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The Association Behaviour of Synthetic Protein-Lipid Complex Analogues

By

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There are a number of people whose help with this project has been invaluable. The first person is my PhD supervisor, Professor Brian Tighe, whose vast knowledge and kind support have carried me through the challenges of researching. I am deeply grateful to Dr. Robert and Dr. Nipapan Molloy for opening my eyes to the world of science and inspiring me to do research. I also wish to thank Dr. Steve Tonge, whose work this project has been based on.

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Executive Summary

Hypercoiling poly(styrene-*alt*-maleic anhydride) (PSMA) is known to undergo conformational transition in response to environmental stimuli. This responsive behaviour makes it possible to mimic structural aspects of native apoproteins. The association of PSMA with lipid *2*-dilauryl-*sn*-glycero-*3*-phosphocholine (DLPC) produces polymer-lipid complex analogues to lipoprotein assemblies found in lung surfactant. These complexes represent a new bio-mimetic delivery vehicle with applications in the cosmetic and pharmaceutical industries.

The primary aim of this study was to develop a better understanding of PSMA-DLPC association by using physical and spectroscopic techniques. Ternary phase diagrams were constructed to examine the effects of various factors, such as molecular weight, pH and temperature on PSMA-DLPC association. ^{31}P-NMR spectroscopy and potentiometric titration were used to further investigate the nature of the complexes. Average size, size distribution and morphological details of PSMA-DLPC complexes were also examined by DLS and negative-staining TEM. Results obtained from this study were expected to provide valuable tool for designing and tailoring properties of the complexes for both pharmaceutical and cosmetic applications.

Abstract

Factors affecting association behaviour of poly(styrene-*alt*-maleic anhydride) (PSMA) and the phospholipid, *2*-dilauryl-*sn*-glycero-*3*-phosphocholine (DLPC) have been investigated. The polymer-lipid association depends on various factors including the polymer/lipid ratio, polymer molecular weight, introduction of hydrophobic methyl ester substituents, heat treatment and pH of the aqueous environment. ^{31}P-NMR spectroscopy suggested that the formation of the mixed complexes involves anisotropic-to-isotropic lipid phase transformation. As the size of PSMA increased and hydrophobic moieties introduced, the conformational transition of PSMA plays lesser important part in achieving lipid phase transformation. Dynamic Light Scattering and TEM data further shows the dependency of complex size and aqueous pH. It was found that as the pH increased from 4 to 7, the complex becomes bigger. This is, possibly, associated with the conformational transition of PSMA from a more compact to an expanded chain conformation.

บทคัดย่อ

งานวิจัยนี้ ได้ทำการศึกษาปัจจัยที่มีผลต่อพฤติกรรมการรวมตัวของ พอลิ(สไตรีน-มาเลอิก แอนไฮไดร์) (PSMA) และ ฟอสโฟลิปิด ชนิด *2*-ไดลอริว-กลีเซอโร-*3*-ฟอสโฟโคลีน (DLPC) ผลการศึกษาพบว่า พฤติกรรมการรวมตัวนี้ ขึ้นอยู่กับหลายปัจจัย อาทิเช่น อัตราส่วนระหว่าง PSMA และ DLPC มวลโมเลกุลของพอลิเมอร์ การเพิ่มหมู่แทนที่ที่ไม่มีขั้ว เช่น เมทิลเอสเทอร์ การให้ความร้อน และค่าพีเอชของสารละลาย การศึกษาโดยเทคนิค ^{31}P-NMR พบว่า การเกิดสารเชิงซ้อนนี้ เกี่ยวข้องกับกระบวนการเปลี่ยนแปลงเฟสของลิปิดจากแบบที่ไม่เป็นระเบียบไปเป็นแบบที่เป็นระเบียบทุกทิศทาง นอกจากนี้ยังพบว่า เมื่อขนาดและหมู่แทนที่ที่ไม่มีขั้วเพิ่มขึ้น การเปลี่ยนโครงรูปของพอลิเมอร์มีความสำคัญต่อการเปลี่ยนแปลงเฟสของลิปิดน้อยลง ผลการศึกษาโดยเทคนิคการกระเจิงแสง และการส่องผ่านโดยใช้กล้องจุลทรรศน์อิเลกตรอน พบความสัมพันธ์ระหว่างขนาดของอนุภาคสารเชิงซ้อน และค่าพีเอชของสารละลาย นั่นคือ เมื่อค่าพีเอชของสารละลายเพิ่มขึ้นจาก 4 เป็น 7 อนุภาคของสารเชิงซ้อนจะมีขนาดใหญ่ขึ้น ทั้งนี้ อาจเกี่ยวเนื่องกับการเปลี่ยนโครงรูปของ PSMA จากแบบม้วนขดงอไปเป็นแบบยืดขยายนั่นเอง

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CHAPTER 1
INTRODUCTION

1.1 Background

Living systems are composed of a variety of macromolecules that can change their conformation and function in response to environmental stimuli. Such dynamic behaviour can be partly reproduced by charged, synthetic macromolecules. The ability of certain polymers to hypercoil or to associate hydrophobically to form excessively compact molecules offers one possible mechanism by which macromolecules could be made to change their conformation, and therefore, their function, in response to local stimuli. Polymers bearing weakly ionizable pendant groups that form weak acids, such as carboxylic acid, are substantially charged above their pK_a values. If the polymer also bears alkyl pendant groups, the resulting ionic repulsion overcomes hydrophobic interactions between alkyl side chains within such polymers and leads to uncoiling of the polymer chain. Conversely, as the pH is lowered the proportion of charged pendant groups falls and hydrophobic interactions between the alkyl side chains become the predominant factor, causing the polymer chain to progressively collapse into distinct hydrophobic microdomains. This effect is known as **hydrophobic association** and is occasionally referred to as **hypercoiling**, a process that ultimately results in the formation of a compact, insoluble, which precipitates from aqueous solution. Hypercoiling or hydrophobically associating polymers are usually surface active, leading to the possibility that surface activity and related functional properties could be 'switched on or off' in response to changes in the pH.[1]

Alternating copolymer of styrene and maleic anhydride (hydrolyzed to maleic acid) (PSMA), structure shown in Fig.1.1, has long been known to exhibit hypercoiling behavior in acidic aqueous solution.[2] In aqueous media, at least below a particular pH range, the associating polymer will generally adopt a helical coil

configuration with the hydrophobic side chain groups presented along one facet and the anionic hydrophilic groups presented along the opposite facet. This smart behaviour can mimic that of native apoproteins that arrange their hydrophobic and hydrophilic groups at opposite facets of α-helical coil to form an amphipathic structure such as that shown in Fig.1.2.[1-3]

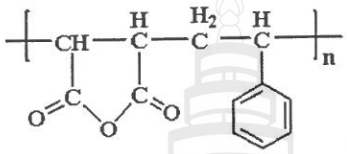

Fig.1.1 Chemical structure of poly(styrene-*alt*-maleic anhydride) (PSMA).

Fig.1.2 Amphipathic coil of PSMA showing hydrophobic (dark grey) and hydrophilic (light grey) facets.

In a similar way to poly(*2*-ethacrylic acid) and other hydrophobically associating polymers, hypercoiling PSMA can interact with phospholipid vesicles to form nanostructural mixed complexes.[1, 4-10] The complexes formed, at least when freshly prepared, have a maximum diameter or cross-sectional dimension of less than 50 nm under physiological conditions. Sizes of the discoidal micellar assemblies appear to be in the range of 10-40 nm in diameter, typically 20 nm, and 5-7 thick[2, 3], as shown in Fig.1.3. This is similar to the dimensions of lipoprotein micellar assemblies found in nature, such as the well characterized system between apolipophorin III and dimyristoyl phosphatidylcholine (DMPC) that has been identified in insects[11], see Fig.1.4. As such, they represent a new biomimetic delivery vehicle for water insoluble substances for both pharmaceutical and cosmetic industries. The molecular arrangement of PSMA-DLPC complexes, initially proposed by Tonge, is illustrated in Fig.1.5.

