การพัฒนาชุดตรวจวินิจฉัยสำหรับการตรวจหาโปรตีนจำเพาะของแบคทีเรียก่อโรคฉี่หนูโดยใช้อนุพันธ์ไคโตซานที่ละลายน้ำ

นางสาวนฤมล สังฆะพงศ์

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Development of diagnostic kit for detection of pathogenic Leptospira- specific protein by water soluble chitosan derivatives

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	Development of diagnostic kit for detection of pathogenic Leptospira- specific protein by water soluble chitosan derivatives
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นฤมล สังฆะพงศ์ : การพัฒนาชุดตรวจวินิจฉัยสำหรับการตรวจหาโปรตีนจำเพาะของแบคทีเรียก่อโรคฉี่หนูโดยใช้อนุพันธ์ไคโตซานที่ละลายน้ำ (Development of diagnostic kit for detection of pathogenic Leptospira- specific protein by water soluble chitosan derivatives) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. วันเพ็ญ เตชะบุญเกียรติ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. พญ. กนิษฐา ภัทรกุล, ผศ. ดร. นพ. อมรพันธุ์ เสรีมาศพันธุ์, 40 หน้า.

พอลิเมอร์ที่มีความสามารถในการตอบสนองต่อสิ่งเร้าได้มีการศึกษาอย่างแพร่หลายเพื่อการนำไปใช้งานในหลายด้าน งานวิจัยนี้มีจุดประสงค์เพื่อนำอนุพันธ์ของไคโตซานที่มีความสามารถละลายน้ำคอนจูเกตกับแอนติเจนหรือแอนติบอดีสำหรับการตรวจหาโปรตีนจำเพาะที่ก่อให้เกิดโรคฉี่หนูอย่างรวดเร็ว ขั้นแรกอนุพันธ์ฟอสฟอริเลตเตดไคโตซานที่ละลายน้ำถูกสังเคราะห์โดยปฏิกิริยาฟอสฟอริเลชันของไคโตซานกับฟอสฟอรัสเพนทอกไซด์ในกรดมีเทนซัลโฟนิค ฟูเรียร์ทรานสฟอร์มอินฟราเรดสเปกตรัมของอนุพันธ์ฟอสฟอริเลตเตดไคโตซานแสดงพีค P=O ที่ 1260 cm^{-1} และ P-O-C ที่ 800 cm^{-1} โดยระดับการแทนที่ของหมู่ฟอสเฟตต่อหน่วยของไคโตซานวิเคราะห์ด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดชนิดแจกแจงพลังงานรังสีเอกซ์มีค่าประมาณ 0.08 LipL32 และโมโนโคลนอลแอนติบอดี (MAb) ได้ถูกเลือกเพื่อใช้เป็นตัวแทนของแอนติเจนและแอนติบอดี จากนั้น LipL32 และ MAb ถูกคอนจูเกตบนฟอสฟอริเลตเตดไคโตซานด้วยพันธะเปปไทด์โดยใช้สารเชื่อมโยง EDC/NHS ในน้ำที่อุณหภูมิ 0-4 องศาเซลเซียสเป็นเวลา 12 ชั่วโมง การเกิดคอนจูเกตระหว่าง LipL32 และ MAb กับอนุพันธ์ฟอสฟอริเลตเตดไคโตซานถูกยืนยันด้วยเทคนิคฟูเรียร์ทรานสฟอร์มอินฟราเรดสเปกโตรสโคปีและ SDS-PAGE เจลอิเล็กโทรโฟริซิส จากฟูเรียร์ทรานสฟอร์มอินฟราเรดสเปกตราของฟอสฟอริเลตเตดไคโตซาน-LipL32 และฟอสฟอริเลตเตดไคโตซาน-MAb พบพีคจำเพาะของ amide I ที่ 1655 cm-1 และ amide II ที่ 1555 cm-1 ชี้ให้เห็นว่าการคอนจูเกชันสำเร็จที่หมู่อะมิโนของฟอสฟอริเลตเตดไคโตซาน นอกจากนี้ SDS-PAGE เจลอิเล็กโทรโฟริซิสแสดงการหน่วงแถบในช่วงกว้างของน้ำหนักโมเลกุลพร้อมกับการลดลงของหมู่อะมิโน ชี้ให้เห็นว่าเกิดพันธะโควาเลนต์ ค่าไอโซอิเล็กทริก (pI) ของฟอสฟอริเลตเตดไคโตซาน-LipL32 และฟอสฟอริเลตเตดไคโตซาน-MAb เลื่อนจากของฟอสฟอริเลตเตดไคโตซานที่ 5.80 ไปเป็น 10.64 และ 9.47 ตามลำดับ ฟอสฟอริเลตเตดไคโตซาน-LipL32 และ คอมเพล็กซ์ของฟอสฟอริเลตเตดไคโตซาน-LipL32กับฟอสฟอริเลตเตดไคโตซาน-MAb แสดงการจับจำเพาะเมื่อมี LipL32 แอนติเจนอิสระ โดยการตกตะกอนอย่างรวดเร็วภายในเวลา 1 นาที จากผลการทดสอบคาดว่าฟอสฟอริเลตเตดไคโตซาน-LipL32 และ คอมเพล็กซ์ของฟอสฟอริเลตเตดไคโตซาน-LipL32 กับฟอสฟอริเลตเตดไคโตซาน-MAb สามารถใช้เป็นชุดตรวจวินิจฉัยรวดเร็วสำหรับตรวจหาแอนติเจนของแบคทีเรียก่อโรคฉี่หนูได้

ภาควิชา	วัสดุศาสตร์	ลายมือชื่อนิสิต .....
สาขาวิชา	วิทยาศาสตร์พอลิเมอร์ประยุกต์และเทคโนโลยีสิ่งทอ	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
ปีการศึกษา	2559	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5672168623 : MAJOR APPLIED POLYMER SCIENCE AND TEXTILE TECHNOLOGY
KEYWORDS: PHOSPHORYLATED CHITOSAN / BIOMEDICAL APPLICATION / CHITOSAN WATER SOLUBLE / DIAGNOSIS / LEPTOSPIROSIS

NARUEMON SANGKAPONG: Development of diagnostic kit for detection of pathogenic Leptospira- specific protein by water soluble chitosan derivatives. ADVISOR: ASST. PROF. WANPEN TACHABOONYAKIAT, Ph.D., CO-ADVISOR: ASST. PROF. KANITHA PATARAKUL, M.D., Ph.D., ASST. PROF. AMORNPUN SEREEMASPUN, M.D., Ph.D., 40 pp.

Responsive polymers have been studied intensively in various field of applications. The aim of this study was to use water soluble chitosan derivatives conjugated with antigen or antibody for rapid detecting Leptospira-specific protein. First, water soluble phosphorylated chitosan (PCTS) was synthesized by phosphorylation of chitosan with phosphorus pentoxide in methane sulfonic acid. Fourier transform infrared (FT-IR) spectrum of PCTS revealed peaks of phosphoric groups at 1260 cm^{-1} (P=O stretching) and 800 cm^{-1} (P-O-C). Degree of phosphate substitution was determined of around 0.08 by scanning electron microscope equipped with energy-dispersive X-ray spectrometer (SEM-EDX). LipL32 and monoclonal antibody (MAb) were selected as antigen and antibody respectively. Then, LipL32 and MAb were conjugated onto PCTS through peptide linkage by using EDC/NHS as coupling agent in DI aqueous solution at 0-4°C for 12 hrs. The conjugation of PCTS with LipL32 and MAb was confirmed by FTIR and SDS-PAGE gel electrophoresis. FTIR spectra of PCTS-LipL32 and PCTS-MAb revealed characteristic peaks of amide I at 1655 cm^{-1} and amide II at 1555 cm^{-1} indicating successful conjugation at amino groups of PCTS. Furthermore, SDS-PAGE gel electrophoresis shown the band retardation in wide range of molecular weight with decreasing in amino groups, indicating covalently conjugation. Isoelectric point of PCTS-LipL32 and PCTS-MAb were also shifted from 5.80 (PCTS) to 10.64 and 9.47, respectively. PCTS-MAb and PCTS-LipL32/PCTS-MAb complex exhibited specific binding in the presence of LipL32 free antigen by rapidly aggregation within 1 min. This can be concluded that PCTS-MAb and PCTS-LipL32/PCTS-MAb complex could be utilized as rapid diagnostic kit for detecting antigen of leptospires.

Department:	Materials Science	Student's Signature .....
Field of Study:	Applied Polymer Science and Textile Technology	Advisor's Signature .....
		Co-Advisor's Signature .....
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Chapter 1
INTRODUCTION

1.1 Background

Polymers have become the most popular materials used in both everyday life and worldwide, and smart polymer that can be tailored to respond to external changes depend on our demands are developed. Responsive polymers have been increasingly studied in many recent years [1, 2]. They have been extensively reported in fields of biomedical features such as biological delivery [3], pharmaceutical potency and safety [4, 5], disease diagnosis and antigen/antibody biomarker detection [3, 6]. The development of materials to respond environment required creative molecular design and much inspiration that might be come from the nature [7, 8]. Biopolymer have been frequently used for the functional polymers due to their properties including biocompatibility, biodegradability, and biological functionality [9]. The challenging of functional biopolymer structure designs are the highest demand for sensitive, efficient system and approaches that respond to fighting disease [3, 10]. The chemical, physical and morphological properties of biopolymer structures can be custom tailored to increase numerous features that were described such as solubility, size, stability, colloidal aggregation and biological capability.

