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FUNCTIONAL GROUP MODIFICATION ON THE SURFACE OF CHITOSAN FILM VIA REACTION OF AMINO GROUP

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A Thesis Submitted in Partial Fulfillment of the Requirements

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นพพงษ์ พงศ์ชัยสิริกุล : การดัดแปรหมู่ฟังก์ชันบนพื้นผิวฟิล์มไคโตซานด้วยปฏิกิริยาของหมู่อะมิโน (FUNCTIONAL GROUP MODIFICATION ON THE SURFACE OF CHITOSAN FILM VIA REACTION OF AMINO GROUP) อ. ที่ปรึกษา: ดร. วราวุฒิ ตั้งพสุธาดล; อ. ที่ปรึกษาร่วม: ดร. วิภาวี โฮเว่น; **62** หน้า. ISBN 974-03-1266-7

ไคโตซานเป็นพอลิเมอร์จากธรรมชาติที่ได้จากการกำจัดหมู่แอซิทิลของไคติน โครงสร้างของไคโตซานประกอบไปด้วยหมู่ไฮดรอกซีและหมู่อะมิโนจำนวนมาก ซึ่งสามารถถูกดัดแปรได้โดยปฏิกิริยาเคมี ในงานวิจัยนี้ได้ศึกษาการทำปฏิกิริยาระหว่างหมู่อะมิโนบนไคโตซานกับสารที่เป็นอนุพันธ์ของกรดคาร์บอกซิลิก ได้แก่ ทาลิกและซักซินิกแอนไฮไดรด์ เมตา-ไอโอโดเบนโซอิลคลอไรด์ สเตียริลคลอไรด์และเฮพตะฟลูออโรบิวทีริลคลอไรด์ เพื่อสร้างพันธะแอมีด การดัดแปรหมู่ฟังก์ชันบนพื้นผิวของฟิล์มไคโตซานทำได้โดยแช่ฟิล์มในรีเอเจนต์ที่เหมาะสม จากการวิเคราะห์ทางพื้นผิวโดยเทคนิคเอ็กซ์เรย์โฟโตอิเล็กตรอนสเปกโตรสโกปี (เอ็กซ์พีเอส) พบธาตุฮาโลเจนซึ่งใช้เป็นตัวบ่งบอกถึงผลสำเร็จในการตรึงสารประกอบที่มีธาตุฮาโลเจนกับไคโตซาน ผลจากการวิเคราะห์โดยการวัดมุมสัมผัสของน้ำบนพื้นผิวฟิล์มพบว่าพื้นผิวของฟิล์มไคโตซานมีความชอบน้ำมากขึ้นหลังจากนำฟิล์มไปทำปฏิกิริยากับทาลิกและซักซินิกแอนไฮไดรด์ แต่สมบัติการชอบน้ำจะลดลงจากเดิมหลังจากนำฟิล์มไปทำปฏิกิริยากับสเตียริกแอซิด นอกจากนี้การสังเคราะห์อนุพันธ์ของหมู่อะมิโนที่อยู่บนพื้นผิวของฟิล์มได้รับการยืนยันโดยเทคนิคเอทีอาร์-เอฟทีไออาร์ และสุดท้ายวิธีการดัดแปรที่ใช้ในงานนี้ส่งผลกระทบบางส่วนต่อความสามารถในการดูดซับโปรตีนแอลบูมินและไลโซไซม์บนพื้นผิวฟิล์ม

ภาควิชา.....เคมี.....ลายมือชื่อนิสิต Nopparng Pongchaisirikol

สาขาวิชา.....เคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

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KEY WORD: SURFACE MODIFICATION/ CHITOSAN FILM/ AMIDE BOND/ PROTEIN ADSORPTION/ HYDROPHILICITY

NOPPONG PONGCHAISIRIKUL: FUNCTIONAL GROUP MODIFICATION ON THE SURFACE OF CHITOSAN FILM VIA REACTION OF AMINO GROUP

THESIS ADVISOR: VARAWUT TANGPASUTHADOL, Ph.D.

THESIS CO-ADVISOR: VIPAVEE P.HOVEN, Ph.D. 62 pp.

ISBN 974-03-1266-7

Chitosan, partially deacetylated form of chitin, is a natural polymer. Structure of chitosan contains a large number of hydroxy and amino groups, which can be modified by various chemical reactions. This research focused on the reactions between the amino groups of chitosan and derivatives of carboxylic acids, e.g., phthalic anhydride, succinic anhydride, *m*-iodobenzoyl chloride, stearyl chloride, and heptafluorobutyryl chloride to form an amide bond. The chemical modification was performed by immersing chitosan films in a selected reagent. Surface analysis by X-ray photoelectron spectroscopy (XPS) revealed traces of halogen atoms, indicating a success in attaching the halogenated compounds to chitosan. Results from contact angle measurement suggested that surface hydrophilicity of chitosan films increased after the reaction with phthalic and succinic anhydride. On the other hand, the hydrophilicity is decreased after the reaction with stearic acid. Moreover, derivatization of the amino groups on the film surface was confirmed by ATR-FTIR. Last, these modification methods somewhat affected the adsorptivity of albumin and lysozyme on the film surface.

Department.....Chemistry.....Student's signature.....Noppong Pongchaisirikul.....

Field of study.....Chemistry.....Advisor's signature.....

Academic year.....2001.....Co-Advisor's signature.....Vipavee Hoven.....

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Furthermore, I would like to thank Department of Chemistry for providing excellent facilities, and Ratchadapiseksompoj Research Fund for the financial support of this project.

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LIST OF ABBREVIATIONS

ATR-IR	Attenuated total reflectance infrared spectroscopy
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Chi	Chitosan
Chi-Cl	Chitosan film after reacting with *p*-chlorobenzoyl chloride
Chi-F	Chitosan film after reacting with heptafluorobutyryl chloride
Chi-I	Chitosan film after reacting with *m*-iodobenzoyl chloride
Chi-Pht	Chitosan film after reacting with phthalic anhydride
Chi-Stea	Chitosan film after reacting with stearyl chloride
Chi-Succ	Chitosan film after reacting with succinic anhydride
DCC	*N,N'*-dicyclohexyl carbodiimide
DCM	Dichloromethane
DMF	Dimethylformamide
%DD	Percent degree of deacetylation
h	Hour
HOBt	Hydroxybenzotriazole
Lyz	Lysozyme
MeOH	Methanol
$\overline{M}_v$	Viscosity-average molecular weight
$\overline{M}_w$	Weight average molecular weight
NMR	Nuclear magnetic resonance
PBS	Phosphate buffer saline
PFNB	Pentafluorophenyl-*p*-nitrobenzene sulfonate

pI	Isoelectric point
ppm	Part per million
RT	Room temperature
TEA	Triethylamine
TGA	Thermal gravimetric analysis
XPS	X-ray Photoelectron spectroscopy

CHAPTER I

INTRODUCTION

1.1 Rationale

Chitosan has been shown to have a number of good properties, e.g. biocompatible, antimicrobial, antithrombogenic and biodegradable. It is therefore, of interest to many chemists to try to modify its chemical composition to match a final usage. This work has focused on modifying the outer surface of chitosan materials that are in the form of cast films. It was hypothesized that surface modification could be achieved by immersing a chitosan film in a chemical reagent. The chemically modified films should therefore exhibit different surface properties from the original films. Consequently, the modified chitosan surface could be applied for use in specific applications, such as biomaterials.