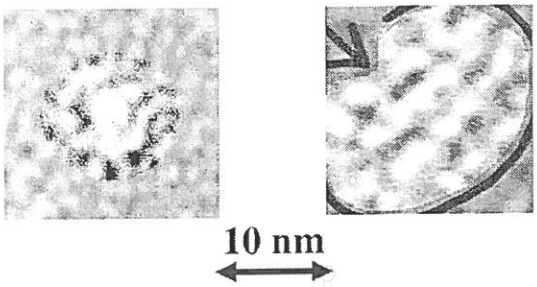

Fig.1.3 Cryo-TEM electron micrographs of PSMA-DLPC vesicles showing axial (left) and lateral (right) views of nanostructures formed (magnification x120,000).[1]

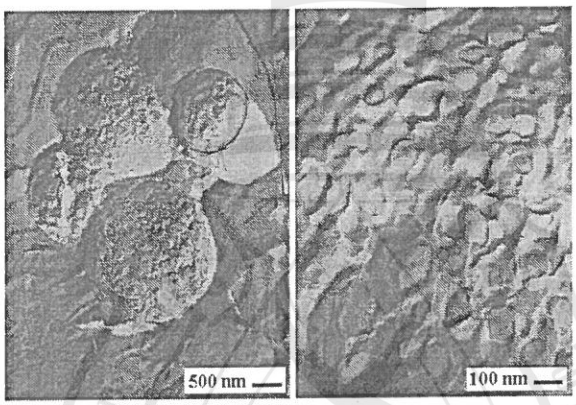

Fig.1.4 Microstructures of (a) *β*-lactoglobulin-phospholipid (DLPC) vesicles (x10,000 magnification) and (b) poly(styrene-*alt*-maleic anhydride)-phospholipid (DLPC) micelles (x50,000 magnification).[3]

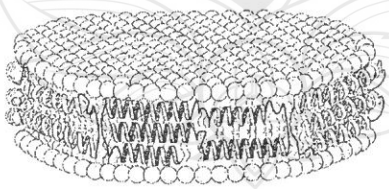

Fig.1.5 Molecular arrangement of PSMA-DLPC complexes with amphipathic polymer arrange around lipid bilayer.[2]

PSMA-DLPC complexes show some interesting properties that have already been reported. In some ways, they are analogous to native lipid-apoprotein assemblies (HDL) present in the blood plasma and responsible for transporting fatty materials around the body.[1, 5, 6, 10, 12] When tested under conditions of dynamic surface

compression, these polymer-lipid complexes showed a high surface activity with surface tensions approaching the values observed with commercially available, animal-derived lung surfactants.[1, 4, 5, 10] Furthermore, the complexes show a remarkably ability to solubilize a wide range of active compounds, ranging from very hydrophobic to amphipathic ones.[5, 6, 9, 13] The nanostructured complexes may be an ideally suited for biomedical and pharmaceutical applications, in particular for ocular drug delivery as it enable oil-soluble active agents to be incorporated into a clear and colorless aqueous composition that is most acceptable to the eye and avoids the use of ointments and emulsion.

The initial work was carried out with a PSMA having a molecular weight of 1,600 with a monomer ratio of 1:1 (styrene to maleic acid). The mixed complexes based on this polymer (PSMA-1.6) show surprisingly surface chemical and solubilization characteristics.[5, 6, 10, 12, 13] For biomedical applications, the formulation should typically be maintained at around pH 5.0-7.5. This requirement can be simply fulfilled by a further increasing of pH solution after the formation of mixed complexes. However, the pH adjustment can lead to instability which may be observed as a loss of clarity over time as the mixed assemblies undergo degradation. Many attempts have been made to stabilize the system, including addition of co-surfactant[9] and applying of the heat treatment[5]. However, none of these enhances the long term stability. Studies of polymer-lipid association phenomena in this system are a necessary step towards stabilizing and utilizing of the complexes in biomedical applications. This study explores the factors elating to the formation of these interesting complexes, particularly the limits of concentration and composition of the precursors including the effect of dramatic increase in the molecular weight and of the partial esterification.

1.2 Objective and Conceptual Framework

This research aimed to explore the factor elating to the formation of PSMA-DLPC complexes through the use of both physical and spectroscopic techniques. Data obtained was believed to provide valuable tool for designing and

tailoring properties of the mixed complexes for both pharmaceutical and cosmetic applications.

1.3 Scope of Research

One approach to obtain a better understanding of PSMA-DLPC association is to investigate the phase behavior of the polymer-lipid system using ternary phase diagram. By varying the type of PSMA used, the effects of other factors, such as polymer molecular weight and chain architecture as well as the hydrophobic moiety content, on the association can readily be exploited. A phase diagram was constructed by measuring %transmittance of formulation at 600 nm and values above 80 are interpreted as successful complexation. The assumption for this was that successful complexation results in complexes that do not scatter light and, as a consequence, produce a homogeneously clear formulation. To examine if the pH neutralization procedure and sterilization process (autoclaving) affects the phase behaviour of the system, phase diagram of solutions, prepared at both pH 4 and 7 with and without autoclaving were also constructed.

In this study, the polymorphic phase transition of hydrated DLPC upon association with PSMA was explored through the use of ^{31}P-NMR spectroscopy. The technique senses the behavior and environment of the phosphorus atom in the phospholipid headgroup and reports on the conformation and structural dynamics of the phosphate group. By analyzing the ^{31}P-NMR spectral line shapes, one should be able to investigate the phase transitions of the phospholipid assemblies upon association with PSMA at different pH.

Another technique used was potentiometry. It was expected that the results obtained would provide useful information about conformational changes of PSMA during ionization and then the effects of these on association with DLPC. Additional details concerning size and size distribution of the complexes were obtained thought the using of Dynamic Light Scattering (DLS) technique and Transmission Electron Microscopy (TEM). It was expected that the techniques would also provide details of how the particle size varies with pH solution.

1.4 Time Frame

The following table shows time frame for this research study. It is worth noting that this research project was conducted mainly at Mae Fah Luang University (MFU) and partly at Chiang Mai University (CMU). The samples for ^{31}P-NMR spectroscopy experiments were freshly prepared and further analyzed at Aston University, UK.

Table 1 Time frame for the research study.

Activity	Months (from June 2009 to January 2010)								
	June	Jul	Aug	Sept	Oct	Nov	Dec	Jan	Remark
1. Characterization of the polymer samples			◆—	—◆					Used data obtained from supplier
2. Hydrolysis of the polymer sample			◆—	—	—	—	—◆		PSMA received 2/Aug/09
3. Synthesis of PSMA-DLPC complexes				◆—	—	—	—◆		DLPC received 3/Sept/09
4. Study of PSMA-DLPC association via phase behavior				◆—	—◆				Performed at MFU
5. Study of PSMA-DLPC association via potentiometric titration			◆—	—◆					Performed at MFU
6. Study of PSMA-DLPC association via ^{31}P-NMR spectroscopy		◆—	—	—	—◆				Performed at Aston University, UK
7. Study of PSMA-DLPC association via DLS and TEM			◆—	—◆				◆—	DLS performed at UK. TEM performed at CMU
8. Review the results and prepare for submission			◆—	—	—	—	—	—◆	Performed at MFU
9. Hand in final report and prepare for presentation								◆→	Performed at MFU

CHAPTER 2
LITERATURE REVIEW

2.1 Interaction of Anionic Hypercoiling Polymers with Phospholipid Bilayers

Among all synthetic polyelectrolytes, polyanions have been investigated for various applications in the medical field. They have been found to inhibit adjuvant arthritis and modulate phagocytic activity.[14] Recently, polyanions have been evaluated as part of drug delivery systems, either as complexes or conjugates with biomolecules or in the preparation of pH-responsive liposomal formulation.[14] The application of anionic hypercoiling polymers to medicine was first suggested by Seki and Tirrell.[2] This work sprang from a need to synthesize phospholipid vesicles or liposomes that could be made to respond to their environment and used, for example, to selectively release drugs. Such liposomes were complexed with various poly(acrylic acid) derivatives to render the vesicle membrane sensitive to pH, whereby, a variation of pH acted as a 'trigger' mechanism to change polymer structure and alter the properties of the liposomal membrane.

Poly(*2*-ethacrylic acid) (PEAA) (0.1%)[15] was found to adsorb onto the surface of dipalmitoylphosphatidylcholine (DPPC) liposome (0.1%) suspended in aqueous solution at pH 7.4, that is, above the pK_a value of the polymer. Such behaviour was explained in terms of hydrogen bonding between the charged carboxylic acid pendant groups within the polymer and the phosphodiester head groups of the phospholipid. Lowering of the pH to 6.5 (towards the pK_a of the polymer) caused a loss of charge within the polymer, at which point hydrophobic interactions between ethyl pendant groups then predominated and led to a collapse of the polymer chain.[15] The resultant hydrophobic domains acted to disrupt the liposomal membrane and caused a release of its contents, e.g. the marker compound carboxyfluorescein.[15] Concomitant differential scanning calorimetry studies showed a sudden broadening of the melting endotherms as the pH of the system was lowered,

indicating a reorganization of vesicle structure.[15, 16] Electron microscopy provided a further insight into these pH-induced structural changes, and showed the presence of small (125-400 Å) diameter micellar particles, similar to those observed in high density lipoprotein.[2] The pH-dependent reorganization of phospholipid vesicle membranes by PEAA can be illustrated in Fig.2.1. Such pH-induced macromolecular reorganization resulted in the formation of optically clear, aqueous suspensions.[15] The pH at which conformational transition in polymer-liposome structure was found to occur depended, not only on the chemical structure of the polymer, but also upon its tacticity. Incorporation of the photophysical probe pyrene, into PEAA, enabled the presence of hydrophobic environments to be identified. Studies using this technique[17] revealed that a large increment in fluorescence occurred as the pH was raised above 6.2 and this was concomitant with a conformational transition of the polymer. Binding of DPPC to PEAA was also found to result in a shift in the pK_a value of the polymer causing a decrease in its apparent acidity[17], i.e. behaved as a weaker polyacid. This shift of pK_a value was believed to attribute to hydrogen-bonding interactions between PEAA and the membrane surface.