Chitosan is a deacetylated derivative of chitin which is the most second abundant of polysaccharides in nature consisting of one amino group and two hydroxyl groups in each unit ,whilst chitin is consisted of acetamide group instead of amino group. Chitosan acquires a great attention as a functionalized biomolecule for several medical applications [11, 12]. Because of its biocompatibility, biodegradability and non-toxicity, they are frequently used as drugs and macromolecules carrier [13-15]. It was our interests to conjugate protein on to chitosan which an approach for biomedical

applications such as cholesterol entrapment, antigen-antibody sensor and so on. The active primary amino group on chitosan molecule at C-2 position being reactive sites providing for an attachment peptide and protein. The combination of two biomolecule showed their enhancement properties such as solubility [16], stability [17, 18], molecular bioactivity [19] and low immunogenicity [20]. The facile derivatization makes chitosan an ideal candidate for biofabrication. Various chemical modification methods used for conjugation such as reactive amidation via amine [21-23], esterification or amidation via carboxylic acid [24-26], and click chemistry methods [27, 28].

However, chitosan can be dissolved only in acidic condition which is the main drawback of using chitosan in physiological condition. Therefore, phosphorylated chitosan (PCTS), a water soluble derivative of chitosan synthesized in our laboratory [29], was conjugated with protein to overcome this limitation. Antigen and antibody were selected as protein model in this study.

We intended to conjugate PCTS with antigen and antibody by coupling reaction using water soluble carbodiimide as a coupling agent. The objective of this study is to investigate the conjugation between PCTS and biomolecule by structural characterization with Fourier Transform Infrared Spectroscopy (FTIR), surface charges alteration by measuring zeta potential, and the increase in biomolecule molecular weight after conjugation by observing band retardation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Specific binding ability of conjugated PCTS is performed by adding LipL32 antigen and monitored by UV-Vis spectrophotometer.

Chapter 2
LITERATURE SURVEY

2.1 Responsive polymers

Responsive polymers (i.e. stimuli-responsive polymer, smart polymer, intelligent polymer) are capable with presented reversible or irreversible changes in physical properties and/or chemical structures under environmental stimuli. These materials are increasing interest and have been intensively studied over the years for a wide range of applications in field of biomedical such as sensors and biosensors [30], controlled and triggered drug/biomolecule delivery [31], diagnostics and imaging tissue engineering [32], and so on [33]. The undergo characteristics of responsiveness leading to design of variations of polymeric structures. Responsive polymer especially in solution can show the alterations of their structure such as size, solubility, stability, colloidal aggregation, secondary structure, degree of intermolecular association [34]. The most famous classification of responsive polymer is defined in three categories; physical, chemical and biological.

2.1.1 Physical- responsive polymer

Physical-responsive polymers belong to the class of stimuli-responsive polymer which change their properties with external stimuli such as temperature, mechanical force, electric-field, magnetic- field, ultrasound, light or other physical stimuli [33, 34].

Thermo-responsive polymers can be functionalized with moieties that bind to specific biomolecules. The polymer-biomolecule conjugate can be precipitated from solution by a small change of temperature. Zhao et al.[35] were studied according to thermo-responsive aggregation behavior (LCST) of poly (ethylene oxide) (PEO) conjugated triterpene cholesterol (CE) and betulin (BE). They found that both cholesteryl–PEO (CE) and betulinyl–PEO (BE) comprising less than 70 %(w/v) PEO

precipitated upon heating in water. CE solutions exhibited thermoprecipitation behaviors at room temperature with their aggregates. BE solutions with short chains PEO exhibited precipitated at high temperature and formed hydrogel at low temperature. Thus, it demonstrated dual thermo-responsive behavior, and BE multi-block copolymer was found to form large aggregates in water due to the hydration of short PEO chains. These results revealed that the structure and chemical composition can be controlled the solution properties of triterpene-PEO.

2.1.2 Chemical-responsive polymer

Majority of chemical-responsive polymers are utilized under the change of pH, ionic strength, redox, and solvents [34]. pH is an important feature especially in biomedical applications due to its change in many specific or pathological compartments. The overall charges were generated by the presence of ion, weak acidic or basic moieties that attach to backbone or pendant groups. For example, Naka et al.[36] synthesized colloidal dispersions of imidazolium cations-modified gold nanoparticles (AuNPs) by using poly (acrylic acid) (PAA) to control pH responsive aggregation in aqueous solution. In pH range between 1.9 and 5.5, due to the electrostatic interaction between the imidazolium cations on AuNPs and the carboxylate anions on PAA, AuNPs were aggregated. When pH was dropped below 1.9, by addition of HCl, the protonation of the carboxylate units of PAA in the precipitates promotes the anion exchange of the imidazolium cations to chloride. Gold nanoparticles were re-dispersed in aqueous solution. When the pH rose above 5.5 by addition of NaOH, the polymer backbones are electrostatically repulsive each other. AuNPs attached on PAA was pushed out to disperse again.

Tsyalkovsky et al.[37] were synthesized fluorescent silica nanoparticles with a responsive shell. Poly (2-vinylpyridine) (P2VP) and poly (ethylene glycol) (PEG) binary polymer brush was synthesized on silica nanoparticles via the “grafting to” technique. Poly (glycidyl methacrylate) (PGMA) labeled with Rhodamine B (RhB) was

used to form a reactive and fluorescent shell on the nanoparticle surface. The aqueous dispersions of the particles are stable at low pH, an increase of pH to 6 caused a slow aggregation as a consequence of the hydrophobic attraction between the collapsed and almost nonprotonated P2VP macromolecules. The aggregation was fully reversible after the decrease in pH. The pH variation did not quench the fluorescence of the colloidal suspensions. The pH-tunable aggregation of the fluorescent nanoparticles could find diverse applications for labeling and contrasting of cells and tissues when the size of the label and the intensity of the optical signals can be tuned by and related to the pH of the host environment.

2.1.3 Biological-responsive polymer

Miyata et al.[38] proposed a strategy for the preparation of smart soft materials that recognize a target biomolecule and induce structural change. The strategy for preparing such biomolecule-responsive hydrogels was to use biomolecular complexes as reversible crosslinks that dissociate and associate in the presence and absence of a target biomolecule, respectively. Based on this strategy, they have developed two types of biomolecule-responsive hydrogels that undergo a change in volume in response to an antigen. One system composed of a biomolecule-crosslinked hydrogel and another composed of a biomolecule imprinted hydrogel. The biomolecule-crosslinked hydrogel swells in the presence of a target biomolecule because of a decrease in crosslinking density resulting from complex exchange. The biomolecule-imprinted hydrogel shrinks in response to a target biomolecule because the crosslinking density increases due to the formation of sandwich-like complexes that act as crosslinker.

Antigen-responsive hydrogels were prepared by using antigen-antibody bonds at crosslinking points in the hydrogel [39, 40]. For example, the antigen-responsive gels with semi-interpenetrated network (semi-IPN) structures were composed of linear polyacrylamide (PAAm) with grafted antibodies (goat anti-rabbit

IgG) and PAAm networks with grafted antigens (rabbit IgG). The complexes of the grafted antibodies and grafted antigens played important roles as stimulti-responsive cross-linking points. The addition of free goat IgG did not produce any change in hydrogel. It meant that the gels having antigen-antibody binding can recognize only rabbit IgG to induce their structural changes. Furthermore, the antigen-antibody gel with a semi-IPN structure was swollen immediately in the presence of free rabbit IgG antigen and shrunken in its absence. Such reversible antigen-responsive swelling behavior of the antigen-antibody semi-IPN gel is due to the fact that its crosslinking density changed reversibly by complex formation and dissociation between grafted antibody and grafted antigen in the absence and presence of free antigen, respectively.

Lectin, which are carbohydrate-binding proteins, interact with glycoproteins and glycolipids on cell surfaces, and induce various effect such as cell agglutination, cell adhesion to surface, and hormone-like action. The unique properties of lectins have been applied to the design of glucose responsive systems. Miyata et al. [41] showed that a polymer with pendant glucose groups, poly (2-glucosyloxyethyl methacrylate)(PGEMA), can form a complex with ConcanavalinA (ConA). The PGEMA-ConA complex was dissociated in the presence of free glucose or mannose by their complex exchange between PGEMA and monosaccharide, but was not dissociated in the presence of free galactose. These results indicated that the PGEMA-ConA complex exhibited monosaccharide recognition functions and could respond to a specific monosaccharide.