1.2 Statement of purpose

The purpose of this research is to modify hydrophobicity and protein adsorptivity of chitosan films. The chemical modification is carried out by means of attaching organic compounds to the films via an amide linkage. The films before and after modification are characterized by XPS and ATR-IR for chemical composition, and by contact angle measurement and protein adsorption study for surface properties.

1.3 Scope of this research

This research was divided as three steps. First, the reactivity of carboxylic acid derivatives towards acylation at amino groups was studied. Aniline and *p*-methyl aniline were chosen as model compounds in this step. Second, the reactions between amino groups on the chitosan films and derivatives of carboxylic acids were studied under heterogeneous condition. The derivatives of carboxylic acids in this step contain halogen atoms that could be detected and, therefore, analyzed for success of the

reaction. Last, the changes of hydrophobicity on the surface of chitosan films after reacting were investigated by using contact angle measurement. Moreover, the protein adsorptivity on the surface of chitosan films before and after modified are studied. The result of proteins adsorption study could be used as information for the development of biomaterials in the future.

1.4 Theory

1.4.1 Chitosan

Chitosan, an *N*-deacetylated[1] derivative of chitin, has a structure similar to cellulose. Chitosan mainly consists of 2-amino-2-deoxy-D-glucose (GlcN) repeating unit with a small amount of 2-acetyl-2-deoxy-D-glucose residues. The amount of GlcN unit in chitosan is generally referred to by percent degree of deacetylation or %DD. Various techniques were used for determination of %DD such as IR,[2] NMR,[3] and metachromatic titration.[4] The structures of chitosan and chitin are shown in Figure 1.1.

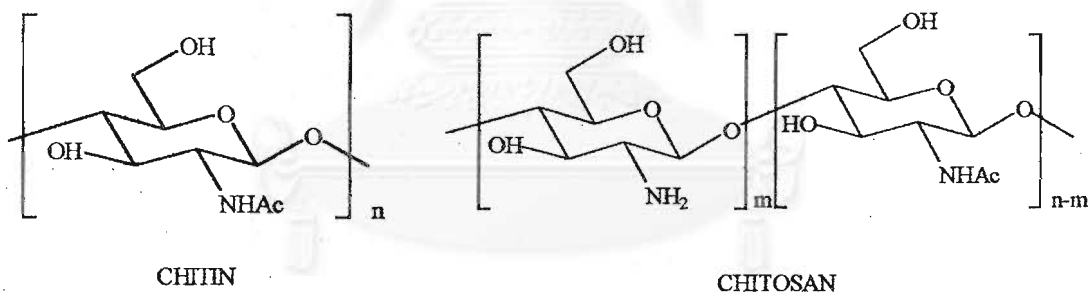


Figure1.1 Structures of chitin and chitosan

Chitosan is dissolved in inorganic acids such as HCl, HNO_3, and organic acids, preferably acetic acid and formic acid. Pure chitosan precipitates from solutions if the pH rises above 6.

Since chitin is the main component of the exoskeleton of crustaceans such as crabs, lobsters, shrimps, and cray fish, chitin and chitosan have recently been interesting choices of materials for various types of health related applications. Both chitin and chitosan are proved to be biologically safe for use in human. Chitosan was also found to be an antimicrobial and antifungal agent. It is therefore used in food industries[5] and

many biomedical related applications [6] such as wound healing patches and drug delivery[7] etc. Besides, chitosan can chelate metal ions so it is used as metal capture[8] in waste water treatment.

1.4.2 Chemical modification of chitosan

Chitosan carries a number of hydroxy (-OH) and amino ($-NH_2$) groups. This prompts many researchers to find ways of modifying its properties either by physical blending or chemical reactions.

Graft-copolymerization

- Poly(ethylene glycol) (PEG) was grafted onto chitosan by Schiff-base formation under homogeneous condition.[6] The resulting chitosan-g-PEG exhibited improved mechanical properties and antithrombogenicity.
- Acrylic acid (AA) and methacrylic acid (MAA) were grafted onto chitosan using a ceric ion initiation technique.[9] The resulting chitosan-g-AA and chitosan-g-MAA showed great potentials to be used as biodegradable drug delivery devices.

Crosslinking

- Chitosan chains were crosslinked with epichlorohydrin or glutaraldehyde for controlled delivery of drugs[10] such as 2,5-dihydroxybenzenesulfonic acid (DHBSA) and 8-hydroxy-7-iodoquiniline-5-sulfonic acid (SQ).

Blend or interpenetrating polymer network (IPN)

- Interpenetrating polymer network (IPN) hydrogels composed of chitosan and poly(acrylic acid) (PAAc)[11] were synthesized by UV irradiation. The IPN polymer showed improvement of mechanical properties. This IPN was intended to be used in biomedical fields
- PVA/chitosan-blended hydrogels are biocompatible materials of considerable promise: they have high water content and facilitate both a high degree of cell attachment and a remarkable cell growth rate.[12]

Moreover, percent elongation and CO_2 and water vapour permeability of chitosan/PVA blends could be modified by adding plasticizer, sucrose and sorbitol. Thus it can be used in food packaging industries.[13]

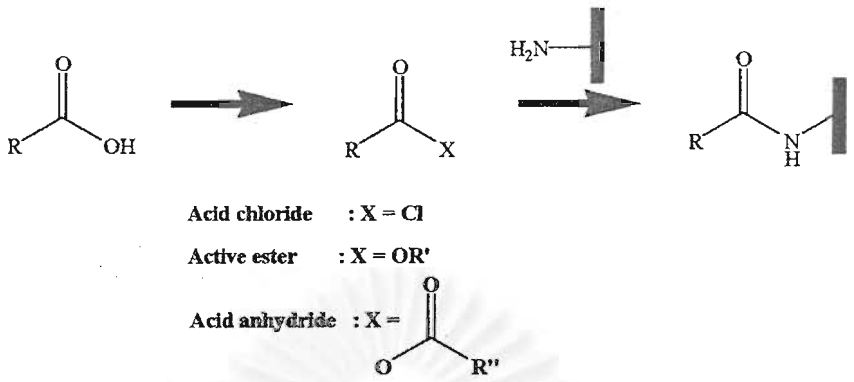
Attachment of chemical compounds

- *N*-benzyl sulfonate derivatives were attached on chitosan in order to improve heavy metals sorption from acidic industrial effluents.[8]
- Attachment of peptide on chitosan was reported. The material was intended to be used as biocompatible and biodegradable materials[14] and may be used as a drug carrier for peptides.[15]

Synthesis of chitosan derivatives can be carried out in a homogeneous or heterogeneous fashion. In homogeneous condition,[16] the solvents used are limited to only acidic solvents. For example, reactions between chitosan and fluorescein-5-isothiocyanate[17] or *p*-formylphenyl-α-melibioside,[18] were carried out in 0.1 M acetic acid. Soluble carbodiimide (WSC) and isonicotinic acid[16] were also used as coupling reagent. In the heterogeneous condition, chitosan powder was suspended in organic solvents such as THF, DMF, and methanol. A number of compounds, including hyperbranched dendritic polyamidoamine[19], were successfully grafted onto the chitosan powder under a heterogeneous condition.