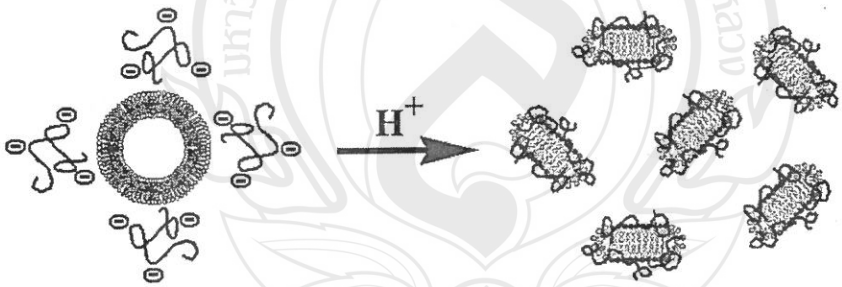

Fig.2.1 pH-Dependent reorganization of phospholipid vesicle by PEAA.[18]

2.2 Synthetic Protein-Lipid Complex

As mentioned earlier in Chapter 1, responsive hydrophobically associating polymers, PSMA, are widely known to undergo conformational transition in response to environmental stimuli. This smart behaviour can mimic that of native apoproteins that arrange their hydrophobic and hydrophilic groups at opposite facets of α-helical coil to form an amphipathic structure such as that shown in Fig.1.2 .[1-3] When

the hypercoiling polymer is combined with film-forming lipids, they associate to produce polymer-lipid nanostructures analogous to lipoprotein assemblies such as HDL. The synthetic nanostructures produced are sub-liposomal in dimension, as illustrated in the micrographs, shown in Fig.1.3 and Fig.1.4.

One of the most important properties of the synthetic protein-lipid complexes with regard to their applications such as, solubilizing agent and artificial lung surfactant, is the manner in which surface tension varies with repetitive changes in surface area. One test method used to obtain such profile is a pulsating bubble technique using a pulsating bubble surfactometer consisting of a sample chamber, pulsator unit and pressure-recording device. The pulsating bubble technique simulates to some extent the contraction and expansion of the alveolar sacks in the lungs and allows the surface tension to be assessed at minimum and maximum bubble volumes. This technique also allows the effect of repeated expansion and compression cycles upon the adsorption of surface active components to be observed and quantified. The technique has been used as a model *in vitro* system for testing the efficacy of synthetic lung surfactants.

Tonge et al. revealed[1-3] that the nanostructure, consisting of PSMA and DLPC, when tested under dynamic surface compression exhibit remarkably high surface activity and approach the values observed with commercially available lung surfactant, see . This finding indicates a high suitability of using these complexes as artificial lung surfactants and as solubilizing agent.

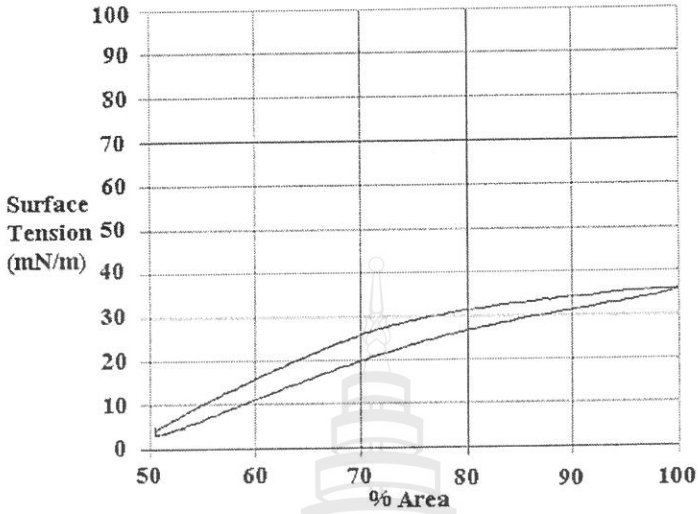

Fig.2.2 Surface activity of the PSMA-DLPC complexes (1.25/0.5%) measured by pulsating bubble surfactometry at 5 min of pulsing.[3]

Some other important characteristics of PSMA-DLPC complexes have already been reported including sizes, surface activity and encapsulation efficacy. Results from optical microscopy provide direct evidence that these synthetic protein-lipid complex analogues possess the ability to solubilize a wide variety of active compounds ranging from very hydrophobic ones to amphipathic ones.[5] The results offer evidence for the practical applicability of these synthetic protein-lipid analogues in biomedical fields.

Studies of PSMA-DLPC association are certainly a necessary step towards tailoring properties of these complexes for biomedical applications. Such properties include chemical and thermal stability, size-distribution, structural configuration, surface lubricity and drug loading efficacy. This research will be the real attempt to systematically study this topic.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

Preliminary studies revealed that the nature of purified water used for suspension of the phospholipid exerted an influence on both phase diagrams and the stability of the complexes. In the work described here, only double-deionized water (conductivity <10x Siemens) was employed.

DLPC (dilauroylphosphatidylcholine) was purchased from Genzyme and was hydrated to a concentration by weight of 2 %, and used in that form for complexation experiments. PSMA copolymers were obtained from commercial sources listed in Table 1. The nomenclature used here denotes the molecular weight as indicated in the table. PSMA-120 was received in the form of the sodium salt and diluted without further treatment to give a final concentration of 2 % by weight. PSMA-1.6 and PSMA-350 samples were hydrolyzed in aqueous solution prior to use. Hydrolysis was carried out by addition of the samples to a 0.5 M NaOH solution (pH 12.5). The samples were maintained at room temperature with agitation until optically clear polymer solutions were obtained. The solution pH was then re-adjusted to pH 7 before use. The final concentration of PSMA-1.6 and PSMA-350 solution was 2 % by weight.

Table 2 Molecular weight (MW) and suppliers of the PSMA copolymers used.

Polymer Designation	MW	Supplier
PSMA-1.6	1,600 (Mn)	Scientific Polymer Products
PSMA-120[a]	120,000 (Mw)	Sigma-Aldrich
PSMA-350[b]	350,000 (Mw)	Sigma-Aldrich

[a] received in the form of sodium salt; [b] contains 10-15 % methyl ester moieties

3.2 Methods

3.2.1 Ternary Phase Diagrams

An appropriate amount of 2 % DLPC was hydrated in double-deionized water. The mixture was then mixed with 2 % PSMA solution at pH 4. The range of concentration for DLPC used to obtain the phase diagram was 0-1 % by weight while that for PSMA was 0-5 % by weight. The phase diagrams were constructed by measuring the percentage transmittance (%T) of formulations at 600 nm using a UV-Vis spectrophotometer (Hitachi). In order to verify if the neutralization procedure affects the phase behaviour of this system, additional experiments were carried out. The pH of the mixtures, comprising DLPC (2 %) and PSMA (2 %), obtained above, were further re-adjusted to 7 by adding 0.1 M NaOH. The phase diagram at pH 7 of the system was then obtained by using the same methods as described for pH 4. To examine if the sterilization process (autoclaving) affects the phase behaviour of the system, phase diagram of solutions, prepared as described above, at pH 4 were subjected to an autoclave cycle of 121°C and 100 MPa for 20 min. After the autoclaving was completed, the solution samples were allowed to cool down at room temperature. The %T of these samples was first measured at pH 4. After that, the pH of the samples was re-adjusted back to 7. The phase diagrams for both pH values of samples subjected to the autoclaving were obtained by using the same methods as described for the untreated samples.

3.2.2 ^{31}P-NMR Spectroscopy

In this study, ^{31}P-NMR was used to examine the polymorphic behaviour of aqueous DLPC dispersions upon associating with the polymer. The PSMA samples used were PSMA-1.6 and PSMA-350 prepared as previously described. The pH-dependent ability of these copolymers to associate with the DLPC was also

investigated at pH 4, 5, 6 and 10. The ^{31}P-NMR spectra were acquired at 121.5 MHz with a Bruker Avance 300 Spectrometer in conjunction with XWIN NMR Version 3.5 software to process the spectra. The spectrometer field was locked during acquisition by using D_2O. The proton-decoupled spectra were obtained using a relaxation delay of 4 sec, spectral width of 18,248 Hz and acquisition time of 1.8 sec; the number of acquisitions was 160 scans. All spectra were recorded at room temperature and the chemical shifts were measured relative to H_3PO_4 (85 %) as an external reference. The ^{31}P-NMR spectrum for aqueous DLPC dispersion was recorded at a lipid concentration of 5 mg/ml, while for the aqueous PSMA solution, a 30 mg/ml polymer concentration was used. For all DLPC-PSMA mixtures, the NMR spectra were recorded at lipid and polymer concentrations of 5 and 30 mg/ml, respectively. The pH of solution was adjusted to the required value by using NaOH or HCl solution.

3.2.3 Potentiometric Titration Study

Potentiometric titration was performed on freshly prepared solutions in glass vessels at room temperature using a Radiometer PHM52 digital pH meter with Radiometer GK2301C combined electrodes. Standardization was checked at pH 4.00 (±0.01), 7.00 (±0.01) and 10.00 (±0.01) with Radiometer buffer solutions. The potentiometric titration was performed with 0.1 M HCl so that the polyelectrolyte concentration did not vary significantly during titration. The polymer used was hydrolyzed PSMA-1.6 (2 % by weight) together with an aqueous suspension of DLPC with a concentration of 2 % by weight. In order to investigate association behaviour between PSMA and DLPC, 10 ml of the mixture comprising both components was initially prepared to give the final concentrations by weight of 0.5 % DLPC and 3.0 % PSMA. The resultant mixture was carefully titrated with HCl solution and the change of pH reading was monitored as a function of HCl added. Each titration was performed with a period of about 5 min between successive readings.