Duncan et al.[42] have reported for Concanavalin A (ConA) to adsorped and mediated aggregation of dextran coated Silica dioxide (SiO_2) colloids that is tunable via a competing ConA–glucose interaction. Video and confocal scanning laser microscopy and quasi-2D optical microscopy were used to characterize ConA adsorption to dextran colloids. Stability was also mediated by adding glucose as a competitive inhibitor that binds ConA more strongly than dextran. The glucose

concentration could be tuned to both lower the ConA adsorbed amount and decrease aggregation of the dextran coated colloids via ConA bridges. Based on a model including bridge formation kinetics, rapid colloidal aggregation occurs only when the ConA exceeds a threshold concentration, whereas slow aggregation is observed at lower ConA concentrations or when glucose is added to reduce ConA bridging rates. These findings reveal a mechanism for tuning colloidal interactions and aggregation kinetics through specific, competitive biomolecular interactions, which lends insights into aggregation phenomena in mixed synthetic-biomaterial and biological systems.

2.1.4 Multi-responsive polymer

Multi-responsive polymers are pronounced significance as they can be responded through several mechanisms. For example, Naik et al.[43] were synthesized a series of poly(propylene oxide)-b-poly(l-lysine) (PPO-PK) block copolymers using Huisgen's 1,3-dipolar cycloaddition, and the polymer self-assembly was studied using transmission electron microscopy (TEM), circular dichroism spectroscopy (CD), and dynamic and static light scattering techniques. The previous studies of poly (lysine)-based block copolymers, PPO-PK exhibited a significant shift in the pH associated with the helix–coil transition of the poly (lysine) block, potentially a result of decreased hydrophobicity in the core PPO block. Morphology changes with pH for spherical micelles were consistent observed by CD spectra, while vesicles showed a larger-than-expected morphology change compared with the helix-coil transition. Given the proximity of the lower critical solution temperature of the PPO block, these materials exhibited both pH and temperature-responsive self-assembly, The unimer-assembly morphology and kinetics were investigated via light scattering and interpreted in terms of changes in the second osmotic virial coefficient, and schizophrenic assembly was demonstrated. Finally, the PPO-PK vesicles was studied for the encapsulation and passive release of hydrophilic drugs.

Azzam et al.[44] modified cellulose nanocrystals via carboxylation by Tetramethylpiperidinyloxy (TEMPO) oxidation and grafting thermo-responsive Jeffamine polyetheramine (M2005) by peptide coupling in water. In this work, CNCs was improved to stabilize water and many organic solvents due to polymer acted as layer on particle. Moreover, CNCs exhibited reversible aggregation by changing pH due to electrostatic force of polymer on surface and by LCST from hydrophobic attractive interaction of polymer chain, and the aggregation number could be tuned by varying the ionic strength and/or the pH of the medium, making the suspension possessed multi-responsive property. The sensitivity of the aggregation properties to temperature, ionic strength, and pH make the polymer-decorated CNC multi-responsive, which paves the way to their use as smart building blocks for the preparation of advanced CNC-based materials.

2.3 Biopolymer

2.3.1 Chitosan

Chitosan [11-15] is the most second abundant natural polymer derived from chitin by deacetylation with alkaline treatment. However, deacetylation of chitin is almost never complete, thus, chitosan still contains acetamide groups to some extent, but generally need to have %acetylation less than 60%. Chitosan is a linear polysaccharide composed of β-1,4-D-glucosamine and β-1,4-*N*-acetyl-glucosamine units. Chitosan usually found in the shells of crustaceans such as shrimp and crab, exoskeletons of insects and mollusks including the cell walls of some fungi. Chitosan provide a great potential in wide range of applications such as food processing, cosmeceutical, pharmaceutical and especially in biomedical applications due to their biocompatibility, biodegradability, antimicrobial activity and nontoxicity. Chitosan is only soluble in most dilute acidic aqueous solution but insoluble in water, it has been a major drawback for using in biomedical applications. Chitosan have a primary and

secondary hydroxyl groups on each repeating unit, and the amine group on each deacetylated unit. These reactivity group can be modified with many other functional groups to improve their water solubility.

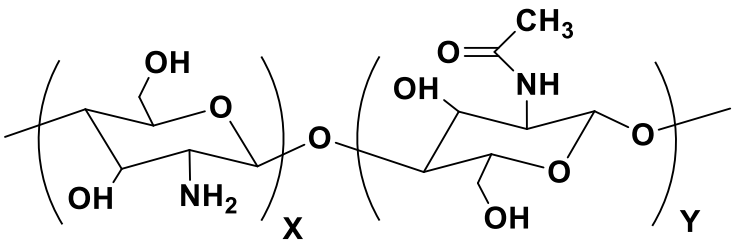


Figure 2.1 Chemical structure of chitosan

- Phosphorylated chitosan

Nishi et al.[45] studied on water soluble chitin derivative. Phosphorus pentoxide was added to the mixture of chitin or DA-chitin (chitosan). The reaction was manually stirred at 0-5°C for 2-3 hrs and the product was precipitated in ether. The residue was wash with ether, acetone, and methanol and then dried. From the results, they found that chitin phosphates are easily soluble in water independent of DS. But DA-chitin phosphates of high DS are insoluble in water while those of low DS are soluble. These phenomena might be occurred by the formation of inter- or intra molecular salt linkage between amino and phosphate groups, although the amino group of the products is a salt with methanesulphonic acid, and might be regarded as a formation of poly-ion complex as generally seen in polyampholites.

Tachaboonyakiat et al.[29] studied on elimination of inter- and intramolecular crosslinks of phosphorylated chitosan by sodium salt formation. Phosphorylated chitosan was synthesized using methanesulfonic acid and phosphorus pentoxide system, and they were destroyed the electrostatic interaction between ammonium groups and phosphoric groups were destroyed by using alkaline condition. Sodium salt formation was carried out by dialyzing both water soluble and water-

insoluble phosphorylated chitosan against 0.1 sodium hydroxide for 12 h and then was neutralized with 0.1 M hydrochloric acid. After neutralization, the obtained mixture was dialyzed again against deionized water for 2 days to remove sodium chloride. Salt of alkali metals shows good solubility in water. Thus, sodium phosphorylated chitosan was thoroughly dissolved in water.

2.4 Biomolecules

2.4.1 Antigen and antibody

Antibodies are proteins manufactured by the body that help to fight against foreign molecules called antigens, such as pathogens and their chemical toxins. The antigens are specifically bound with antibodies to form an antigen-antibody complex. When an antigen enters the body, the immune system responds and then produces the antibodies to attach or bind themselves with antigen and inactivates it. The antigen-antibody reaction is widely used in laboratory diagnostics. It is a reversible chemical reaction based on noncovalent bonds such as electrostatic forces, hydrogen bond, hydrophobic and van der Waals interactions. The unique features of antibodies are associated with the immune responses, many researcher using antibodies as a variety of immunological assays with the specificity and versatility necessary to detect biological substances. They have been many studies used antigen or antibody complex as smart materials to detect other biological molecule [38, 39].

Leptospirosis is an infection caused by bacteria called *Leptospira interrogans*. Leptospires are thin, highly motile, slow-growing obligate aerobes with an optimal growth temperature of 30 °C and can be distinguished from other spirochaetes on the basis of their unique hook or question mark-shaped ends[46]. Leptospires have an outer membrane protein and LipL32 is the most abundant lipoprotein exposed on the surface of *Leptospira* spp.

- LipL32

LipL32 is the major outer membrane lipoprotein expressed *in vitro* by pathogenic but not by saprophytic leptospires, these bacteria is caused of infection called Leptospirosis [47]. LipL32 is a 272 amino acid protein with a 19 residue lipidation signal sequence [48]. Thus, monoclonal antibody (MAb) was produced by immunized the LipL32 in mice. MAbs produced can be useful for the development of diagnostic tests based on detection of LipL32 leptospiral antigen in biological fluids [49].

- Monoclonal antibody (MAb)

MAb is a mouse monoclonal antibody that was produced by hybridoma technique. MAb is very specific to recognize only one epitope on an antigen. MAb were conjugated to peroxidase and evaluated in a native protein enzyme-linked immunosorbent assay (ELISA) with intact and heat-treated leptospiral cells, conjugated to fluorescein isothiocyanate (FITC) for indirect immunofluorescence with intact and methanol fixed cells and were used for LipL32 immunoprecipitation from leptospiral cells [49].

Our approach for the development of antigen responsive polymer based on principle of antigen-antibody bindings. Water soluble polymer which conjugated with antigen or antibody was applied as phenomena amplification. In this research, water soluble derivative of chitosan, phosphorylated chitosan (PCTS) was used to conjugate with antigen or antibody. LipL32 and MAb were selected as model antigen and antibody, respectively. With the fact that antigen can form specific complex with antibody. The formation of antigen-antibody complex in which conjugation on water soluble polymer would cause polymeric aggregation. Therefore, the MAb conjugated on PCTS (PCTS-MAb) as well as the PCTS-LipL32/PCTS-MAb complex should detect free antigen of LipL32, then would be aggregated. Therefore, the PCTS-MAb and PCTS-LipL32/PCTS-MAb complex would be applicable to diagnose the infection early in the course of disease.

