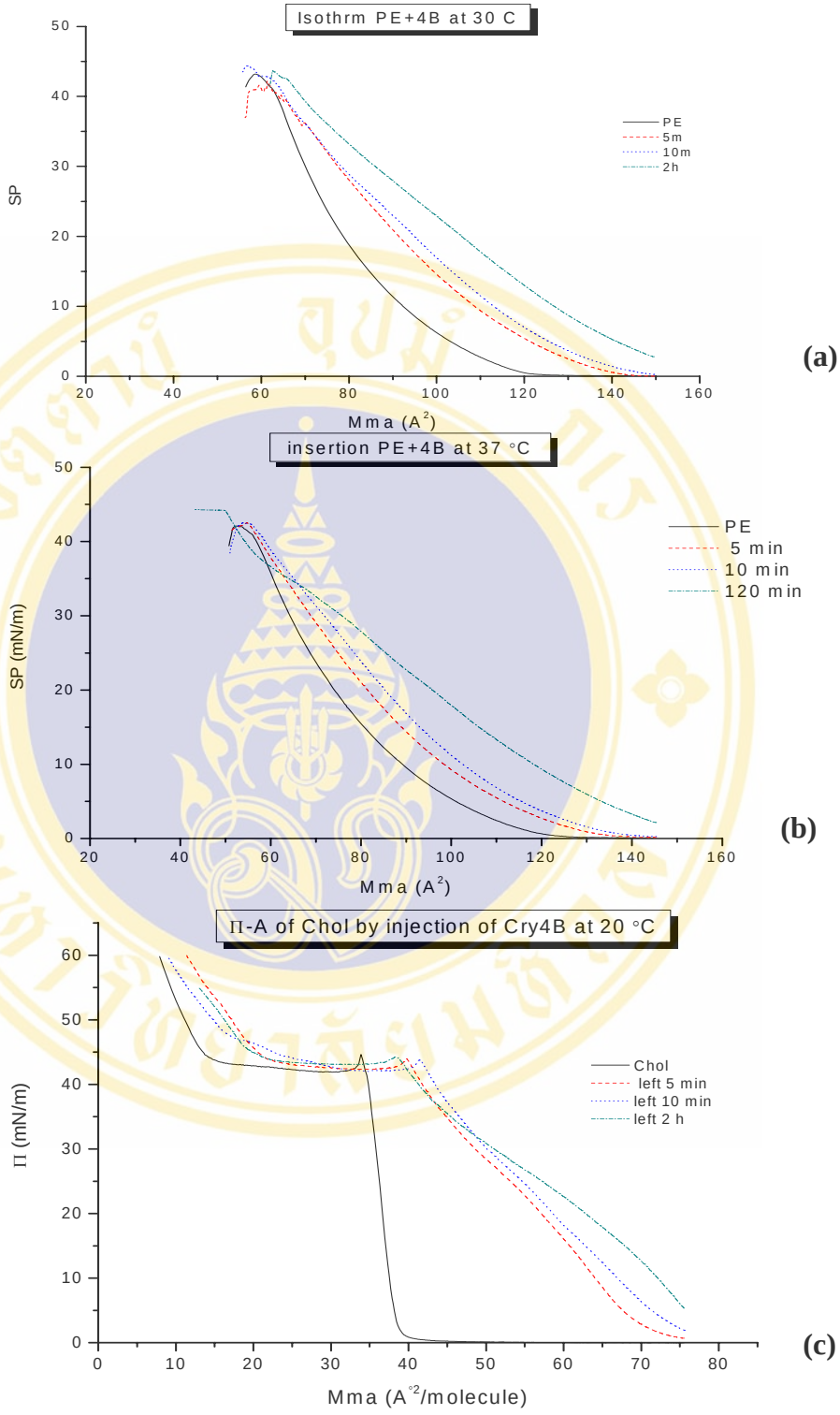

Figure C3 Π-Mma isotherm of mix
(a) DOPE-Cry4Ba at 30 °C (b) DOPE-Cry4Ba at 37 °C
(c) Chol-Cry4Ba at 20 °C