3.2.4 Measurement of Particle Size

The average diameter and size distribution (polydispersity index) of PSMA-DLPC complexes were measured by Dynamic Light Scattering (DLS). DLS was performed at room temperature in deionized water with a scattering angle of 90° on the 90Plus Particle Size Analyzer (Brookhaven Instruments Corp., Holtsville, NY, USA). Three 3 min sub-runs per sample were collected and averaged. The aqueous dispersions of the complexes were prepared at pH 4 by mixing 5 mg/ml of DLPC suspension (2 % by weight) with 30 mg/ml of hydrolyzed PSMA (2 % by weight) and were suitably diluted with deionized water. In order to examine if the process of neutralization affects the size of the complexes, an additional experiment was performed. The same samples previously obtained at pH 4 were neutralized by adding 0.1 M NaOH, further diluted and then analyzed as described above.

3.2.5 Transmission Electron Microscopy

Morphological examination of mixed complexes was performed using a Transmission Electron Microscope (JEOL, JEM2010 Electron Microscope) after negative staining with saturated uranyl acetate (UA) solution.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Ternary Phase Diagrams

It was known that clear solutions of PSMA-DLPC complexes could be formed with various PSMA samples and over a range of concentrations and ratios. In order to gain a more quantitative understanding of the factors affecting the association behaviour of PSMA-1.6 (which had been first used to demonstrate the unusual surface chemical and solubilization characteristics of the complexes [6, 10, 12]), attention was first concentrated on establishing limits for the phase behaviour of this material.

The approach adopted involved the creation of ternary phase diagrams, which have the advantage that factors affecting the association can be conveniently visualized. It allows various environmental conditions such as pH, type of water, temperature as well as the molecular weight, chain architecture and the hydrophobic moiety content of the PSMA to be investigated. The approach involves construction of phase diagrams by measuring the %T of the various formulations at 600 nm and interpreting values above 80% as successful complexation. The assumption was that successful complexation results in complexes that do not scatter light and, as a consequence, produce a homogeneously clear formulation. Each dot in the diagram represents a successful complexation at conditions given by the coordinates of the dot. To avoid various complications that may occur during preparation of the high-viscosity formulations, the concentration of DLPC, used to construct phase diagrams in this study, was in the range from 0 to 1% by weight and that of PSMA was from 0 to 5% by weight.

Fig.4.1a-d shows the ternary phase diagrams of Water/DLPC/PSMA-1.6 systems. The shaded areas in the phase diagrams correspond to a transparent single phase and two tie-lines have been drawn on each diagram representing a constant ratio

of PSMA and DLPC. The formulations that fall within the shaded area show a successful complexation between PSMA and DLPC. As mentioned earlier, this assumption is based on the fact that the complexation causes both components to reorganize and form mixed complexes that do not scatter the light, and as a consequence, produce a homogeneously clear formulation.

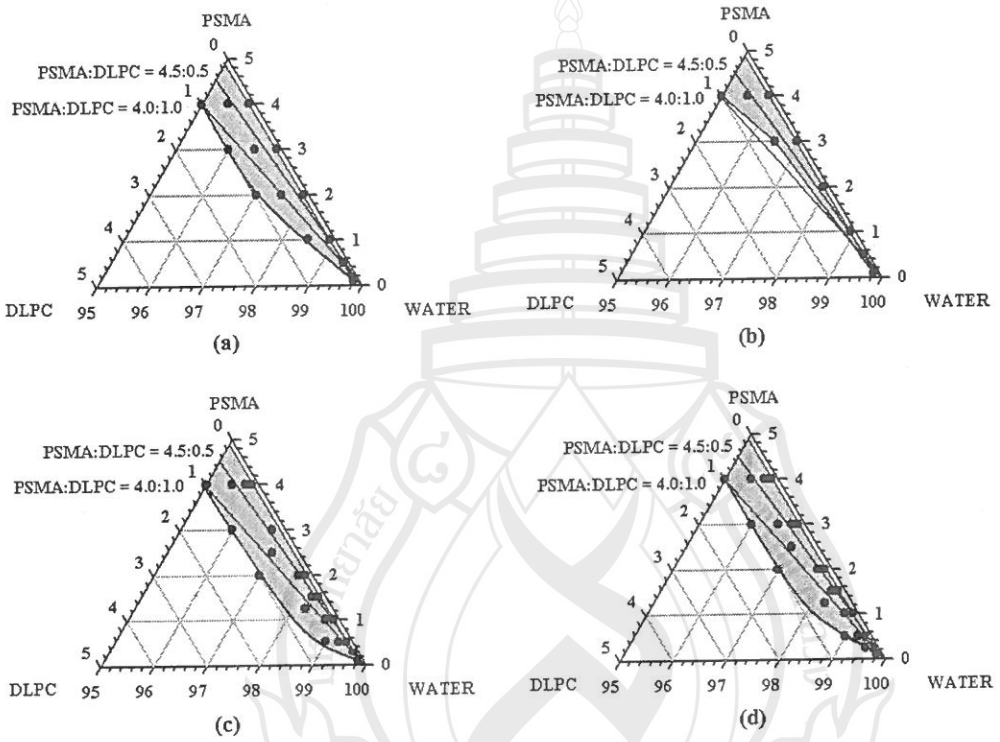

Fig.4.1 Ternary phase diagrams of Water/DLPC/PSMA-1.6 systems at room temperature at (a) pH 4 and (b) pH 7. The ternary phase diagrams of the system subjected to steam sterilization (autoclaving) at (c) pH 4 and (d) pH 7 were also shown in comparison. The shaded area corresponds to a single transparent phase and the tie-lines represent the constant ratios of PSMA to DLPC.

The tie-lines shown in Fig.4.1a-d, correspond to constant ratios of PSMA and DLPC = 4.5:0.5 and 4.0:1.0. The effect of dilution on the complexation, when keeping the PSMA/DLPC ratio constant, may be seen as moving down along these tie-lines toward 100 % water. Fig.4.1a indicates that the complexes with these ratios and at pH 4 are not affected by dilution, showing that they are dependent on the polymer/lipid ratio and not their concentrations. The effect of neutralizing the solution on the phase behaviour of the system can be seen in Fig.4.1b. Comparing with

Fig.4.1a, the shaded region observed is narrower, implying that the obtained complexes are less stable at pH 7. At pH 7, the polymer chains are more ionized than at pH 4. An increase in the degree of ionization thus, enhances the ionic repulsion between PSMA chains and modifies the hydrogen bonding with water molecules. This opens the compact coils of PSMA and weakens the hydrophobic association with the lipid, causing the complexes to swell and eventually scatter light.

The effect of autoclaving on the phase behaviour of Water/DLPC/PSMA-1.6 systems can be observed in Fig.4.1c-d. It is found that the sterilization process enlarges the shaded areas, particularly at pH 7, indicating that the process not only enhances complex formation at pH 4 but also allows the complexes to maintain their stability during the process of neutralization. A reasonable explanation is that energy input (either sonication or heating) applied during sterilization enhances the intermingling of PSMA chains in the DLPC assemblies. This strengthens hydrophobic interaction between the compact coils of PSMA and the lipid aliphatic side chains and so reduces the size of the complexes.

It is apparent from inspection that whereas the ratio of PSMA to phospholipid can be increased without apparent loss of stability, there are definite discontinuities as the ratio is decreased. An important question arises. If the ratio by weight of PSMA to phospholipid is maintained constant but the molecular weight is dramatically increased (say by a factor of around 100), will the position of that discontinuity be dramatically affected? This point was investigated by preparation of ternary phase diagrams in an identical fashion, maintaining, as far as possible the alternating fully carboxylated PSMA structure but increasing the molecular weight from 1.6K to 120K.

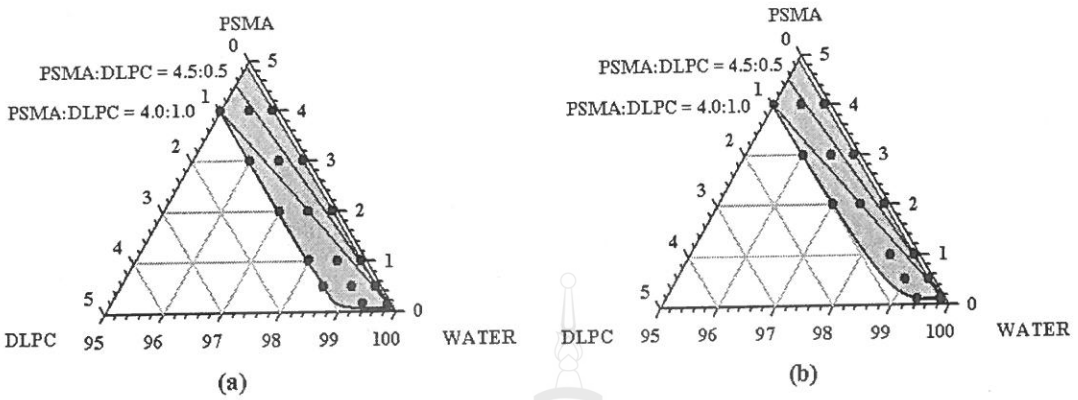

Fig.4.2 Ternary phase diagrams of Water/DLPC/PSMA-120 systems at room temperature at (a) pH 4 and (b) pH 7. The shaded area corresponds to a single transparent phase. The shaded area corresponds to a single transparent phase and the tie-lines represent the constant ratios of PSMA to DLPC.