Chapter 3
EXPERIMENTAL

3.1 Materials and chemicals

Chitosan flake with degree of deacetylation (DD) 85% was purchased from Seafresh Industry Public Company Limited, Bangkok Thailand and used for preparing water soluble chitosan. Methanesulfonic acid (analytical reagent grade) was purchased from Sigma-Aldrich Co., Paris la defense, France and used as the solvent and protective agent for the amino group of chitosan. Phosphorus pentoxide (synthesis grade) was purchased from Acros organics, Morris Plains, New Jersey, USA and used as substitution group in phosphorylation reaction. LipL32 antigen and monoclonal antibody (mAb82) were gifted from Department of Microbiology, Faculty of Medicine, Chulalongkorn University, and used as representative model of antigen and antibody, respectively. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC-HCl) (synthesis grade) was purchased from Sigma-Aldrich Co., Missouri, USA, and used as a coupling agent for synthesis. *N*-Hydroxysuccinimide (NHS) (synthesis grade) was purchased from Sigma Aldrich, Tokyo, Japan, and used as a proton donor for synthesis. Dialysis tubing cellulose membrane (MWCO 14,000) was purchased from Sigma Aldrich and used to dialyze the chitosan derivatives products. Acetone (commercial grade) was purchased from RCI Labscan Co, Ltd., Bangkok, Thailand, and used to precipitate the chitosan derivatives product. Ethanol (commercial grade) was purchased from RCI Labscan Co, Ltd., Bangkok, Thailand, and used to purify the chitosan derivatives product. Deionized water was used in the whole experiment.

3.2 Experimental procedure

3.2.1 Synthesis of phosphorylated chitosan (PCTS)

Phosphorylated chitosan (PCTS) was synthesized in our laboratory and have been described elsewhere[29]. In brief, chitosan with degree of deacetylation 85

was dissolved in phosphorus pentoxide and methanesulfonic acid with continuous flowing nitrogen gas under 0°C for 3 h. The crude mixture was precipitated in large amount of acetone, washed with ethanol following by dialyzed in distilled water following by neutralization, and lyophilized. PCTS was kept in desiccator after lyophilization for further used.

3.2.2 Conjugation of PCTS with antigen and antibody

Antigen or antibody (1mg/ml, 100µl, 8×10^{-4} mmol) was dissolved in deionized water (1 mL) under nitrogen atmosphere at 4 °C. EDC-HCl (23 mg, 0.12 mmol) and NHS (13.8 mg, 0.12 mmol) were then added to the solution following by continuous stirring. After that, 1 mL of PCTS aqueous solution (10 mg, 0.06 mmol) was added dropwisely, the reaction mixtures were carried on for 12 h under the same condition. The product was dialyzed against DI water to eliminate unreacted chemicals and stored at 4 °C for further characterization.

3.2.3 Characterization

3.2.3.1 Fourier Transform Infrared Spectroscopy (FT-IR)

The functionalities of PCTS was characterized by FTIR (Nicolet 6700, Thermo Scientific, Waltham, USA). The samples (chitosan and PCTS) were ground with potassium bromide (KBr). FTIR spectra were recorded in range of 400 to 4000 cm-1 with 64 scans. The FTIR spectra were recorded in % absorbance. In addition, the aliquot of PCTS conjugated antigen or antibody samples were dropped on the zinc selenide (ZnSe) and dried before recorded in the same range of previous samples.

3.2.3.3 Scanning Electron Microscope equipped with Energy Dispersive X-Ray Spectrometer (SEM-EDX)

The degree of phosphate substitution was determined by SEM-EDX (JEOL JSM-7610F, JEOL Ltd., Tokyo, Japan). PCTS samples were ground and placed on SEM carbon holders with carbon tape. SEM images were viewed under pressure

with a voltage of 20 kV and a magnification of 2500. An electron beam was set at a constant distance of 15 μm. The percentage of elements present in samples was recorded. The degree of phosphoric substitution to chitosan can be calculated using the following equation:

$$\text{Degree of substitution} = \frac{\%P}{\%N} \quad \text{.....(1)}$$

Where %P=phosphorus content of phosphoric groups and

%N=nitrogen content in chitosan molecules.

3.2.3.4 Dynamic light scattering (DLS)

In order to determine point of isoelectricity (pI), zeta potential of PCTS and PCTS conjugates were evaluated by DLS (NanoZS, Malvern Instrument Ltd., Worcestershire, UK) at 25°C in a variation of pH (5-12). A 0.1 mL aliquot of sample (PCTS, PCTS conjugated LipL32 or PCTS conjugated MAb) was diluted in DI water to a final concentration of 1×10^{-2} %v/v. All experiments were collected three times with standard deviation (S.D.).

3.2.3.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

In order to confirm the conjugation, the protein bands were observed on SDS-PAGE gel electrophoresis. SDS-PAGE in 8% acrylamide separating gel and 5% stacking gel was performed under reducing conditions. The protein molecular weight marker and all of samples were dissolved in buffer solution and incubated at 100°C for 5 min. Total protein loaded in gel slots was 4 μg. The electrophoresis was conducted at 100 volt for 1.30 h until the solutions moved to the bottom of the gel. Gel was stained with Coomassie Brilliant Blue R-250 solution and destained in mixed solution composed of 10% methanol and 10% acetic acid. The obtained molecular mass was estimated by comparison with PageRuler unstained protein ladder. In all

experiment, antigen, antibody PCTS and PCTS with conjugating agent were used as control.

3.2.4 Monitoring the association of antigen and antibody

To investigate the specific binding ability, the conjugated polymers were mixed as showed in **Table 3.1**. All of Mix solutions were prepared in the final volume at 500 µl consisted of PCTS-LipL32 and PCTS-MAb, and the 15 µl (8×10^{-4} mmol) of LipL32 was added to Mix 4, Mix 5 and Mix 6 to observe the antigen responsive behavior by UV-vis spectrophotometer.

Table 3.1 Preparation of PCTS-LipL32 and PCTS-MAb solution to monitoring turbidity before (Mix 1, Mix 2, Mix 3) and after adding of LipL32 free antigen (Mix 4, Mix 5, Mix 6)

Sample	PCTS-LipL32 (4 mg/ml)	PCTS-MAb (4 mg/ml)	LipL32 (1 mg/ml)
Mix 1	500 µl	-	-
Mix 2	-	500 µl	-
Mix 3	250 µl	250 µl	-
Mix 4	500 µl	-	15 µl
Mix 5	-	500 µl	15 µl
Mix 6	250 µl	250 µl	15 µl

3.2.4.1 Ultraviolet-visible spectroscopy

UV-vis spectrophotometer (Cary 300 UV-vis spectrometer, Agilent Technologies, USA) was used to determine the absorption of visible light at 500 nm. A 500 µl of each samples were added to glass cuvette. DI water was used as

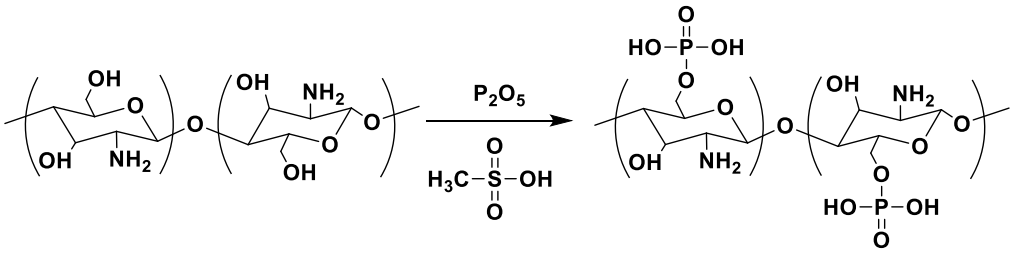
reference. All samples were collected three times with standard deviation (S.D.) at 0 min, 1 min, 1 day and 3 days.

Chapter 4
RESULTS AND DISCUSSION

The aim of this research is to prove concept of monitoring the polymer structural change under go triggered by specific interaction between antigen and antibody by using water soluble chitosan as an amplifier. Chitosan acquires a great attention as a functionalized biopolymer for using in several medical applications [11, 12]. However, chitosan can only be dissolved in acidic solution which is the main drawback for using in some biomedical applications. In order to apply chitosan as water soluble amplifier, chitosan should be chemically modified with some functional groups which leading chitosan becomes water soluble. Chitosan modified with phosphoric group was a great candidate due to its abilities to using in wide range of biomedical applications [12, 29]. LipL32 antigen and monoclonal antibody (MAb) were used to represent as a model of antigen-antibody. Therefore, the conjugation of antigen and/or antibody onto phosphorylated chitosan was easily performed via peptide formation using water soluble carbodiimide.

4.1 Synthesis of water soluble phosphorylated chitosan (PCTS)

Phosphoric group was reacted with hydroxyl groups of chitosan by esterification to obtain phosphorylated chitosan (PCTS). Chitosan was dissolved in methansulfonic acid which protected amino group of chitosan in ice bath, following by added phosphorus pentoxide and the reaction was carried out at 0-4 °C for 3 hours to obtain PCTS. The phosphate group substitution would occur at hydroxyl group of chitosan because the amino group was protected with sulfonic group of methansulfonic acid as solvent. The systhesis pathway was shown in **Figure 4.1**



Chitosan Phosphorylated chitosan

Figure 4.1 The systhesis pathway of PCTS

4.2 Synthesis of PCTS-LipL32 antigen or PCTS-MAb antibody

PCTS-LipL32 or PCTS-MAb were synthesized by conjugation between carboxylic group of LipL32 (or MAb) and amine group of PCTS by using EDC·HCl as a conjugating agent and NHS as a proton donor. First, carboxyl group of LipL32 (or MAb) was activated by EDC·HCl to obtain unstable reactive o-acylisourea ester intermediate following by adding of NHS to obtain semi-stable amine-reactive NHS ester which then reacted with amine group of PCTS to form stable amide bond. The remaining by-product would be removed by dialysis against cold DI water. The reaction was shown in **Figure 4.2**.