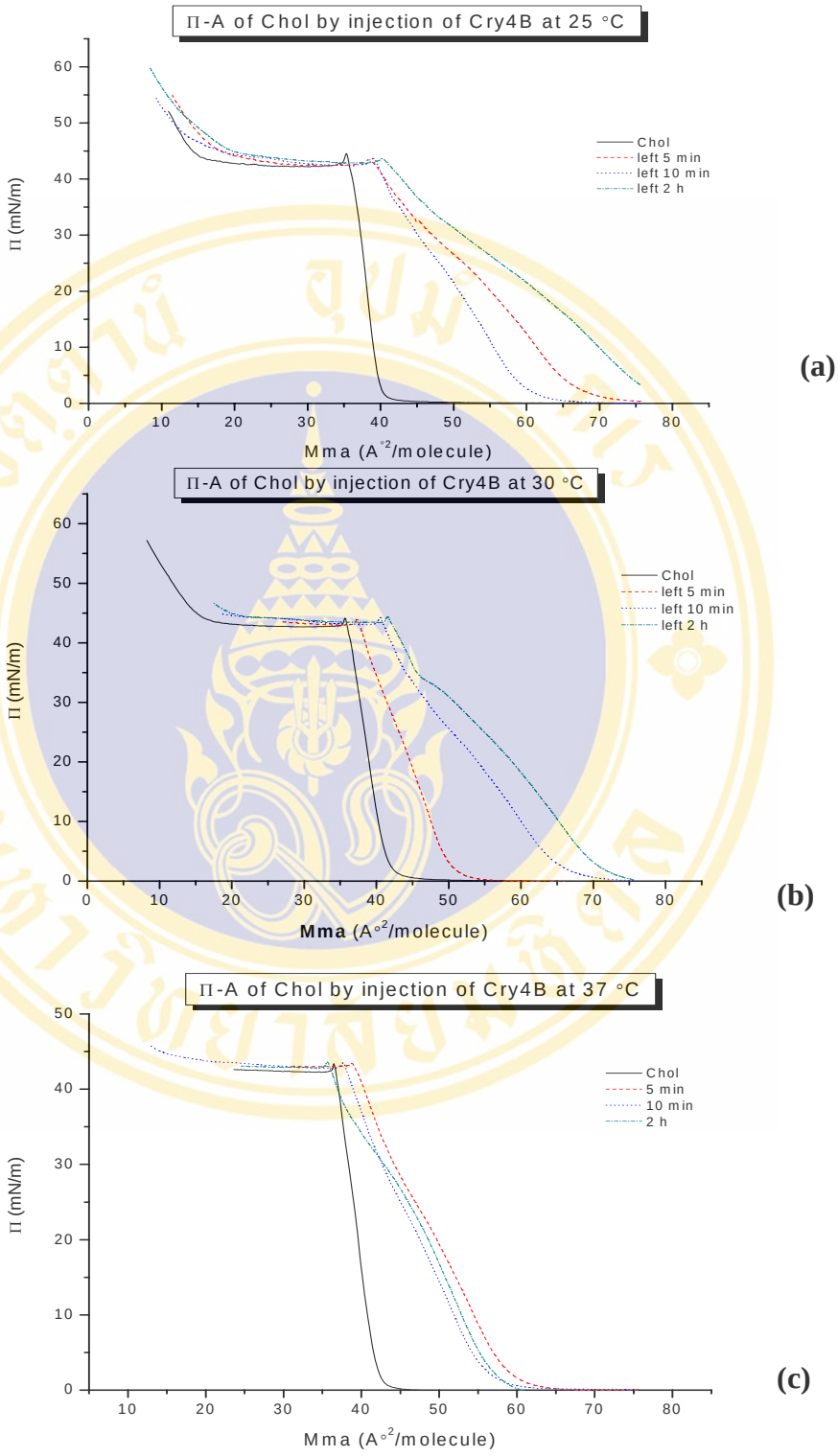

Figure C4 Π-Mma isotherm of mix
(a) Chol-Cry4Ba at 25 °C (b) Chol-Cry4Ba at 30 °C
(c) Chol-Cry4Ba at 37 °C