Fig.4.2a-b show the ternary phase behaviour at room temperature of Water/DLPC/PSMA-120 systems. There are three logical observations. The first and most superficial point is that there is broad correlation between the behaviour of the 120K and 1.6K copolymers in that the 120K compatibility diagram is of the same form. The second is that, comparing Fig.4.1b with Fig.4.2b, in the critical region where discontinuities occur with the 1.6K copolymer, the 120K shows enhanced complex-forming capability. A logical proposition is that the extended alternating sequences in the higher molecular weight polymer form more extensive and more accessible hydrophobic domains on loss of charge. The third point, which underpins this, is that in both 1.6K and 120K systems raising the pH diminishes complexation capability at the high lipid/polymer ratios. This is presumably a feature of the reduction in hydrophobic domains as charge is regained by the PSMA.

4.2 ^{31}P-NMR Spectroscopy

In pursuing the nature of the PSMA-phospholipid interaction, an additional technique to investigate the polymorphic phase transition of hydrated DLPC upon association with PSMA was employed. ^{31}P-NMR spectroscopy is particularly well suited for the study of lipid behavior because the naturally abundant ^{31}P atom provides a sensitive indicator for the structure and dynamics of the phospholipid headgroup. The technique senses the behaviour and environment of the phosphorus atom in the phospholipid headgroup enabling investigation of the conformation and structural dynamics of the phosphate group. It reveals the phospholipid membrane structure without labeling and without disturbing the membrane assembly.[19-24] In general, there are two main ways to obtain information about phospholipid aggregates through ^{31}P-NMR. One involves measurements of the spin-lattice (T_1) and spin-spin (T_2) relaxation times which give the amplitudes and time scale of different motions present in the system. The other requires an analysis of the spectral line shape, which characterizes the topology of each aggregate.[20, 21]

In this study, ^{31}P-NMR spectroscopy was used to examine the polymorphic behaviour of hydrated DLPC upon association with PSMA at different pH values. Since the observed ^{31}P-NMR spectra are characteristic of different lipid phases [19-26], therefore it should be possible to investigate the phase transitions of the phospholipid assemblies upon association with PSMA at different pH by analyzing the ^{31}P-NMR spectral line shapes.

Fig.4.3a-d show proton-decoupled ^{31}P-NMR spectra of aqueous DLPC-PSMA mixtures at room temperature and at pH 10, 6, 5 and 4, respectively. The ^{31}P-NMR spectra of pure DLPC and pure PSMA at pH 4 are also shown for comparison in Fig.4.3e and Fig.4.3f, respectively.

As can be observed in Fig.4.3a, a mixture of DLPC and PSMA at pH 10 shows two spectral peaks, one at chemical shift (δ) around -10 ppm and another at zero chemical shift. This suggests that there are two distinct populations of lipid

experiencing different motional environments. The NMR spectral line shape at δ= -10 ppm, Fig.4.3a, indicates a lamellar organization for the DLPC in the presence of PSMA. This type of spectrum is typically characteristic for phosphatidylcholine in excess water.[20, 24, 26-28] The narrow symmetrical NMR spectrum at δ=0 ppm observed in Fig.4.3a also suggests the presence of another isotropic phase (e.g. micellar and small vesicular). This isotropic phase is a phase where phosphate heads undergo rapid isotropic averaging motion, which produces a narrow symmetrical ^{31}P-NMR spectrum. Narrow signals at isotropic shift value have also been observed in phospholipid systems by many authors and under various conditions.[20, 21, 23, 26]

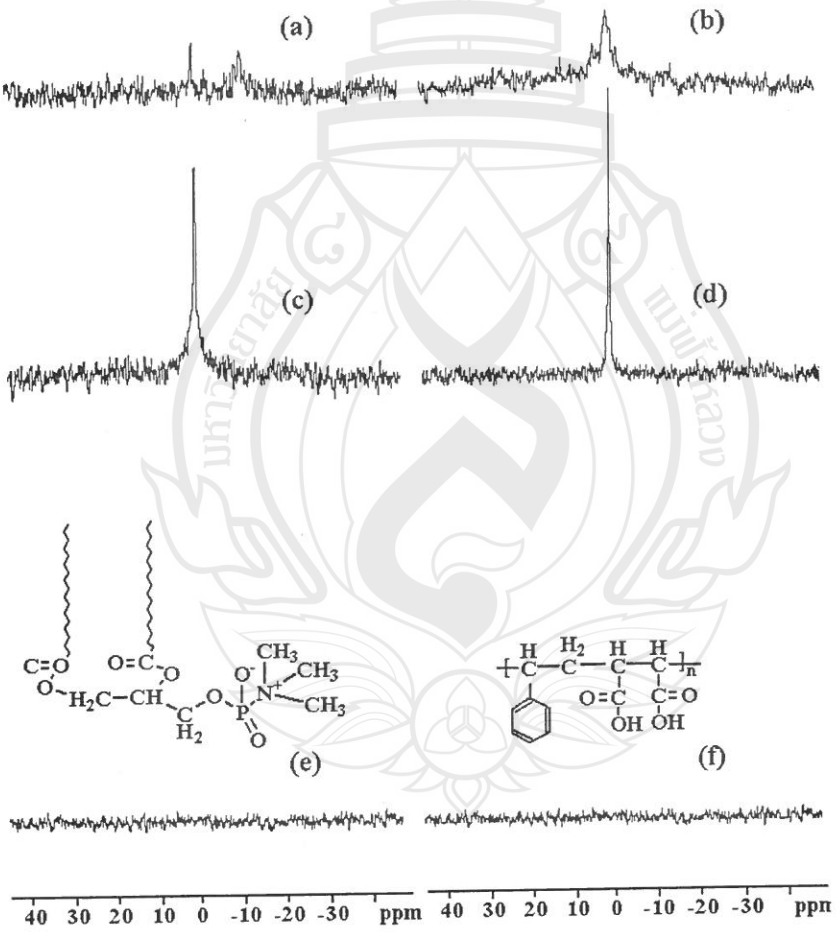

Fig.4.3 Proton-decoupled ^{31}P-NMR spectra of aqueous mixtures of DLPC and PSMA-1.6 at room temperature and at (a) pH 10, (b) pH 6, (c) pH 5 and (d) pH 4. Spectra for all mixtures were recorded at lipid and polymer concentrations of 5 and 30 mg/ml, respectively. The ^{31}P-NMR spectra of pure DLPC (5 mg/ml) and pure PSMA (30 mg/ml) at pH 4 were also shown for comparison along with their chemical structures in (e) and (f), respectively.

Upon lowering the solution pH from 10 to 6, a broad ^{31}P-NMR peak appears at δ between +10 and -5 ppm, (Fig.4.3b). The peak observed at pH 6 is believed to be a result of the superposition of individual peaks; one corresponding to isotropic phase (δ=0 ppm) and another corresponding to anisotropic lipid phase (δ= +10 ppm). The isotropic phase coexists once again with anisotropic phase. However, this time the isotropic phase possibly coexists with other type of anisotropic phase in stead of a lamellar. This is evidenced by the disappearance of the lamellar peak at around δ= -10 ppm, as observed in Fig.4.3b. It is important to note that apart from the micellar structure, other isotropic structures that give rise to a narrow isotropic peak include the small vesicles, cubic, rhombic and inverted micellar structures.[19, 22, 26]

Further decreasing the pH of the DLPC-PSMA mixture towards pH 5 causes the anisotropic peak to disappear and only the isotropic peak at δ=0 ppm remains, (Fig.4.3c). As the pH of the mixture is further decreased to pH 4, these changes are even more pronounced, as illustrated by a sharper isotropic resonance with higher peak intensity, (Fig.4.3d). The fact that the ^{31}P-NMR spectra for DLPC-PSMA at pH 4 and pH 5 shows only narrow signals at isotropic shift value demonstrates that at these pH values, the lipid-polymer complexes favor forming micelles, small vesicles or other isotropic structures. The anisotropic phases may no longer exist under these conditions. The ^{31}P-NMR spectra of unsonicated DLPC dispersion (5 mg/ml) and pure PSMA solution (30 mg/ml) at pH 4 are presented in Fig.4.3e and Fig.4.3f, respectively. It is notable that neither sample shows any detectable NMR resonance. The obvious explanation for the PSMA sample is that it does not have any phosphorus nuclei to give rise to a ^{31}P-NMR spectrum. The case of the DLPC sample may be explained by the molecular packing constraint of the lipid.

The pH-dependent membrane disruptive activity of PSMA-1.6 is believed to be associated with the conformational transition of the polymer. The protonation of the free carboxylic groups of PSMA triggers this transition from an expanded conformation at high pH values to a relatively hydrophobic globular coil in acidic solution. The collapsed polymer chain then provides an increased number of hydrophobic sites which enhance polymer adsorption to the phospholipid and so the

ability of PSMA to disrupt the DLPC assembly. The reorganization of phosphatidylcholine vesicles upon adsorption of polymer has been observed earlier in many different systems.[29, 30] The question of whether increasing the polymer hydrophobic moiety (achieved by either increasing polymer chain length or introducing of different ratios of esterified alkyl chains of various lengths to PSMA) affects the pH-dependent membrane disruptive activity is of interest. To investigate this question, further experiments were carried out, replacing PSMA-1.6 with PSMA-350. It is important to mention that this polymer contains 10-15 % methyl ester moieties and has a chain length more than 100 times that of PSMA-1.6. On the basis of the foregoing phase equilibrium studies it was anticipated that PSMA-350 would provide more hydrophobic binding sites for the lipid and so possess a greater ability to destabilize the lipid assemblies.