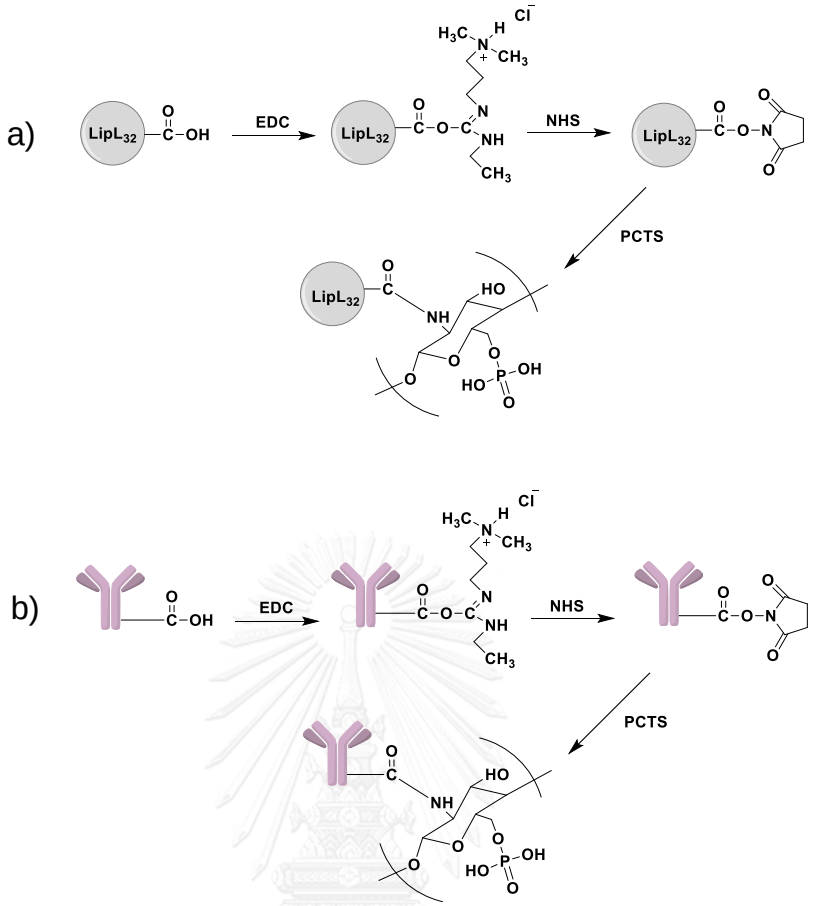


Figure 4.2 a) Synthesis pathway of PCTS-LipL32 and b) Synthesis pathway of PCTS-MAb

4.3 Structural characterization of water soluble chitosan derivatives

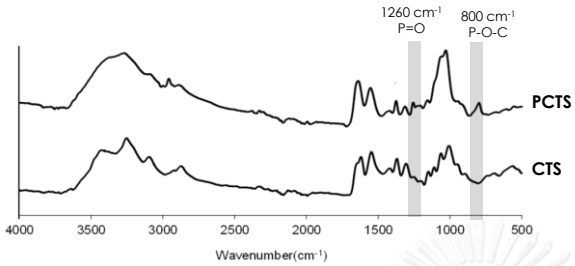
4.3.1 FT-IR analysis

The chitosan and PCTS were characterized by FT-IR (**Figure 4.3** a). Chitosan shown characteristics peak at 3450 cm^{-1} (O-H stretching and N-H stretching), 2960 cm^{-1} (C-H stretching), 1560 cm^{-1} (N-H bending) and 1072 cm^{-1} (C-O stretching). PCTS shown the new characteristic peaks of phosphoric groups at 1260 cm^{-1} (P=O stretching) and 800 cm^{-1} (P-O-C).

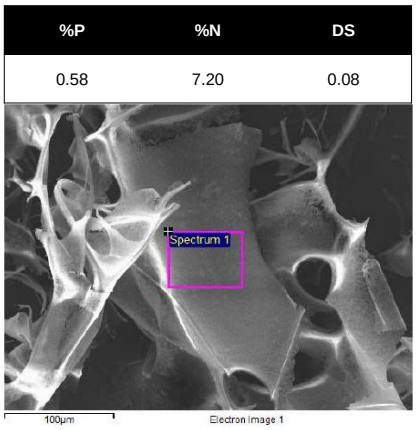
4.3.2 Degree of substitution

The degree of phosphoric groups substituted onto chitosan backbone was obtained from SEM-EDX. The element percentage of phosphorus and nitrogen

were calculated by using equation (1) (**Figure 4.3** b). Thus, the degree of substitution of phosphoric group per chitosan unit was 0.08.



a) FT-IR spectra of phosphorylated chitosan and chitosan.



b) Degree of phosphoric substitution of PCTS.

Figure 4.3 a) FT-IR spectra of phosphorylated chitosan and chitosan b) Degree of phosphoric substitution of PCTS.

4.4 Conjugation characterization

4.4.1 FT-IR analysis

From the FTIR spectra as shown in **Figure 4.4**, PCTS-LipL32 and PCTS-MAb shown the characteristic peaks of amide I appeared at 1644-1645 cm^{-1} and amide II appeared at 1551-1556 cm^{-1}. The observed peak positions of amide I and amide II were changed in PCTS-LipL32 and PCTS-MAb spectra in which of the decreasing intensity ratio of amide II to amide I. This can predict that the content of amide I band in PCTS-LipL32 and PCTS-MAb extended due to the amide linkage formation between PCTS and LipL32 or MAb. The results from FTIR spectra supports the amide linkage was form between PCTS and LipL32 or MAb.

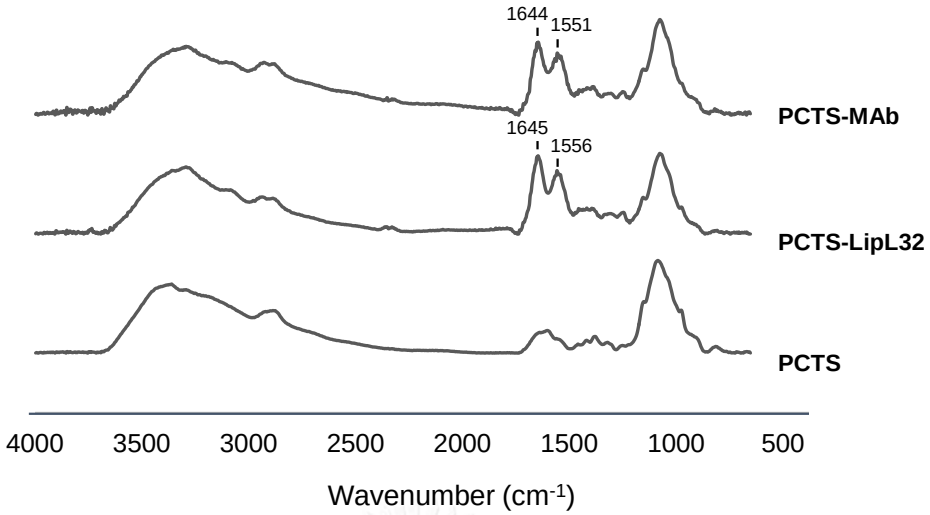


Figure 4.4 FTIR spectra of PCTS, PCTS-MAb and PCTS-LipL32

4.4.2 Determination of point of isoelectricity (pI)

The zeta-potential under the condition of various pH in range of 5-12 was used to determine the point of isoelectricity (pI) of the conjugated PCTS before and after the conjugation. The pI of PCTS changed from 5.8 to 10.6 after conjugating with LipL32 meanwhile changed from 5.8 to 9.5 after conjugating with MAb. This evidence might come from the neutralization of the different charged moieties revealed that the net charge on molecules were changed indicated that successful antibody conjugation [50]. In order to prove the PCTS-LipL32 and PCTS-MAb conjugation, the increasing of molecular weight after conjugation should be observed by electrophoresis band retardation.