1.4.3 Surface modification

In this research, chemical modification of chitosan was focused on amide bond formation under a heterogeneous condition. Chitosan in the form of films were allowed to react with various types of carboxylic acid derivatives. It was postulated that these active derivatives could be attached to the surface of chitosan film at the amino groups, thus via amide linkages (Scheme 1.1). It was also expected that surface properties of chitosan films could be varied depending on the polarity of the derivatives used. Many factors were considered; molecular weight of chitosan, type of medium, types of reagents, reaction time and temperature.



Scheme 1.1 Attachment derivatives of carboxylic acid compounds onto the surface of chitosan film at the amino groups via amide linkage

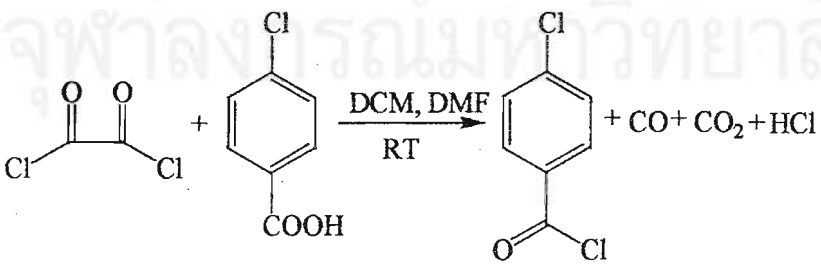
1.4.4 Amide formation[20]

In this study, four amide formation methods were investigated.

Reaction between amino groups and acid chlorides

In order to convert a carboxylic acid into acylating agents, its hydroxy group must be replaced by an electron-withdrawing substituent (X) to enhance the polarization of the carbonyl group and thereby the electrophilicity of its carbon atom. Thus the nucleophilic attack by the amino group on chitosan is greatly facilitated. Chlorine is an obvious choice for the role of the electron-withdrawing moiety (X).

In this study, conversion of carboxylic acid to acid chloride was carried out with oxalyl chloride (Scheme 1.2).



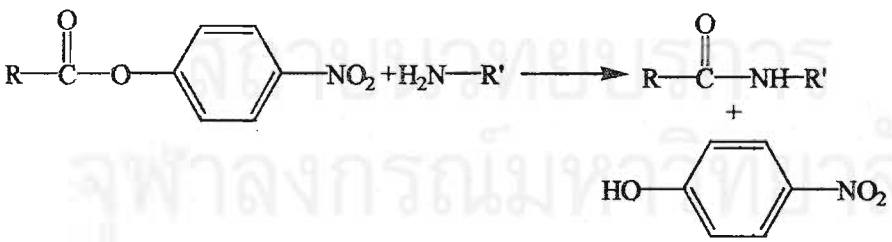
Scheme 1.2 Conversion of carboxylic acid to acid chloride

CO, CO_2 and HCl generated in the reaction readily escape. *p*-Chlorobenzoic acid, *m*-iodobenzoic acid and stearic acid are choices of the carboxylic compounds selected in this work.

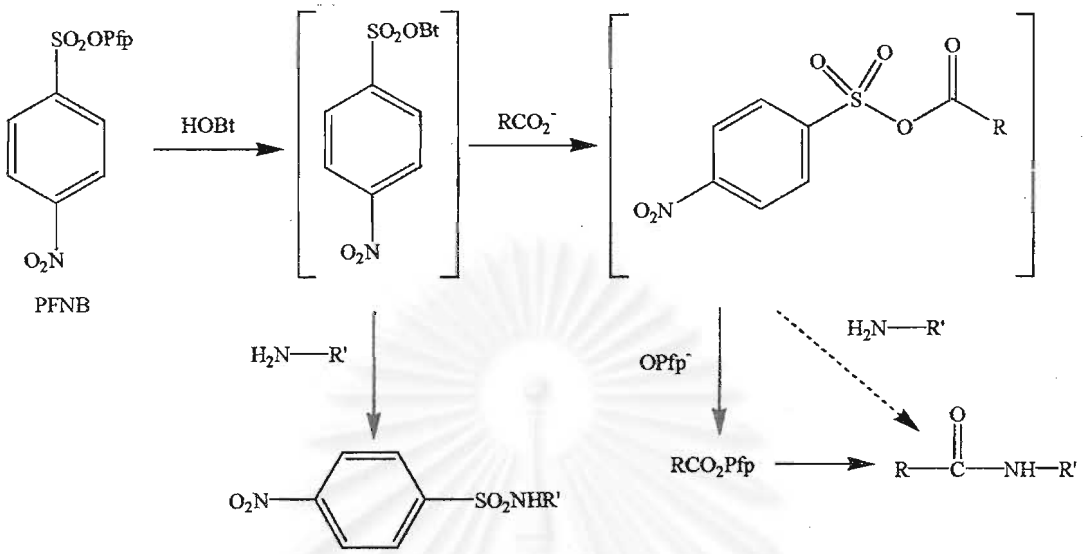
Reaction between amino groups and active esters

Various active esters were used for peptide synthesis such as aminolysis of cyanomethyl ester (Scheme 1.3) and aminolysis of *p*-nitrophenyl ester (Scheme 1.4). In 2001, Pudhom and Vilaivan have reported using pentafluorophenyl-*p*-nitrobenzene sulfonate (PFNB)[21] as peptide coupling agent and 1-hydroxybenzotriazole (HOBt) as catalyst for amide formation. Using PFNB can reduce reaction time. Product obtained can be easily purified (Scheme 1.5).

Scheme 1.3 Aminolysis of cyanomethyl ester



Scheme 1.4 Aminolysis of *p*-nitrophenyl ester

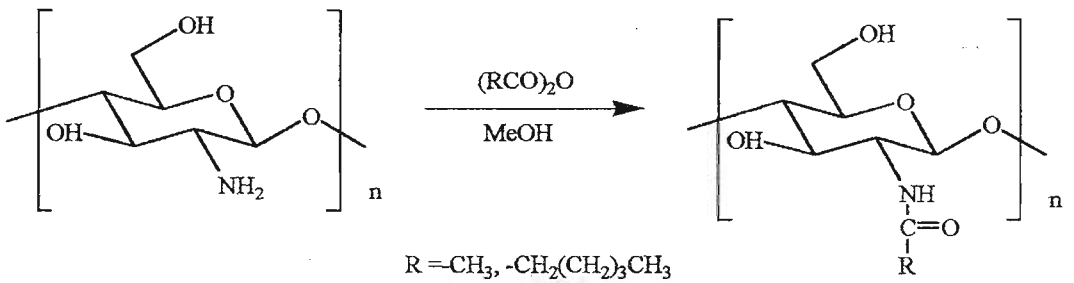


Scheme 1.5 Using pentafluorophenyl 4-nitrobenzene sulfonate (PFNB) as a peptide coupling agent and 1-hydroxybenzotriazole (HOBt) as a catalyst for amide formation

In this study, *p*-chlorobenzoic acid was reacted with PFNB to form an active ester. The active ester then reacted with amino groups on chitosan by using HOBt as catalyst under heterogeneous reaction.