Fig.4.4a and Fig.4.4b show proton-decoupled ^{31}P-NMR spectra of the mixtures containing PSMA-350 at room temperature at pH 7 and pH 4, respectively. As can be observed in Fig.4.4a-b, the mixtures at both pH give only the sharp isotropic peaks at δ=0 ppm, demonstrating that at these pH values PSMA-350 completely destabilizes and then associates with the lipid assemblies. The results suggest that in both cases the mixed complexes favor forming micellar or other isotropic structures. In contrast to the results obtained in the DLPC/PSMA-1.6 system, PSMA-350 can successfully induce the lipid to undergo anisotropic-to-isotropic phase transition even at physiological pH values, confirming a greater ability of PSMA-350 to destabilize the lipid assemblies. As expected, hydrophobic interaction is thus one of the factors dominating the association, and therefore, the formation of DLPC-PSMA complexes.

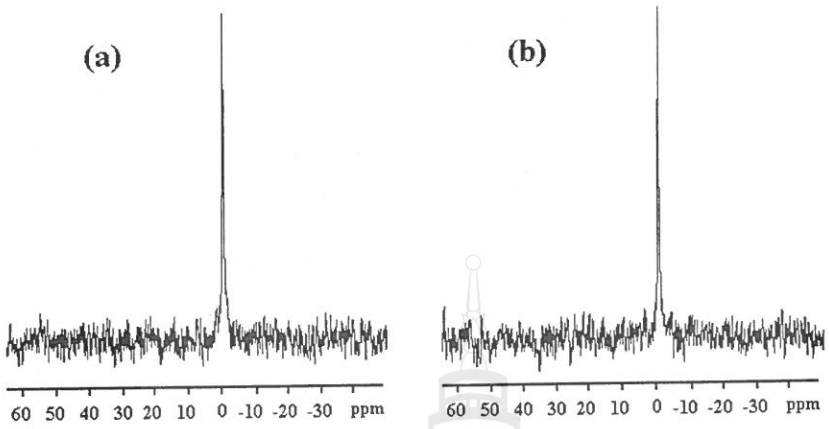

Fig.4.4 Proton-decoupled ^{31}P-NMR spectra of aqueous mixtures of DLPC and PSMA-350 at room temperature and (a) at pH 7 and (d) at pH 4. Spectra for all mixtures were recorded at lipid and polymer concentrations of 5 and 30 mg/ml, respectively.

4.3 Potentiometric Titration Study

The association behavior of PSMA-1.6 and DLPC was examined using potentiometric titration. It was expected that the results obtained would provide useful information concerning conformational changes of PSMA during ionization and then the effects of these changes on association with DLPC. It is worth mentioning here that titration of PSMA solution with HCl solution, which generally results in reduction of polymer charge density, induces the polymer to undergo conformational transition from an uncoiled to a more compact conformation. A number of studies of PSMA compact coils have shown that hydrophobic interaction has a definite role to play in the stabilization of the globules. Intramolecular hydrogen-bonding of the polymer can also contribute to the stability.

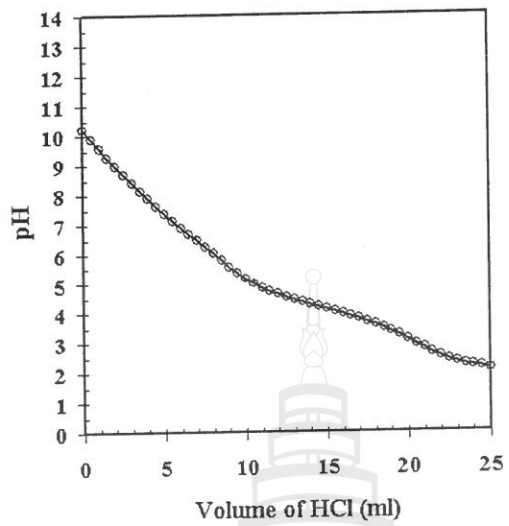

Fig.4.5 Titration curve of PSMA-DLPC mixture with 0.1 M HCl as titrant. Concentrations by weight of PSMA-1.6 and DLPC used are 3.0 and 0.5%, respectively.

Fig.4.5 shows the titration curve of DLPC/PSMA-1.6 mixture with 0.1 M HCl solution. It is found that the titration curve of the mixture shows a second inflection point at pH~4.5, similarly to that of hydrolyzed PSMA solution (3.0 % weight by volume) (data not shown). The fact that PSMA-DLPC system shows no shift of inflection point suggests that PSMA-DLPC association does not occur through intermolecular hydrogen-bonding between carboxyl group of the polymer and the phosphodiester of the lipid head groups. Instead, it is consistent with the view that the cooperative association occurs through hydrophobic interaction between the distinct hydrophobic microdomains of the polymer and aliphatic side chains of DLPC. The work by Watanabe et al.[31] draws the opposite conclusion for the poly(*2*-ethylacrylic acid) (PEAA) and dipalmitoylphosphatidylcholine (DPPC). Their results showed that DPPC significantly shifted the inflection point of PEAA from 6.1 to 6.6, implying that the polymer associates with the lipid membrane surface through hydrogen-bonding interaction. The origin of this difference in behaviour of PSMA and PEAA is almost certainly associated with the increased hydrophobic character of the PSMA in comparison with PEAA.

4.4 Particle Size and Morphological Observation

The average diameter and size distribution (polydispersity index) of selected PSMA-DLPC complexes were measured by Dynamic Light Scattering (DLS). Examination of Fig.4.1a-d indicates that, although for PSMA-1.6 a ratio of polymer/lipid of 1:1 gave stable complexes under some conditions, higher ratios were necessary to ensure stability under all conditions. A PSMA/DLPC ratio of 6:1 was therefore selected as representative of a universally stable composition. The results are shown in Table 3.

Table 3 Composition and physicochemical properties of DLPC/PSMA-1.6 complexes.

Composition (mg/mL)		Polymer/Lipid		pH 4		pH 7	
DLPC	PSMA	Mass ratio	Mole ratio	Diameter (nm)	PDI	Diameter (nm)	PDI
5	30	6 : 1	2:1	16 414	0.324 0.586	53 543	0.252 0.221

The first set of data to consider are those relating to DLPC/PSMA-1.6 systems, at pH 4. The complexes appear at 16 nm with a polydispersity index (PDI) of 0.324. These correspond to the simplest mixed assembly. A "disc-like" model has previously been used to illustrate this simplest molecular arrangement. The model, supported by Cryo-Transmission Electron Microscopy work[4, 10, 12], envisages that the mixed assembly consists of minute sheets of lipid surrounded by the amphiphatic polymer in a doughnut arrangement with the lipid contained within the center of the structure. The schematic illustration of this doughnut arrangement is shown in Fig.1.5. A mean diameter of around 16 nm for PSMA-DLPC assemblies (at pH 4) obtained from this study is consistent with the previous work. The dynamic light scattering data for the system at pH 4 also shows a second type of aggregation. This aggregate, characterized by a higher mean diameter (~400 nm) with a broad size distribution, may be formed by further association of the simplest entities into lager structures. The pH neutralization of DLPC/PSMA-1.6 (e.g. by raising the pH solution to 7) increases the sizes from 16 to 53 nm for a single particle and from 414 to 543 nm for the

agglomerated particle (Table 3). The explanation for this phenomenon is logically associated with the conformational changes in the PSMA molecule, from a more compact to an expanded chain conformation upon deprotonation of carboxylic acid groups along the polymer backbone.

Fig.4.6 shows negative staining TEM micrographs of DLPC/PSMA complexes at pH 4. A single nanostructure of the complexes, pointed by the black arrows in Fig.4.6, appears disk-like in structure with the size ranging between 15 and 25 nm, which is consistent with the average diameter of 16 nm obtained from dynamic light scattering. The aggregation of such small nanostructures into larger structures can be observed as interconnected white regions in Fig.4.6a-b. The mechanism of such aggregation is not well known yet. It is possible that the simplest structures may connect with each other through hydrophobic attraction between the unassociated polymer segments. This natural occurring phenomenon, observed from TEM, supports the potentially aggregation of simplest nanostructures into larger supramolecular structure (414 nm) previously reported by a dynamic light scattering measurement (Table 3).