APPENDIX D

PENETRATION KINETICS

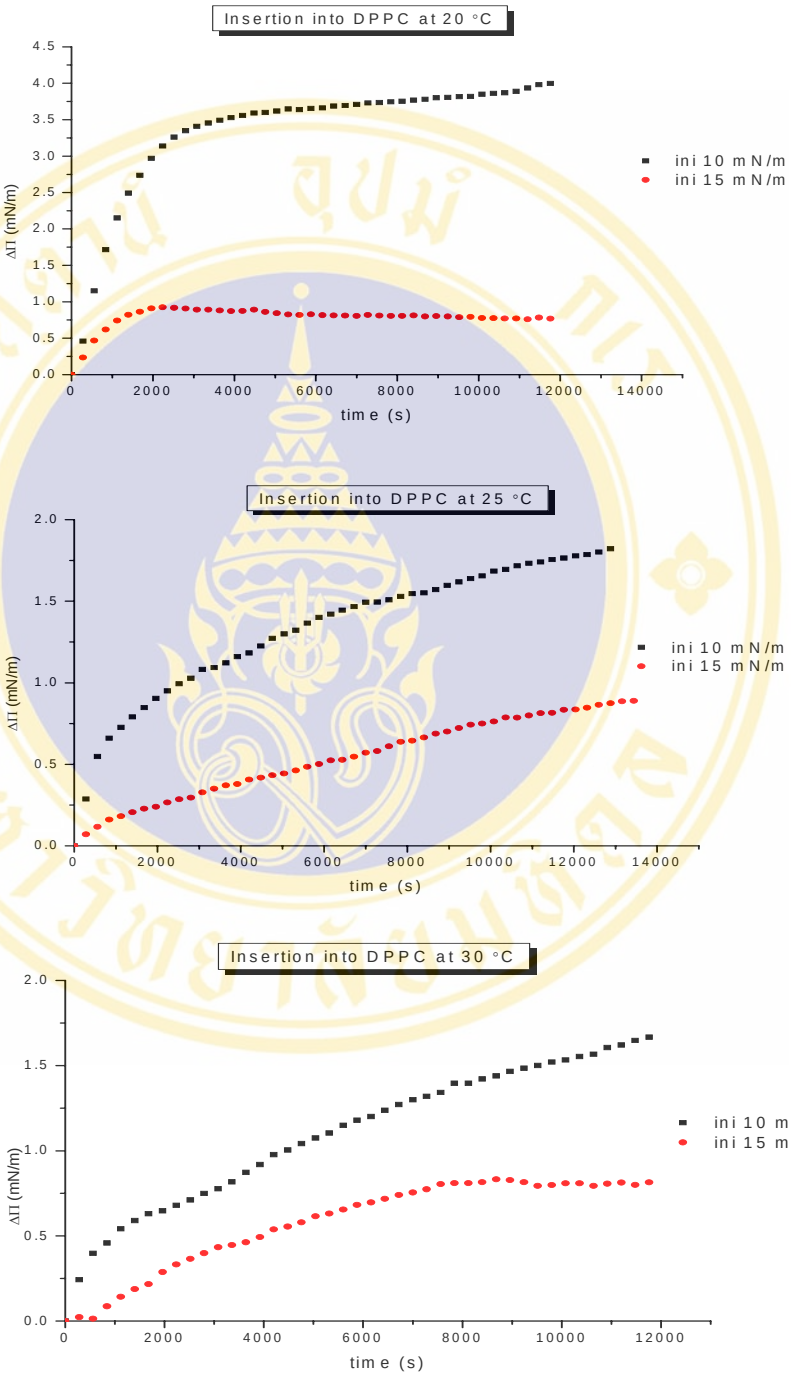

Figure D1 ΔΠ-t of insertion kinetics of Cry4Ba into DPPC layer at (a) 20 °C (b) 25 °C (c) 30 °C