Reaction between amino groups and acid anhydrides

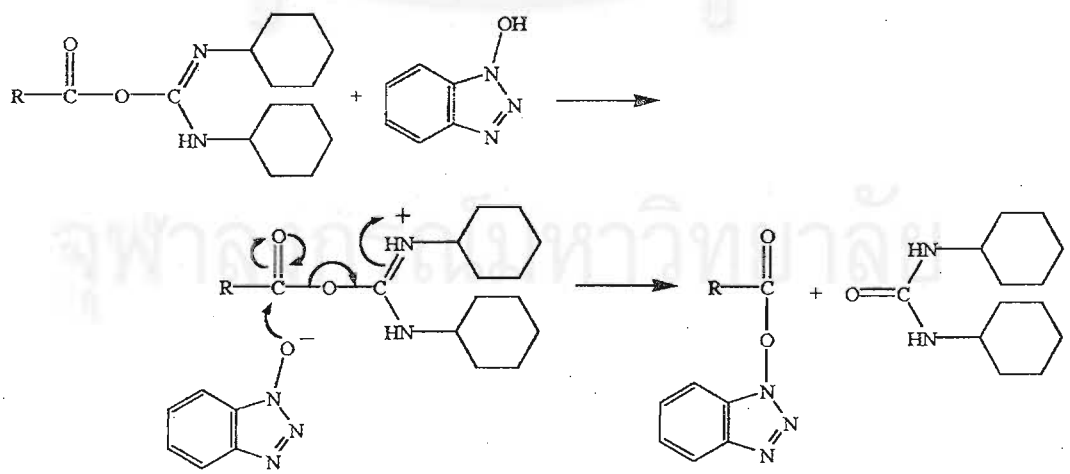
Modifications of chitosan by acid anhydrides have been reported. Acetylated and hexanoylated chitosan films were prepared using acetic anhydride and hexanoic anhydride in methanol under heterogeneous condition (Scheme 1.6).[22] Partially deacetylated chitin derivative via a ring opening reaction with cyclic acid anhydride were prepared in lithium chloride and DMA under homogeneous condition.[23] In this study, phthalic and succinic anhydride were chosen to react with chitosan under heterogeneous condition.



Scheme 1.6 Acetylated and hexanoylated chitosan films

Reaction between amino groups and carboxylics acid in the presence of coupling reagents

Coupling reagents, such as dicyclohexylcarbodiimide (DCC), could react with carboxyl component to form O-acyl-isourea. These intermediates contain a N=C group that provides powerful activation for coupling with amine. The speedy execution of activation and coupling in a single operation and the sample removal of the insoluble by-product, *N,N'*-dicyclohexylurea (DCU), all contributed to the popularity of the DCC method. Both racemization and *N*-acylurea formation can be suppressed by the addition of auxiliary nucleophiles such as 1-hydroxybenzotriazole (HOBt). Coupling reaction mechanism using DCC/HOBt system was shown in Scheme 1.7.



Scheme 1.7 Coupling reaction mechanism using DCC/HOBt

In this research, DCC was selected as a coupling reagent and HOBt as auxiliary nucleophile. Stearic acid is a model of carboxylic acid compounds that was attached on chitosan surface. The reaction was carried out under heterogeneous condition.

1.4.5 Modifying surface properties of polymers

Recently, polymers have been increasingly used in various applications. One way to match polymer properties to its usage is to modify its surface either by chemical or physical methods. Therefore a number of surface properties, such as surface reactivity towards chemical reaction, compatibility between interface, surface hydrophobicity, and adhesion property are of interest among many researchers nowadays.

Techniques of physical modification on polymer surface include flame, plasma, UV, laser treatment, and sputtering. In chemical modification, polymer surfaces were modified by direct chemical reactions with substances that can be either small molecules or macromolecules. Figure 1.2 shows some of the methods used to modify polymer surfaces.

Considering chitosan structure, the hydroxyl and amino groups provide several possibilities for derivatization or grafting of desirable bioactive groups. By grafting hyperbranched dendritic polyamidoamine to chitosan, chitosan powder surface was found to turn from hydrophobic to hydrophilic.[19] The grafting was carried out by repeating two processes, Michael addition of methyl acrylate (MA) to the amino groups of chitosan powder and amidation of resulting ester with ethylenediamine. This grafting product was postgrafted by living polymer cation of 2-methyl-2-oxazoline (MeOZO) and isobutyl vinyl ether (IBVE). Solubility of chitosan in water is increased after grafting with MeOZO onto the chitosan powder.[24] In another study, the rate of enzymatic degradation of chitosan films were increased by acetylation at the amino groups on the film surface using acetic anhydride.[22] This reaction in fact turned glucosamine units on the film surface to *N*-acetyl-glucosamine, which is more susceptible to enzymatic hydrolysis than glucosamine. Therefore, regenerating chitin chain at the film surface dramatically enhanced rate of film biodegradation.

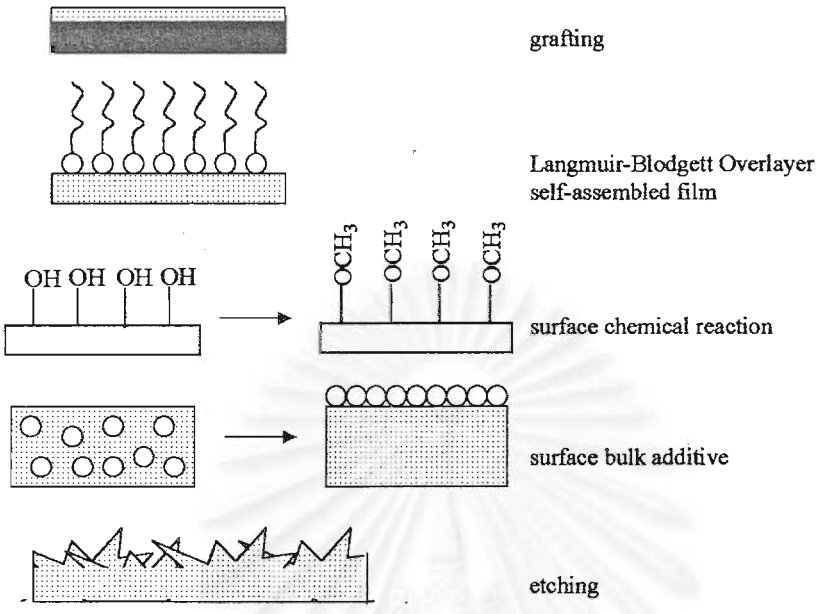


Figure 1.2 Methods used to modify polymer surfaces

1.4.6 Surface characterization

Surface characterization is a method for analyzing chemical and physical properties of material surface. In this research, surface of polymers, before and after modification, were analyzed for atomic composition, hydrophobicity, and protein adsorptivity. Various techniques were used as follow:

X-ray photoelectron spectroscopy (XPS)

XPS is a surface analysis method that provides information on atomic composition of the first 10 nm layer of material surface. In general, the sample is put inside a high-vacuum chamber (pressure 10^{-5} Pa), and irradiated with soft X-rays, usually Mg Kα (1253.6 eV) or Al Kα (1486.6 eV). The primary event is photoemission of a core electron as shown in Figure 1.3. Electrons are also photo-emitted from molecular orbitals occupying the valence band, but with much lower intensity. Spectrum is obtained by passing the emitted electrons into an electrostatic energy analyzer. The binding energies, E_B, of the photoelectron are obtained via the Einstein relation: $E_B = h\nu - E_K - \phi$ where $h\nu$ is the X-ray photon energy, E_K is the electrostatic

energy and ϕ is the sample work function. Peak intensities are proportional to the number of atoms sampled, and with the aid of appropriate sensitivity factors, atomic compositions can be calculated, with detection limits of ~0.2 atom%. In this research, halogens were used as labeling atoms for checking the success of functional group modifications on the surface of chitosan films.

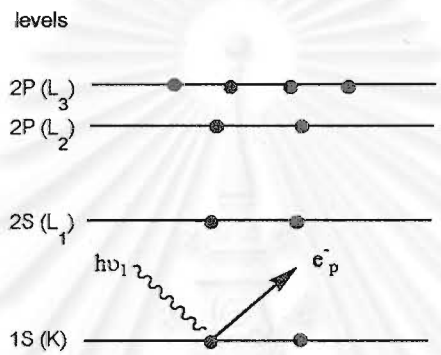


Figure 1.3 photoemission of a core electron

In XPS, take-off angle can be adjusted in order to analyze atomic composition at each depth from the outmost surface. The higher the take-off angle is, the deeper film layer the analysis can be carried out (Figure 1.4).

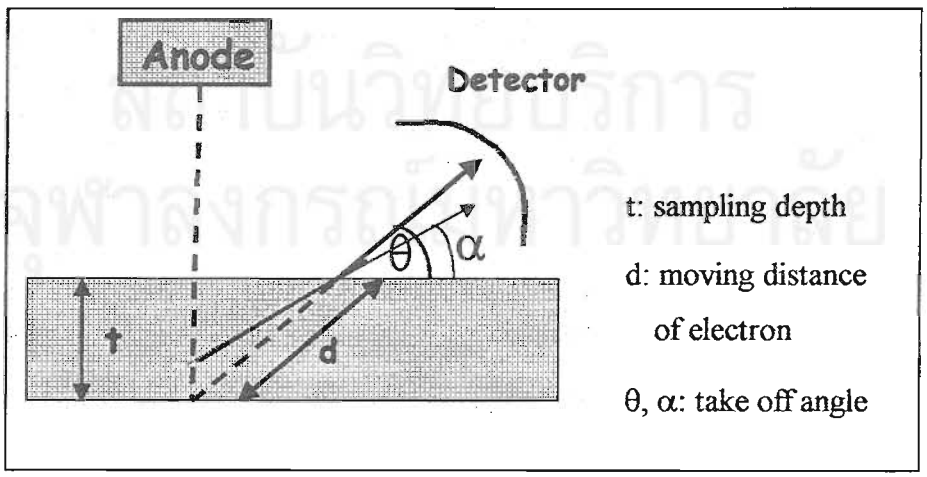
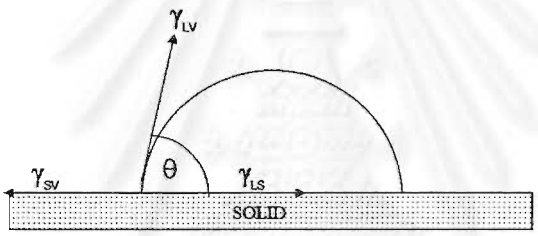


Figure 1.4 Effect of take off angle to depth profile

Contact angle measurement

Contact angle measurement is probably the most common method of surface tension measurement of solids. Contact angle data, especially in the case of polymeric materials, can be obtained with low-price instruments and with simple techniques. The basis of the measurement of solid surface tension by contact angle is the equilibrium of the three-phase boundary, shown in Figure 1.5. As the surface becomes more hydrophobic, θ will be larger.

In this research, air-water contact angle was primarily used for determining hydrophobicity of the chitosan surface.



$\gamma_{SV} - \gamma_{LS} = \gamma_{LV} \cos\theta$: 3-phase equation

γ_{LV}: interfacial tension between liquid and vapour phases

γ_{SV}: interfacial tension between solid and vapour phases

γ_{LS}: interfacial tension between liquid and solid phases

Figure 1.5 Equilibrium of the three-phase boundary on solid surface

Attenuated total reflectance infrared spectroscopy (ATR-IR)

The infrared beam from the spectrometer is focused onto the beveled edge of an internal reflection element (IRE). The beam is then reflected, generally numerous times, through the IRE crystal, and directed to a detector (Figure 1.6).

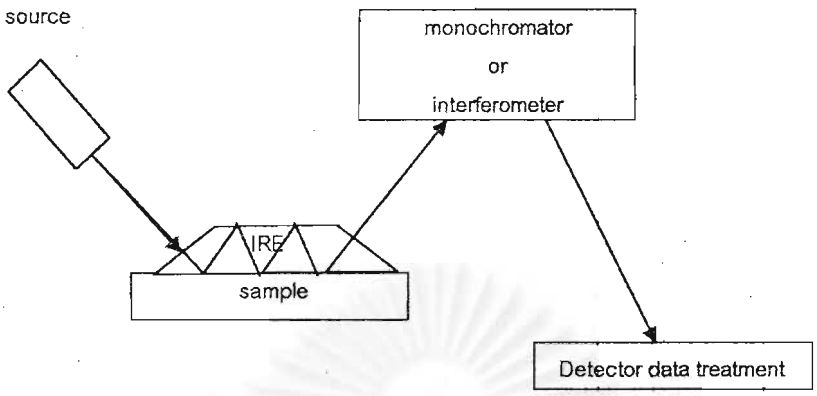


Figure 1.6 Diagram of ATR-IR

The radiation can penetrate a short distance into the sample, thus interact with any functionalities existed within that depth. The depth of penetration (d_p, defined as the distance from the IRE-sample interface where the intensity of the evanescent wave decays to 1/e of its original value) can be calculated using the formula in Equation:

$$d_p = \frac{\lambda}{2\pi n_p(\sin^2\theta - n_{sp}^2)^{1/2}}$$

where λ = wavelength of the radiation in the IRE, θ = angle of incidence, n_{sp} = ratio of the refractive indices of the sample vs. IRE, and n_p = refractive index of the IRE. Practically, the sample is placed in close optical contact with one of the crystal. In this study, ATR-IR was used for identifying functional groups on the surface of chitosan films before and after chemical modifications. Sampling depth of characterization is 1-1.5 μm.