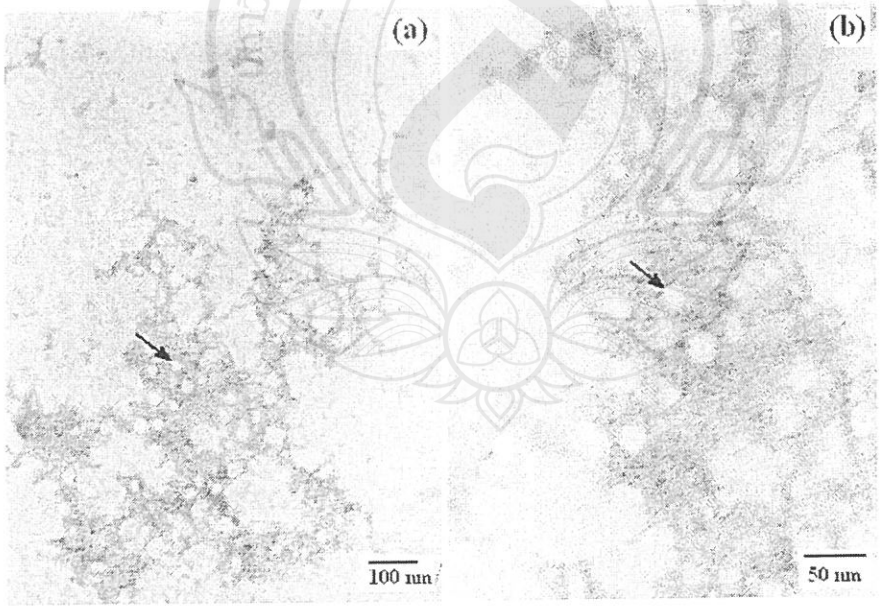

Fig.4.6 Negative staining TEM micrographs of uranyl acetate stained DLPC/PSMA-1.6 complexes at pH 4 (mass ratio of polymer/lipid = 6:1). Black arrows point the single nanostructure of the complexes in (a) and (b). The pictures indicate aggregation of single nanostructures into larger interconnected white regions.

CHAPTER 5
CONCLUSIONS AND FURTHER WORKS

5.1 Conclusions

Hypercoiling polymers can be said to mimic the behaviour of some of the functions that account for the essential living processes, and therefore, be suited for application to living systems and in particular to biomedicine. In aqueous media, at least over a particular pH range, the associating polymers such as poly(styrene-*alt*-maleic anhydride) (PSMA) will generally adopt a helical coil configuration with the hydrophobic side chain groups presented along one facet and the anionic hydrophilic groups presented along the opposite facet. This smart behaviour can mimic that of native apoproteins that arrange their hydrophobic and hydrophilic group at opposite facets of α-helical coil to form an amphipathic structure. When these hypercoiling polymers are combined with film-forming lipid such as dilauroylphosphatidylcholine (DLPC), they associate to produce polymer-lipid nanostructures analogous to lipoprotein assemblies such as HDL (high density lipoprotein) present in the blood plasma and responsible for transporting the fatty materials around the body. As such, they represent a new biomimetic delivery vehicle for fatty or water insoluble substances for both pharmaceutical and cosmetic industries.

One goal of this study was to develop a better understanding of polymer-lipid association, especially in the system of PSMA and DLPC. The details concerning this interaction may lead to the development of a more efficient drug delivery system, particularly, for poorly water-soluble drugs. Such details were previously described in Chapters 4. The ternary phase diagrams of the water-DLPC-PSMA system were constructed as a mean to represent the formation of polymer-lipid complexes and to examine the effects of various factors such as neutralization procedure (via increasing of pH solution), sterilization (via autoclaving) and nature of the polymer (e.g., hydrophobicity, chain length etc.), on the association of lipid and

this pseudo protein. Results showed that the DLPC-PSMA association was dependent on the molecular ratios between polymer and phospholipid, heat treatment, pH solution. Moreover, the polymer architecture and the polymer molecular weight have also been found to play an important role for the formation of these complexes.

Other physical and spectroscopic techniques such as the ^{31}P-NMR and potentiometric titration were also used in this study. ^{31}P-NMR was found to be very useful in elucidating the different phases of phospholipid (DLPC) in the presence of PSMA copolymer under different conditions. More specifically, it was found that the strong interactions between the polymer and DLPC result in the disappearance of the bilayer structure and the formation of the isotropic phases which give rise to the isotropic peaks in the ^{31}PNMR spectra. Changes in lipid molecular shapes, which are considered as the driving force for the bilayer-nonbilayer phase transition of DLPC, were assumed to be correlated with the changes in the packing parameters. This alteration should lead to a bilayer structural change as well as a lipid phase transition. Results from potentiometric titration confirmed that the association between PSMA and DLPC under acidic conditions occurs mainly through hydrophobic interaction between the distinct hydrophobic microdomains of the polymer and aliphatic side chains of DLPC.

Average size, size distribution and morphological details of PSMA-DLPC complexes were obtained by DLS and negative-staining TEM. The results obtained proved the formation of nanoscale PSMA-DLPC complex, in consistent with previous works, and the aggregation of such complex into larger supramolecular assemblies. Furthermore, particle size and molecular arrangement of the complexes were found to be dependent on pH solution, as evidenced by the DLS and TEM results.

5.2 Suggestions for Further Works

Some important properties of the polymer-lipid complexes, such as phase behavior, lipid phase transformation, size and morphological details have been

established through this research, however, other crucial information will obviously be needed in order to gain a better understanding and provide a better scientific foundation for future development. To obtain such details, additional experiments will need to be performed and these will be outlined in the subsequent topics.

5.2.1 *In Vitro* Cytotoxicity of the Complexes

Cytotoxicity studies of PSMA and DLPC have shown that these compounds are biologically safe. However, it is also necessary to establish the non-toxicity of PSMA-DLPC complexes. Cytotoxicity can be measured by the MTT assay, Trypan blue (TB) assay, Sulforhodamine B (SRB) assay, WST assay and clonogenic assay. A colorimetric assay using MTT was first introduced by Mossman as a quantitative measurement of mammalian cell survival and proliferation. It is a laboratory test and a standard colorimetric assay for measuring cellular activity. MTT assay is used to determine cytotoxicity of potential medicinal agents and other toxic materials. MTT is a yellow tetrazolium salt which can be converted into a blue formazan by dehydrogenases of a live cell. The assay is based on the principle that the amount of formazan produced, which can be determined by a spectrophotometer, is directly proportional to the number of live cells

5.2.2 Study of Drug Release

In order to determine if the drug can be encapsulated and then diffuse out of the polymer-lipid complexes, additional drug release experiments will need to be performed. An alternative method is dialysis, in which the loaded vesicles are separated from the bulk medium by a dialysis membrane. The drug release into the bulk medium occurs through the membrane. At a predetermined period of time, a sample is withdrawn and the concentration of the drug present in the sample is then determined by spectrophotometry. Dialysis membranes with varying molecular weight exclusion cutoffs (MWCOs) and compositions are widely used in a variety of fields.

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Bibliography

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2. Education

2.1 Schools

Period	Institution
1983-1988	Primary school at Anuban Chiang Mai school, Chiang Mai, Thailand
1989-1991	Secondary school (M1-M3) at Mukdahan school, Mukdahan, Thailand
1992-1994	Secondary school (M4-M6) at Yuparaj Vittayalai School, Chiang Mai, Thailand

2.2 Higher Education

Period	Degree	Institution	Year of Graduation
1995-1998	B.Sc. (Honors) Major Chemistry	Chiang Mai University, Thailand	1998
1999-2001	M.Sc. Major Chemistry	Chiang Mai University, Thailand	2001
2003- 2008	PhD in Polymer Science	Aston University, UK	2008

Note: From 1999-2001, I received a scholarship from National Metal and Materials Technology Center, Thailand.

From 2003 to 2008, I was funded by Mae Fah Luang University and The Royal Thai Government for my PhD study.

3. Works and Training Experience

- *From October 1998 to December 1998*, I was trained at Teijin Polyester (Thailand) Co., Ltd. in the manufacturing, the quality control and the product inspection units. I was assigned to solve the instability problem of the polyester chips in the manufacturing unit. From that, I gained a wonderful experience presenting my solution to a group of senior Japanese researchers.
- *On August 18, 1999 (National Science Day)*, I did a laboratory demonstration at Chiang Mai University. On that day, I gained a valuable experience of teaching a large group (over 300) of young scientists.
- *From June 2000 to September 2000*, I worked as postgraduate laboratory assistant at Chiang Mai University, Thailand. I gave the short lectures 'General Chemistry' and 'Advance Organic Chemistry'.

4. Previous and Current Jobs

- *From February 2002-August 2003*, I was a lecturer of Chemistry at Mae Fah Luang University, Thailand.