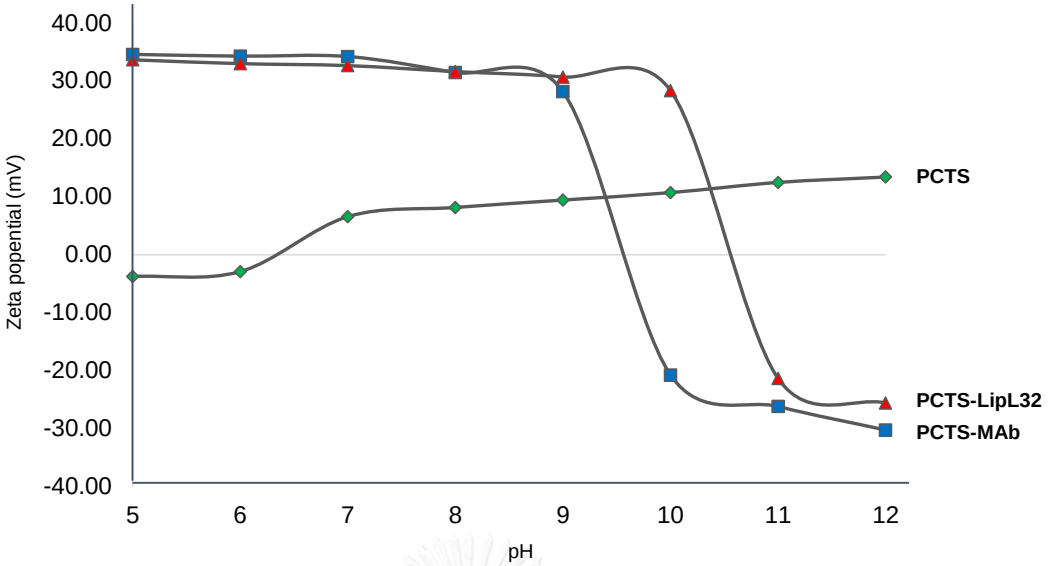


Figure 4.5 The effect of pH on the zeta potential of PCTS, PCTS-LipL32 and PCTS-MAb

4.4.3 SDS-PAGE analysis

The conjugation of PCTS-LipL32 and PCTS-MAb were confirmed by band retardation. Since the mobility of LipL32 and MAb along on SDS-PAGE gel would be retarded by increasing the molecular weight. The increasing in molecular weight might come from two evidences, (i) polyelectrolyte complexation and (ii) covalently conjugation between PCTS and LipL32 or MAb. Therefore, SDS-PAGE was used to prove that LipL32 and MAb covalently bonded with PCTS under coupling reaction and increasing its molecular weight. The amount of protein in samples were illuminated with Coomassie Brilliant Blue R-250. PageRuler unstained protein ladder (Lane 1) was used as discrete known molecular weight marker. The monomeric band of LipL32 (Lane 2) was shown at 32 kDa, indicated that the molecular weight of LipL32. MAb (Lane 3) was shown 3 different bands that indicated near the molecular weight of monoclonal antibody (150 kDa), heavy chain fragment (between 50-60 kDa) and light chain fragment (around 25 kDa). PCTS (Lane 4) exhibited a pale continuous band from the bottom of gel to over 200 kDa, indicated that variation of molecular weight of PCTS . PCTS with EDC/NHS (Lane 5) was shown a pale continuous band as well as

PCTS. The mixture of PCTS and LipL32 (Lane 6: PCTS+LipL32) was used to confirm the polyelectrolyte complexation between PCTS and LipL32 that might retard the LipL32 band. It was found that both PCTS and LipL32 band separately appeared, indicating no electrostatic interaction between PCTS and LipL32, likewise the mixture of PCTS and MAb (Lane 7: PCTS+MAb). The electrophoresis band of PCTS conjugated with LipL32 (Lane 8: PCTS-LipL32) and MAb (Lane 9: PCTS-MAb) migrated broader and more retarded than Lane 2 and 3, indicating the stained of PCTS-LipL32 and PCTS-MAb conjugates appeared polydispersity with a broad molecular weight distribution. It should be confirmed that the LipL32 and MAb conjugated onto PCTS main chain through a covalent bond. However, the shade of Coomassie brilliant blue R-250 was indicated to the remainning of amino groups in samples. The Coomassie dyes bind to proteins through ionic interactions between dye sulfonic acid groups and positive protein amine groups as well as through Van der Waals attractions, the mild band in Lane 8 and 9 refered that amino group of chitosan were used to covalently linked with protein.

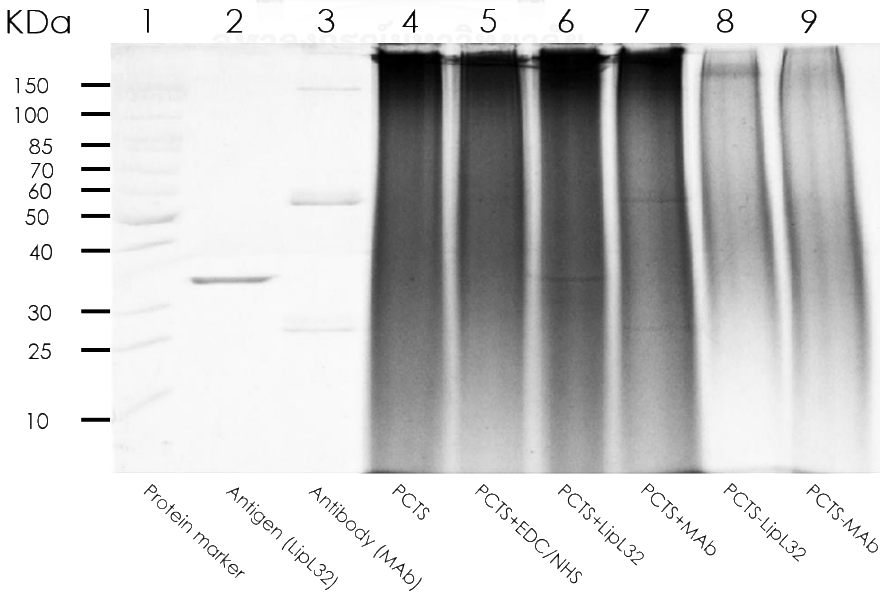
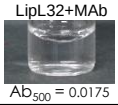
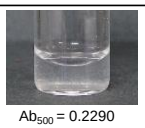
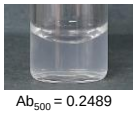
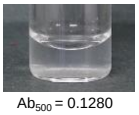
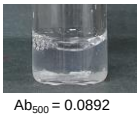
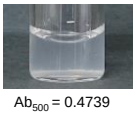
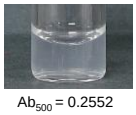
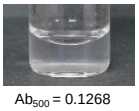
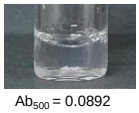


Figure 4.6 SDS-PAGE visualized by coomassie blue staining in UV-vis excitation (lane 1, protein marker: lane 2, LipL32 antigen: lane 3, MAb; lane 4, PCTS; lane 5, PCTS with coupling agent; lane 6, PCTS mixed with LipL32; lane 7, PCTS mixed with MAb; lane 8, PCTS conjugated LipL32; lane 9, PCTS conjugated MAb)

4.5 Monitoring the association of antigen and antibody

To investigate the specific binding ability of PCTS-MAb and PCTS-LipL32, the conjugated polymers were mixed as shown in **Table 4.1**. Polymer conjugated LipL32 (Mix 1) amd polymer conjugated MAb (Mix 2) with a concentration of 4 mg/ml shown a bit of turbidity due to the wide range of molecular weight from SDS-PAGE (**Figure 4.5**), the turbidity of polymer solution was slightly increased after mixing of Mix 1 and Mix 2 resulting in Mix 3. LipL32 free antigen was added to PCTS-LipL32 and PCTS-MAb solution (Mix 4 and Mix 5). The turbidity of Mix 4 was not changed but the in the Mix 5 was precipitated as well as in Mix 6. The rapid change of turbidity was determined by UV-vis spectroscopy as shown in **Figure 4.6**.

Table 4.1 Specific binding ability of PCTS-MAb and PCTS-LipL32

Control LipL32+MAb  Ab_{500} = 0.0175	**Mix 1** PCTS-LipL32	**Mix 2** PCTS-MAb	**Mix 3** PCTS-LipL32 PCTS-MAb	**Mix 4** PCTS-LipL32 LipL32	**Mix 5** PCTS-MAb LipL32	**Mix 6** PCTS-LipL32 PCTS-MAb LipL32
0 min	 Ab_{500} = 0.2477	 Ab_{500} = 0.5105	 Ab_{500} = 0.4710	 Ab_{500} = 0.2557	 Ab_{500} = 0.3387	 Ab_{500} = 0.4543
1 min	 Ab_{500} = 0.2464	 Ab_{500} = 0.5121	 Ab_{500} = 0.4687	 Ab_{500} = 0.2555	 Ab_{500} = 0.2290	 Ab_{500} = 0.2538
1 day	 Ab_{500} = 0.2489	 Ab_{500} = 0.5085	 Ab_{500} = 0.4722	 Ab_{500} = 0.2552	 Ab_{500} = 0.1280	 Ab_{500} = 0.0892
3 day	 Ab_{500} = 0.2489	 Ab_{500} = 0.5067	 Ab_{500} = 0.4739	 Ab_{500} = 0.2552	 Ab_{500} = 0.1268	 Ab_{500} = 0.0892