1.4.7 Protein adsorption on polymer surface

The study of protein adsorption has attracted considerable attention in the last few decades.[25, 26] The adhesion of proteins to surfaces is particularly important in such fields like bioengineering and bioscience. The driving force for protein adsorption may include, van der Waals, hydrophobic interactions, hydrogen bonding, and electrostatic interactions between oppositely charged surface and protein domains.

In this work, protein adsorption on the modified chitosan films surfaces was studied using albumin (bovine serum albumin) and lysozyme (chicken egg white). Albumin is one of the more abundant blood proteins and its biological functions are transport and maintenance of colloid osmotic pressure. Lysozyme is an antimicrobial protein. An isoelectric point (pI) of lysozyme is 11 and contains larger amount of –OH, $-NH_2$ than –COOH groups. Albumin has a pI of 4.8. It is therefore a –COOH rich protein. Adsorptivity of proteins on the chitosan surface before and after modified was compared. These results could be used to design a surface that can control the amount of protein adsorption.

CHAPTER II

EXPERIMENTAL

2.1 Materials

Dimethylformamide (DMF) was distilled over CaH_2 and under reduced pressure. Dichloromethane (DCM) was distilled over CaH_2. Methanol was distilled over molecular sieve type 4A. DCC, HOBt, phthalic anhydride, succinic anhydride, *p*-chlorobenzoic acid, *m*-iodobenzoic acid, and stearic acid were purchased from Fluka Chemika. Bovine serum albumin, lysozyme, bicinchoninic assay kit, PBS, and TEA were purchased from Aldrich Chemical Co. Chitosans $\overline{M}_v$ = 645,535 and $\overline{M}_v$ = 108,630 were obtained from Seafresh Chitosan (Lab) Co., Ltd. and Taming Enterprises Co., Ltd, respectively.

2.2 Equipments

2.2.1 Nuclear magnetic resonance (NMR)

NMR spectra of chitosan were obtained from 1% chitosan dissolved in 1% CD_3COOD in D_2O using 200 MHz (^1H, ^{13}C) on Bruker BZH 200. Chemical shift are in ppm.

2.2.2 Attenuated total reflectance infrared spectroscopy (ATR-IR)

All spectra were collected at resolution of 4 cm^{-1} and 16 scan co-addition using Bruker vector 33 FT-IR spectrometer equipped with a DTGS detector. A multiple attenuated total reflection (MATR) accessory with 45° zinc selenide (ZnSe) IRE (spectra Tech, USA) and a variable angle reflection accessory (Seagull™, Harrick Scientific, USA) with a hemispherical ZnSe IRE were employed for all ATR spectral acquisitions.

2.2.3 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectra (XPS) were obtained on a Perkin-Elmer Physical Electrons 5100 using Mg K_{α} excitation (15 kV, 400W). Atomic composition data were determined using sensitivity factors obtained from samples of known composition: C_{1s}, 0.210; O_{1s}, 0.560; N_{1s}, 0.380; F_{1s}, 1.000; Cl_{2p}, 0.650; I_{3d5}, 6.000. In this study, the take off angle at 15°, 75° were chosen and the approximate of depth profile is 10Å and 40Å respectively.

2.2.4 Contact angle measurement

Deionized water was dropped onto film surface by a Gilmont syringe with a 24-gauge flat-tipped needle. Images of water droplets on film surface were taken with a digital camera (Sony, Model F707). The contact angles were measured with Adobe Photoshop 6.0 software.

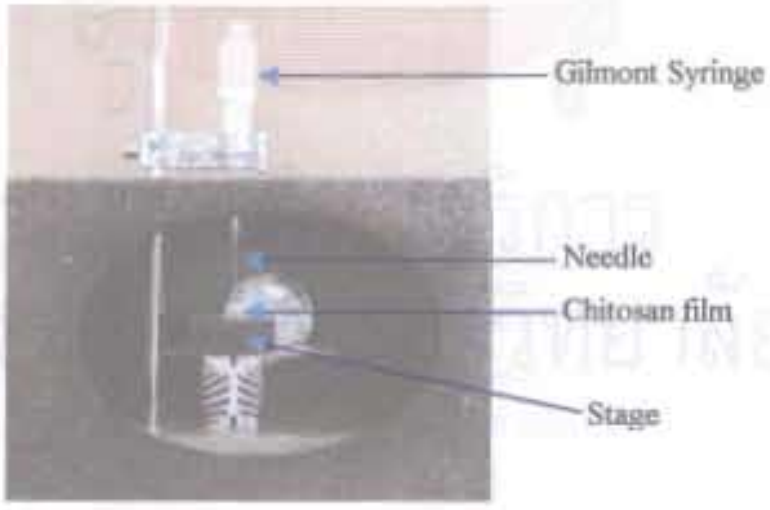


Figure 2.1 Instrument set up for measurement of water contact angle

2.2.5 UV-spectroscopy

UV-spectrometer, JENWEY 6405 UV/VIS spectrometer, was used for determining the amounts of adsorbed protein.

2.3 Determination of % deacetylation (%DD) of chitosan by ^{1}H NMR

Chitosan was dissolved in 1% deuterated acetic acid (CD_3COOD) in D_2O. Percent deacetylation of chitosan was calculated from peak areas of –C<u>H</u>NH_2 at 2.68 ppm and –C<u>H</u>$_3$ of *N*-acetyl group at 1.55 ppm.

2.4 Preparation of chitosan films

Chitosan (2 g) was dissolved in 0.1 M acetic acid (100 mL). After stirring for 24 h, the solution was filtered through a medium pore size sintered glass to remove insoluble substances. The chitosan solution was then cast into film on a Teflon-coated mold (8x8 inch in size). The solvent was allowed to evaporate in air for 4-5 days. The chitosan film was peeled off and immersed in 0.1 M NaOH/methanol (1:1) and methanol/water (1:1) to neutralize the acid used as a solvent. The film was dried under vacuum for more than 1 day to remove solvents. Film thickness was between 40 to 100 μm.

2.5 Molecular weight determination of chitosan by solution viscometry method

Chitosan (0.1 g) was dissolved in 1 M acetic acid solution (10 mL). Water (60 mL) was then added and the solution was stirred overnight. To the solution, 1 M NaCl solution (20 mL) was added and stirred overnight. Last, water was added to the solution to make a 100 mL stock solution. The chitosan solution was filtered through cellulose acetate membrane with pore size 0.8 μm. All viscosity measurements were done in an Ubbelohde tube at 25 °C. Molecular weight of chitosan was calculated from Mark-Houwink equation.