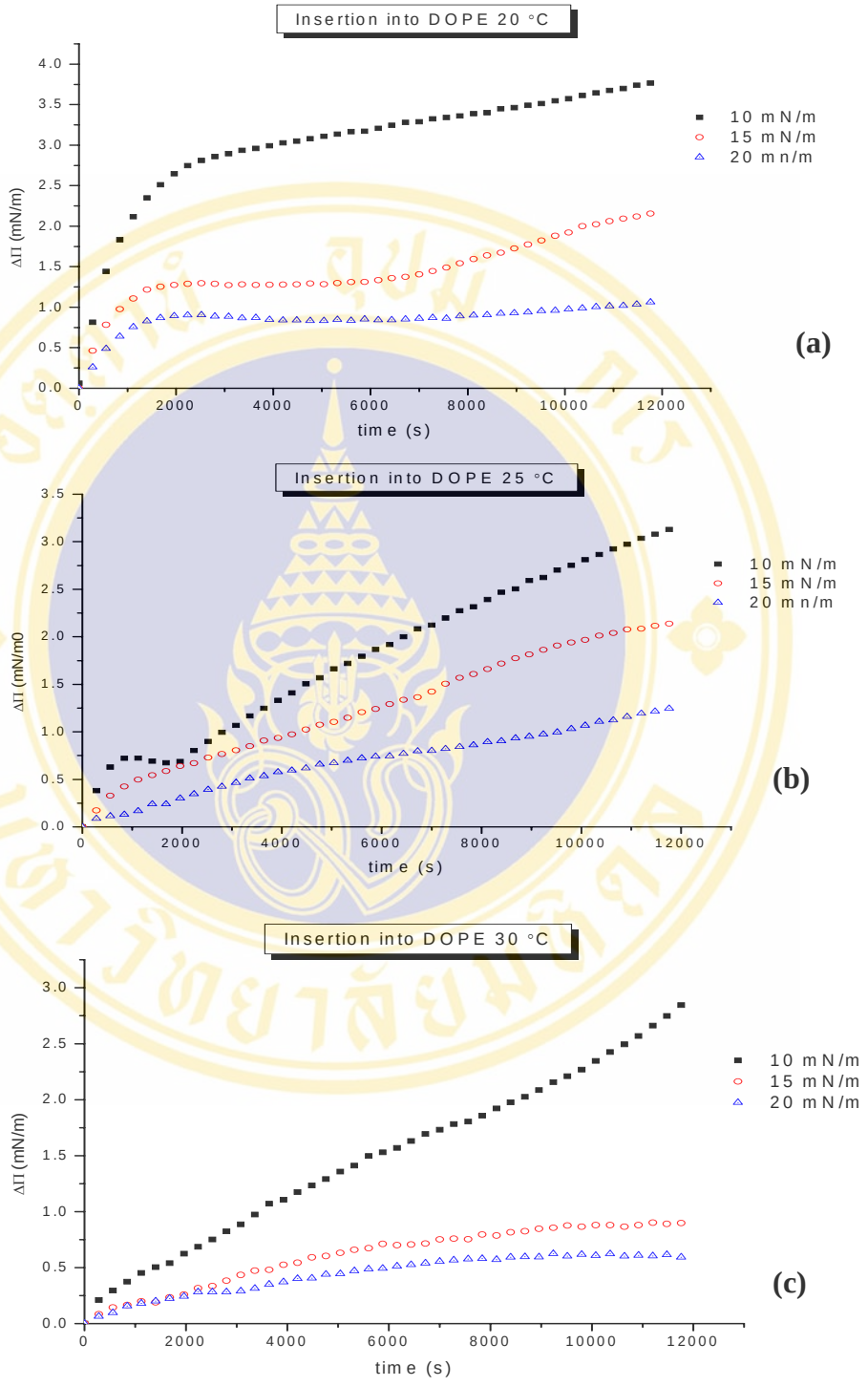

Figure D2 ΔΠ-t of insertion kinetics of Cry4Ba into DOPE layer at (a) 20 °C (b) 25 °C (c) 30 °C