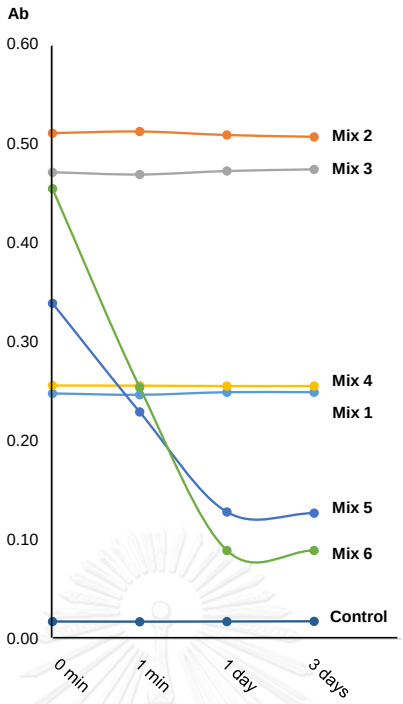


Figure 4.7 The change of turbidity of PCTS-LipL32, PCTS-MAb, PCTS-LipL32/PCTS-MAb complex before and after adding LipL32 free antigen

From the main purpose of this research, we were introduced the approach of tunable aggregation of polymer conjugated with antigen and antibody complex that can be dissociated with the presence of free antigen. Both PCTS-LipL32 (Mix1) and PCTS- MAb (Mix2) were water soluble with UV-vis absorption at 500 nm of 0.24 and 0.51, respectively. With the assumption that in the absence of free antigen, the solution of PCTS- LipL32 mixed with PCTS-MAb (PCTS-LipL32/PCTS-MAb complex) exhibited in aggregated colloidal form, and should be switched to the soluble form in the presence of LipL32 free antigen by exchange of the PCTS-LipL32 for LipL32 free antigen [5, 6]. In constrast, the results from **Table 4.**1 was shown some evidense. The PCTS-LipL32/PCTS-MAb complex (Mix 3) was not formed the aggregation. In the presence of LipL32 free antigen (with the concentration of 1 mg/ml, 15 µl), theLipL32 free antigen did not change the absorption of PCTS-LipL32 (Mix 4, Ab_{500} = 0.25), indicating that LipL32 free antigen did not form any interaction with PCTS-LipL32. Whilst, the addition

of LipL32 free antigen into PCTS-MAb (Mix 5) and PCTS-LipL32/PCTS-MAb complex (Mix6), the aggregation form within 1 min with the absorption decrease to 0.22 and 0.25, respectively. These results reveal the tunable aggregation of PCTS-MAb when associated with LipL32 free antigen. Conjugation of LipL32 onto PCTS might be leading of the decreasing in LipL32 activity [5], which may be the cause of non binding between PCTS-MAb and PCTS-LipL32. To avoid the non specific binding between amine group of PCTS and carboxylic group of LipL32 free antigen, the mixture of PCTS and LipL32 free antigen was prepared as a control and found that the UV-vis absorption at 500 nm was 0.1124, it was not changed when compared with PCTS.

With the limitation of the results, it can be concluded that PCTS-MAb and PCTS-LipL32/PCTS-MAb complex could be utilized as rapid diagnostic kit for detecting specific LipL32 antigen of Leptospires of Leptospirosis infected patients. However, the capability to detect lowest concentration of free antigen of LipL32 will be further investigated.

Chapter 5
CONCLUSION

Phosphorylated chitosan (PCTS) was successfully synthesized via phosphorylation by using methanesulfonic acid/phosphorus pentoxide system which obtain degree of phosphoric group substitution of 0.08. PCTS shown the absorption band of phosphoric group that indicated the substitution occurred on hydroxyl group of chitosan.

LipL32 and MAb was successfully conjugated onto PCTS via amide linkage formation between carboxylic group of LipL32 (or MAb) and amine groups of PCTS using EDC·HCl as conjugating agent and NHS as a proton donor. FTIR analysis shown both absorption bands of amide I and amide II in spectra of PCTS-LipL32 and PCTS-MAb. The conjugation was confirmed by the band retardation of SDS-PAGE, indicating that LipL32 (or MAb) was successfully covalently conjugated with amine groups of PCTS that influenced to the shift of isoelectric point (pI).

Specific binding ability of antibody conjugated PCTS was observed. PCTS-MAb and PCTS-LipL32/PCTS-MAb complex were aggregated and precipitated with the presence of LipL32 free antigen while the activity of LipL32 that was conjugated with PCTS (PCTS-LipL32) was lower than free LipL32 antigen. This can be concluded that MAb still retain the specific binding ability to LipL32 after conjugated with PCTS.

Suggestion:

In order to utilize PCTS conjugated proteins as Leptospirosis specific antigen diagnosis kit, lowest concentration of free antigen LipL32 detected by PCTS-MAb and PCTS-LipL32/PCTS-MAb complex will be further investigated. PCTS-MAb and PCTS-

LipL32/PCTS-MAb complex will be tested with ELISA by various type of other outer membrane protein of leptospires such as LigA, LigB, LipL21, LipL29 etc. to find out the specific selectivity against LipL32. The aggregates of PCTS-MAb and PCTS-LipL32/PCTS-MAb complex will be monitored with Transmission electron microscopy (TEM) and the conjugation will be ensured by using Quartz Crystal Microbalance (QCM).

In order to detect the presence of free antigen after aggregation of PCTS-MAb or PCTS-LipL32/PCTS-MAb complex with free antigen, various free antigens including LipL32 will be labeled with fluorescence dye (fluorescein isothiocyanate, FITC). Then, the free antigen within the aggregation will be observed under confocal scanning laser microscope.

Moreover, PCTS-LipL32 or PCTS-LipL32/PCTS-MAb complex will be further investigated as kit for free antibodies detection by the same methods as mentioned above for antigen detection, in order to prove that PCTS-LipL32/PCTS-MAb complex has more advantage in specific binding for both antigen and antibody detection, which will be expected as specific antigen and antibody diagnostic kit.

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APPENDIX

APPENDIX A

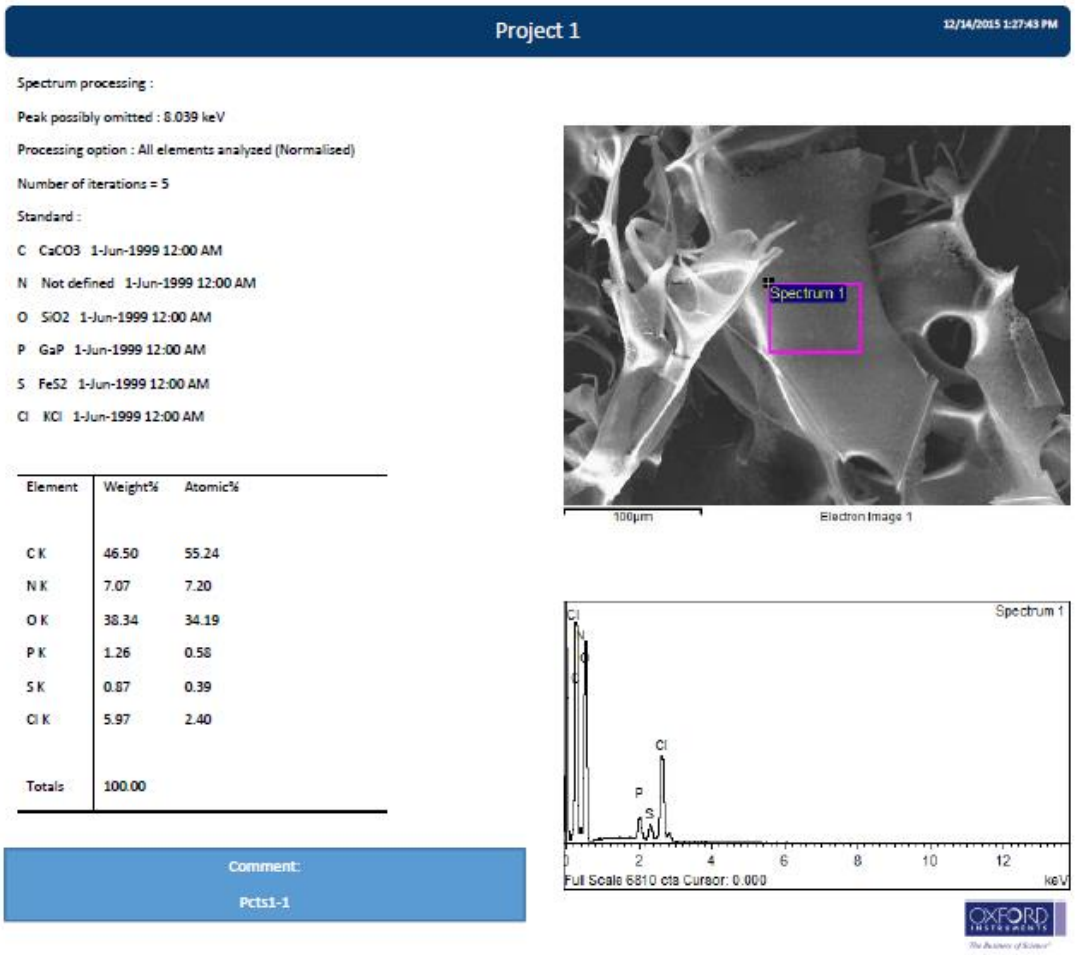

Figure A1 SEM image and percentage of elemental composition of PCTS.