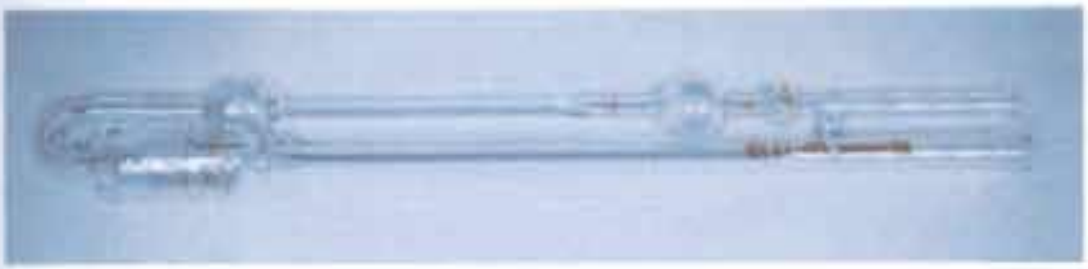


Figure 2.2 Ubbelohde tube

2.6 Synthesis of *m*-iodobenzoyl chloride

Oxalyl chloride (1.5 mL, 16 mmol) was added to a solution of *m*-iodobenzoic acid (3.97 g, 16 mmol) in DCM (7 mL) at room temperature under N_2 atmosphere. DMF (0.1-0.2 mL) was added to the solution and was stirred at room temperature under N_2 gas for 3 h. The organic solvent was evaporated under vacuum. The product was washed by DCM (10 mL, 3 times) and dried under vacuum.

2.7 Reaction between *p*-methyl aniline and *m*-iodobenzoyl chloride

m-Iodobenzoyl chloride (0.37 g, 1.4 mmol) was dissolved in DMF (5 mL). The solution was transferred into a Schlenk flask containing *p*-methyl aniline (1.4 mmol) and TEA (2 mmol) at 0 °C via a cannular. The solution was stirred at room temperature under N_2 gas for 18 h. This solution was diluted with DCM. The solution was extracted with 5% HCl, 5% $NaHCO_3$ and H_2O and then dried over $NaSO_4$. The dried solution was evaporated under reduced pressure. ^{1}H NMR δ 2.3 (3H, s, CH_3), 7.1-7.2 (2H, d, Ar) 7.1-7.2 (1H, t, Ar), 7.4-7.5 (2H, d, Ar), 7.7-7.8 (2H, d, Ar), 8.1 (1H, s, Ar)

2.8 Reaction between chitosan films and *m*-iodobenzoyl chloride

m-Iodobenzoyl chloride (4.26 g, 16 mmol) was dissolved in DMF (10 mL). The solution was transferred into a Schlenk flask containing chitosan films (0.16 g,

0.8 mmol) and TEA (2.2 mL, 16 mmol) at 0 °C via a cannular. The solution was stirred at room temperature under N_2 gas for 48 h. The films were rinsed with DMF (10 mL, 2X, 24 h soaking period), methanol (10 mL, 2X, 24 h soaking period) and were dried under vacuum before characterization.

2.9 Synthesis of stearyl chloride

Stearyl chloride was prepared using the same procedure for preparing *m*-iodobenzoyl chloride, but stearic acid was used as the starting material instead of *m*-iodobenzoic acid.

2.10 Reaction between aniline and stearyl chloride

Stearyl chloride (5 mmol) was dissolved in DMF (5 mL). The solution was transferred into a Schlenk flask containing aniline (5 mmol) and TEA (7 mmol) at 0 °C via a cannular. The solution was stirred at room temperature under N_2 gas for 15 h. This solution was diluted with DCM. The solution was extracted with 5% HCl, 5% $NaHCO_3$ and H_2O and then dried over $NaSO_4$. The dried solution was evaporated under reduced pressure. ^{1}H NMR δ 0.8 (3H, t, CH_3), 1.2 (26H, m, CH_2), 1.6 (4H, m, CH_2), 2.2 (2H, t, $C\underline{H}_2C{=}O$), 7.0-7.5 (5H, m, Ar)

2.11 Reaction between chitosan films and stearyl chloride

The procedure of this reaction is the same as the reaction of chitosan films and *m*-iodobenzoyl chloride, except stearyl chloride was used instead of *m*-iodobenzoyl chloride.

2.12 Reaction between chitosan film and heptafluorobutyryl chloride

DMF (10 mL) was added into a Schlenk flask containing chitosan films. TEA (0.2 mL, 1.4 mmol) and heptafluorobutyryl chloride (0.2 mL, 1.4 mmol) were added into the Schlenk flask via syringes. The solution was stirred at room temperature under N_2 gas for 48 h. Then the films were rinsed with DMF (2X, 24 h soaking

period) and methanol (2X, 24 h soaking period). The films were dried under vacuum before characterization.

2.13 Reaction between chitosan films and phthalic anhydride

Anhydrous DMF or methanol (10 mL) was added into a Schlenk flask containing chitosan films and phthalic anhydride (20 equivalents of amino groups in chitosan films). The reaction was stirred at room temperature, 50 °C for methanol, and 80 °C for DMF under N_2 gas. The reaction proceeded for 48 h. The films were rinsed with DMF or methanol (2X, 24 h soaking period), to remove unreacted anhydride. The films were dried under vacuum before characterization.

2.14 Reaction between chitosan films and succinic anhydride

The procedure of this reaction is the same as reaction between chitosan film with phthalic anhydride, except succinic anhydride was used instead of phthalic anhydride.

2.15 Reaction between chitosan films and stearic acid using DCC as a coupling agent

Stearic acid (1.4225 g, 5 mmol), HOBt·H_2O (0.7657 g, 5 mmol) and DCC (5 mmol, 1.0317 g) were dissolved in DMF (15 mL). Chitosan films (0.06 g, 0.25 mmol of NH_2) were added to the solution. The solution was stirred at room temperature under N_2 gas for 48 h. The films were then rinsed by DMF (2X, 24 h soaking period) and methanol (12 h soaking period). The films were dried under vacuum before characterization.

2.16 Reaction between chitosan films and *p*-chlorobenzoic acid using DCC as a coupling agent

The procedure of this reaction is the same as reaction between chitosan films with stearic acid, except *p*-chlorobenzoic acid was used instead of stearic acid.

2.17 Reaction between chitosan and *p*-chlorobenzoic acid using PFNB

PFNB (0.63 g, 1.7 mmol), HOBt·H_2O (0.23 g, 1.7 mmol) and *p*-chlorobenzoic acid (0.27 g, 1.7 mmol) were dissolved in DMF (5 mL). TEA (0.24 mL, 1.7 mmol) was added to the solution. The solution was stirred at room temperature under N_2 gas. After 2 h, the solution was transferred via a cannular to a Schlenk flask containing chitosan films (0.017 g, 0.087 mmol of NH_2) under N_2 atmosphere. Let reaction proceed for 48 h at room temperature. The films were rinsed by DMF (2X, 24 h soaking period), DCM (24 h soaking period), and dried under vacuum before characterization.