- *From July 2002 August 2003*, I gave the special lectures for final year students at Chiang Rai Rajchabath University in Physical Chemistry.
- *From March 2008-Present*, I have been a lecturer of Polymer Science at Mae Fah Luang University, Thailand

5. Teaching Experience

- General Chemistry
- Principles of Chemistry
- Principles of Chemistry (Laboratory)
- Physical Chemistry
- Analytical Chemistry
- Organic Chemistry
- Science for Life
- Introduction to Material Science
- Introduction to Applied Chemistry
- Structure and Physical Properties of Polymer
- Polymer Synthesis and Characterization (Laboratory)
- Polymers in Solutions
- Specialty Polymers
- Biomedical Polymers

6. Academic Thesis

6.1 Bachelor Program's Special Project

Special Project Title:

Synthesis and Characterization of Block Copolymer of Caprolactone and *L*-Lactide

Abstract:

Diblock and triblock copolymers of ε-caprolactone and *l*-lactide were synthesized by ring-copolymerization of ε-caprolactone and *l*-lactide (1:9 mol ratio) using stannous

octoate as catalyst with *n*-butanol and diethylene glycol as initiators. In the first stage of synthesis, ε-caprolactone was polymerized to give a polycaprolactone prepolymer with hydroxyl end groups. This was then followed, in the second stage, by polymerization of l-lactide initiated by the prepolymer to give the respective block copolymers. The reaction was carried out under a dry nitrogen atmosphere. Determination of the average molecular weight gave $\overline{M_n}$ and $\overline{M_v}$ values for the butanol-initiated polycaprolactone of 5,145 and 9,690 g/mol compared with 6,688 and 12,670 g/mol for the diethylene glycol-initiated polycaprolactone. The $\overline{M_n}$ and $\overline{M_v}$ values for the diblock final copolymers were 8,060 and 13,770 and for the triblock final copolymers were 12,160 and 15,370 g/mol. Compositional analysis of the diblock and triblock copolymers by IR spectroscopy gave mole ratios of *l*-lactide:ε-caprolactone of 9.3:0.7 and 9.0:1.0 while ^{1}H-NMR gave values of 8.4:1.6 and 8.3:1.7, respectively. A combination of TG and DSC thermal analysis techniques showed the presence of copolymers but more information is needed to confirm whether the final products are pure block copolymers or contain some homopolymeric poly(*l*-lactide) in admixture.

6.2 Master Program's Thesis

Thesis Title:

In Vitro Hydrolytic Degradation Studies of Absorbable Monofilament Surgical Sutures

Abstract:

In this research project, the in vitro hydrolytic degradation of 3 commercial synthetic absorbable monofilament surgical sutures, marketed under the trade names of MONOCRYL, MAXON and PDS II, were studied and compared with a random terpolymer of *l*-lactide, ε-caprolactone and glycolide. The terpolymer, poly(*l*-lactide-*ran*- ε-caprolactone-*ran*-glycolide), PLCG (68:21:11 mol %), was melt spun into a monofilament fiber of approximate diameter 0.3 mm using a small-scale fiber extrusion apparatus. The samples were immersed in a phosphate buffer saline (PBS) solution at an initial physiological pH of 7.40 ± 0.01 and maintained at a temperature of 37.0 ± 1.0°C in an incubator. Their hydrolytic degradation was followed via the

changes in weight, tensile strength, melting point, heat of melting and surface appearance which occurred with time. From the results obtained, both the PLCG and MONOCRYL samples showed similar rates of weight and tensile strength reduction, both faster than MAXON and PDS II. Based on these results, a mechanism for the in vitro hydrolytic degradation could be described in terms of the physical and chemical processes taking place. The differences in the property loss-time profiles of the PLCG and commercial sutures could be related to their differences in chemical microstructure and semi-crystalline morphology.

6.3 Doctoral Program's Thesis

Thesis Title:

Synthetic Analogues of Protein-Lipid Complexes

Abstract:

Hypercoiling poly(styrene-*alt*-maleic anhydride) (PSMA) is known to undergo conformational transition in response to environmental stimuli. This responsive behavior makes it possible to mimic structural aspects of native apoproteins. The association of PSMA with lipid *2*-dilauryl-*sn*-glycero-*3*-phosphocholine (DLPC) produces polymer-lipid complex analogues to lipoprotein assemblies found in lung surfactant. These complexes represent a new bio-mimetic delivery vehicle with applications in the cosmetic and pharmaceutical industries. The primary aim of this study was to develop a better understanding of PSMA-DLPC association by using physical and spectroscopic techniques. Ternary phase diagrams were constructed to examine the effects of various factors, such as molecular weight, pH and temperature on PSMA-DLPC association. ^{31}P-NMR spectroscopy was used to investigate the polymorphic changes of DLPC upon associating with PSMA. The Langmuir Trough technique and surface tension measurement were used to explore the association behavior of PSMA both at the interface and in the bulk of solution, as well as its interaction with DLPC membranes. The ultimate aim of this study was to investigate the potential use of PSMA-DLPC complexes to improve the bioavailability and therapeutic efficacy of a range of drugs. Typical compounds of ophthalmic interest range from new drugs such as Pirenzepine, which has attracted clinical interest for the control of myopia progression, to the well-established family of non-steroidal anti-

inflammatory drugs. These drugs have widely differing structures, sizes, solubility profiles and pH-sensitivities. In order to understand the ways in which these characteristics influence incorporation and release behavior, the marker molecules Rhodamine B and Oil red O were chosen. PSMA-DLPC complexes, incorporated with marker molecules and Pirenzepine, were encapsulated in hydrogels of the types used for soft contact lenses. Release studies were conducted to examine if this smart drug delivery system can retain such compounds and deliver them at a slow rate over a prolonged period of time.

7. Research Interests

- Hydrogel-based systems for controlled drug release
- Therapeutic applications of contact lenses
- Pharmaceutical applications of synthetic protein-lipid complex analogues
- Self-assembling of macromolecules for controlled release of poorly water-soluble drugs
- Silk proteins for biomedical applications

8. Publications

- P. Punyamoonwongsa and B. Tighe, A Smart Hydrogel-Based System for Controlled Drug Release, *Chiang Mai Journal of Science*, 32 (2005) 471-478.
- P. Punyamoonwongsa and B.Tighe, Association Behavior of Synthetic Protein-Lipid Complex Analogues, *submitted to Journal of Colloid and Polymer Science*.
- P. Punyamoonwongsa, Molecular Interactions between Styrene Maleic Anhydride Copolymers and Phospholipid Membrane in solution and at the Air-Water Interface, *to be submitted to Langmuir*.
- P. Punyamoonwongsa, Biomimetic Protein-Lipid Complex Analogues in Ophthalmic Drug Delivery Applications, *to be submitted to Advance Drug Delivery Review*.

9. Proceedings

- P. Punyamoonwongsa and B.Tighe, Therapeutic Applications of Contact Lenses in Myopia Control. Proceedings of "28th Conference of the British Contact Lens Association", Birmingham, UK, May 24-27, 2004. Appeared in *Ophthalmic Res.* 2004; 36: (S1):83. ISSN 0030-3747.
- P. Punyamoonwongsa and B.Tighe, Therapeutic Applications of Contact Lenses in Myopia Control (**prize winner poster**). Proceedings of "Annual European Eye and Vision Research Meeting", Vilamoura, Portugal, September 24-27, 2004. Appeared in *Contact Lens and Anterior Eye*. 2004; 27:125.
- P. Punyamoonwongsa and B. Tighe, Therapeutic Application of Contact Lenses: Myopia and Allergic Eye Disease. Proceedings of the "29th Conference of the British Contact Lens Association", Brighton, UK, June 3-5, 2005. Appeared in *Ophthalmic Res.* 2005; 37: (S1):31. ISSN 0030-3747.
- P. Punyamoonwongsa and B. Tighe, pH-Responsive Hydrophobically-Associating Micellar Structure for Ophthalmic Drug Delivery. Proceedings of the "13th Symposium on the Material Science and Chemistry of Contact Lenses", New Orleans, US, November 29-December 1, 2006.
- P. Punyamoonwongsa and B. Tighe, Therapeutic Applications of Contact Lenses: pH-Responsive Hydrophobically Associating Micellar Structure for Ophthalmic Delivery of Pirenzepine. Proceedings of the "31st Conference of the British Contact Lens Association", Manchester, UK, May 31-June 3, 2007. Appeared in *Ophthalmic Res.* 2007; 39: (S1). ISSN 0030-3747.

10. Conference Presentation

- *May 2004,* poster presentation in "Therapeutic Application of Contact Lenses in Myopia Control" at the "28th Conference of the British Contact Lens Association", Birmingham, UK,
- *September 2004,* oral and poster presentations in "Therapeutic Application of Contact Lenses in Myopia Control" at the "European Association for Vision and Eye Research", Vilamoura, Portugal.

- *January 2005,* oral presentation in "A Smart Hydrogel-Based System for Controlled Drug Release" and poster presentation in "Processing – Microstructure-Property Relationships in Monofilament Polyester Fibers for Use as Absorbable Surgical Sutures" at the "International Conference on Smart Materials", Chiang Mai, Thailand.
- *February 2005*, invited speaker, 'A Smart Hydrogel-Based System for Controlled Drug Release: Nanotechnology Approach', at Chiang Mai University, Thailand.
- *June 2005*, poster presentation in "Therapeutic Applications of Contact Lenses: Myopia and Allergic Eye Disease" at the "29th Conference of the British Contact Lens Association", Brighton, UK,
- *September 2005*, poster presentation in "pH-Responsive Hydrophobically-Associating Micellar Structures for Ophthalmic Delivery of Pirenzepine" at an "International Conference on Bionanotechnology Research", Brighton, UK.
- *December 2006*, poster presentation in "pH-Responsive Hydrophobically-Associating Micellar Structures for Ophthalmic Drug Delivery" at the "13th Symposium on the Material Science and Chemistry of Contact Lenses", New Orleans, US
- *May 2007*, poster presentation in "Therapeutic Applications of Contact Lenses: pH-Responsive Hydrophobically Associating Micellar Structures for Drug Delivery" at the "31st Conference of the British Contact Lens Association", Manchester, UK.