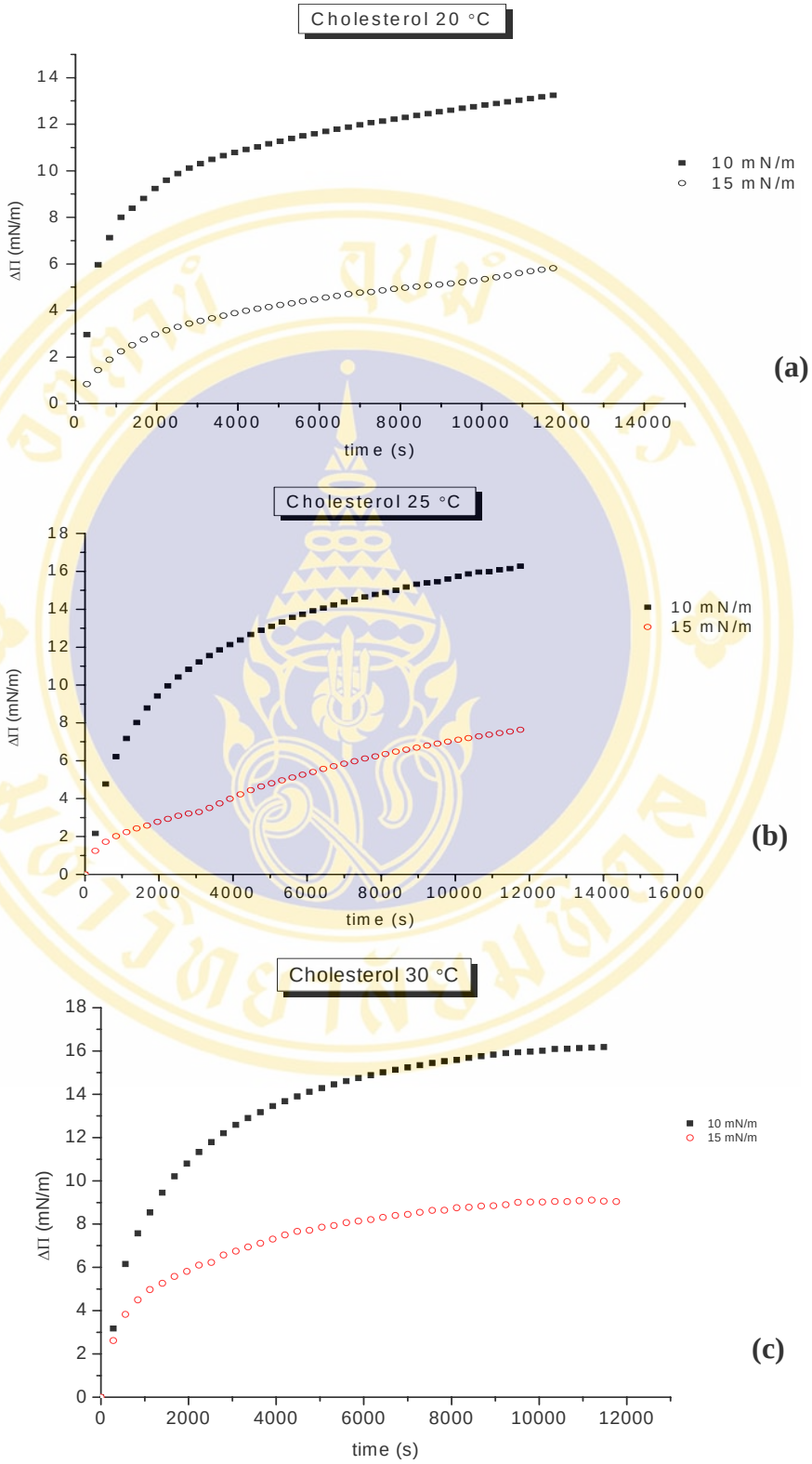

Figure D3 ΔΠ-t of insertion kinetics of Cry4Ba into Chol layer at (a) 20 °C (b) 25 °C (c) 30 °C

BIOGRAPHY

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