APPENDIX B

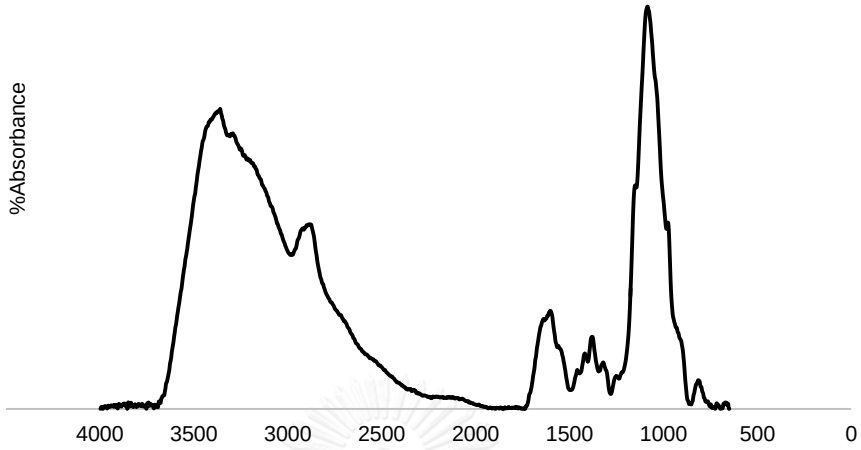

Figure B1 FTIR spectrum of PCTS

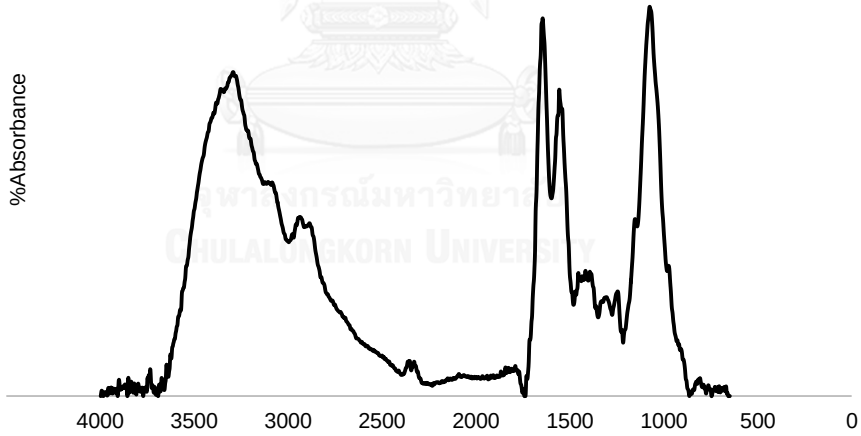

Figure B2 FTIR spectrum of PCTS-LipL32

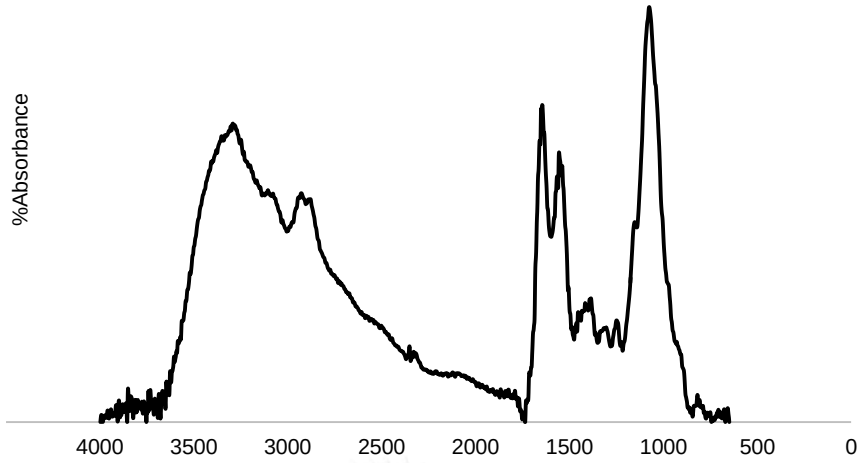

Figure B3 FTIR spectrum of PCTS-MAb

APPENDIX C

Table C1 Absorbance at 500 nm of sample in solution 0 min and 1 min

	0min					1min				
	1	2	3	avg	std	1	2	3	avg	std
PCTS-LipL32	0.2461	0.2468	0.2502	0.2477	0.0022	0.2448	0.2458	0.2483	0.2463	0.0018
PCTS-Mab	0.5087	0.5108	0.5119	0.5105	0.0016	0.5104	0.5128	0.5131	0.5121	0.0015
PCTS-MAB/PCTS-LipL32	0.4697	0.471	0.4722	0.471	0.0013	0.4676	0.4683	0.4701	0.4687	0.0013
PCTS-LipL32/LipL32	0.2558	0.2554	0.2559	0.2557	0.0003	0.2554	0.2557	0.2555	0.2555	0.0002
PCTS-Mab/LipL32	0.332	0.348	0.336	0.3387	0.0083	0.2289	0.2299	0.2281	0.229	0.0009
PCTS-LipL32/PCTS-Mab/LipL32	0.452	0.446	0.465	0.4543	0.0097	0.2502	0.2543	0.257	0.2538	0.0034
LipL32/Mab	0.0167	0.0182	0.0175	0.0175	0.0008	0.017	0.0177	0.0173	0.0173	0.0004
PCTS	0.1072	0.1094	0.1095	0.1087	0.0013	0.1065	0.1087	0.1056	0.1069	0.0016
PCTS/LipL32	0.1147	0.1098	0.1126	0.1124	0.0025	0.1106	0.1129	0.1099	0.1111	0.0016

Table C2 Absorbance at 500 nm of sample in solution 1 day and 3 days

	1day					3day				
	1	2	3	avg	std	1	2	3	avg	std
PCTS-LipL32	0.2483	0.2469	0.2516	0.2489	0.0024	0.2481	0.2492	0.2494	0.2489	0.0007
PCTS-Mab	0.5065	0.5096	0.5095	0.5085	0.0018	0.505	0.5077	0.5075	0.5067	0.0015
PCTS-MAB/PCTS-LipL32	0.4706	0.4724	0.4736	0.4722	0.0015	0.4731	0.4739	0.4746	0.4739	0.0008
PCTS-LipL32/LipL32	0.255	0.2553	0.2552	0.2552	0.0002	0.2551	0.2552	0.2552	0.2552	0.0001
PCTS-Mab/LipL32	0.1278	0.1277	0.1286	0.128	0.0005	0.1268	0.1262	0.1274	0.1268	0.0006
PCTS-LipL32/PCTS-Mab/LipL32	0.0862	0.0896	0.0918	0.0892	0.0028	0.0876	0.0888	0.0912	0.0892	0.0018
LipL32/Mab	0.017	0.0176	0.0179	0.0175	0.0005	0.0172	0.0176	0.0182	0.0177	0.0005
PCTS	0.1067	0.1045	0.1053	0.1055	0.001114	0.1066	0.1048	0.1044	0.105267	0.001172
PCTS/LipL32	0.1115	0.1142	0.1076	0.1111	0.003318	0.1125	0.1087	0.1103	0.1105	0.001908

APPENDIX D

Table D1 Zeta potential of samples

	PCTS					PCTS-Mab					PCTS-LipL32				
pH	1	2	3	avg	std	1	2	3	avg	std	1	2	3	avg	std
5	-3.85	-3.82	-3.56	-3.74	0.16	34.30	34.70	34.70	34.57	0.23	33.40	33.70	33.80	33.63	0.21
6	-3.03	-2.86	-2.81	-2.90	0.12	34.10	34.60	34.10	34.27	0.29	32.80	33.10	33.02	32.97	0.16
7	6.25	6.85	6.63	6.58	0.30	34.40	34.20	34.00	34.20	0.20	32.60	32.60	32.70	32.63	0.06
8	8.20	8.05	8.24	8.16	0.10	31.30	32.30	30.70	31.43	0.81	31.10	31.60	32.10	31.60	0.50
9	9.43	9.03	9.85	9.44	0.41	28.10	28.20	28.10	28.13	0.06	30.60	30.80	30.60	30.67	0.12
10	10.85	10.62	10.70	10.72	0.12	-20.34	-21.10	-20.80	-20.75	0.38	28.20	28.50	28.30	28.33	0.15
11	12.60	12.04	12.80	12.48	0.39	-25.30	-26.90	-26.30	-26.17	0.81	-21.72	-21.40	-20.90	-21.34	0.41
12	13.22	13.41	13.60	13.41	0.19	-30.20	-30.10	-30.40	-30.23	0.15	-25.20	-25.60	-25.90	-25.57	0.35

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International Conferences:

1. N. Sangkapong, W. Tachaboonyakiat, "Effect of charges on drugs encapsulation within phosphorylated chitosan oil-in-water emulsion" The 6th International Polymer Conference of Thailand (PCT-6), Pathumwan Princess Hotel, Bangkok, Thailand, June 30-July 1, 2016.

2. N. Sangkapong, K. Patarakul, A. Sereemaspun, W. Tachaboonyakiat, "Synthesis of water soluble chitosan-protein conjugate" The 252nd American Chemical Society National Meeting & Exposition (Fall 2016 Philadelphia ACS National Meeting), Pennsylvania Convention Center, Philadelphia, Pennsylvania, USA, August 21-25, 2016.