2.18 Protein adsorption on the surface of chitosan films

Protein solutions were freshly prepared by dissolving BSA or lysozyme phosphate buffer saline (PBS) at pH 7.4 to give a final concentration of 0.1 mg/mL. The film substrate was immersed in PBS solution for 2 h prior to adsorption. The adsorption experiments were performed in a close polyethylene bottle, by immersing the film in 3 mL protein solution at 37 °C. After three hours, the films were removed and rinsed with 4x10 mL PBS solution to remove reversibly adsorbed protein. To remove irreversibly adsorbed protein from the film surface, each film was transferred to another vial containing 2 mL of 1.0 wt% sodium dodecylsulfate (SDS), and soaked for 1 h at room temperature, followed by sonication for 10 min. To determine the total amount of protein adsorbed on the substrates, micro-bicinchoninic acid (BCA) protein assay was utilized. The 0.5 mL solution was withdrawn from the vial and mixed with 0.5 mL BCA working solution in a test tube. The mixed solution was left stand at room temperature for 16 h to maximize color development. Measure the absorbance of the solution at 562 nm by UV-VIS spectroscopy. The amount of adsorbed protein was determined by comparison of the absorbance of the samples to a calibration curve. Three repetitions were performed for all samples.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Characterization of chitosan

3.1.1 Determination of %degree of deacetylation (%DD) of chitosan by ^{1}H NMR

%DD is defined as the ratio of glucosamine unit and *N*-acetyl glucosamine. ^{1}H NMR was used to determine the %DD of chitosan (Figure 3.1 and 3.2). The signal for $-CH_3$ of *N*-acetyl glucosamine units (1.55 ppm) and the proton at C-2 of glucosamine units (2.68 ppm) were integrated to determine %DD (Table 3.1 and 3.2).

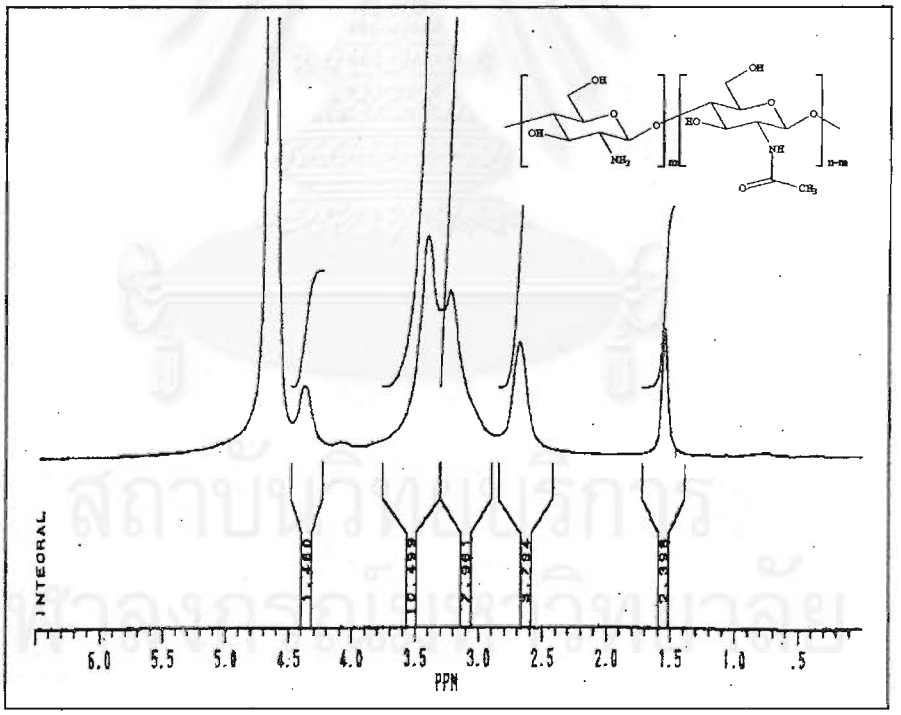


Figure 3.1 ^{1}H NMR spectrum of chitosan from Taming Enterprises Co., Ltd. (solvent: 1% CD_3COOD in D_2O, 25 °C)

Table 3.1 Information obtained from ^{1}H NMR spectroscopy of chitosan from Taming Enterprises Co., Ltd.

	δ (ppm)	Integration	Number of units in chitosan
-C<u>H</u>NH$_2$ of GlcN	2.68	3.79	3.79
-C<u>H$_3$</u> of GlcNAc	1.55	2.39	2.39/3=0.80

From the data in Table 3.1, %DD could be calculated as follows. The total amount of GlcN and GlcNAc units, in chitosan, are equal to 3.79+(2.39/3) = 4.59 units. If the total repeat units in chitosan are 100%, thus %DD is $\frac{3.79 \times 100}{3.79 + (2.39/3)}$ = 82.57%.

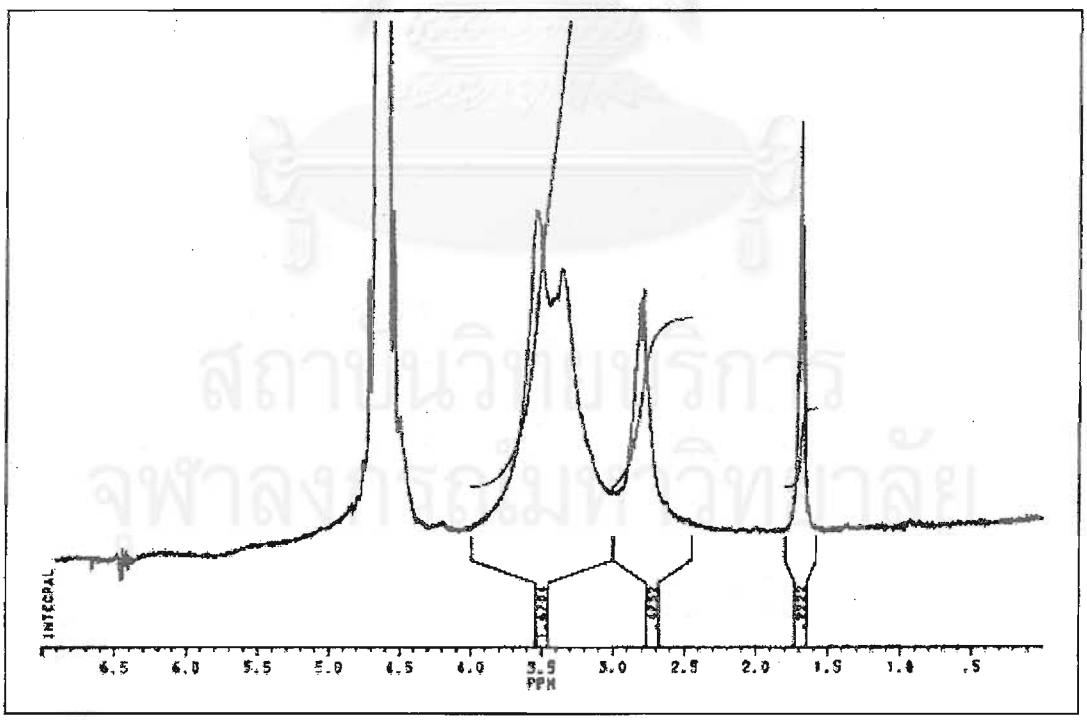


Figure 3.2 ^{1}H NMR spectrum of chitosan from Seafresh Chitosan (Lab) Co., Ltd. (solvent: 1% CD_3COOD in D_2O, 25 °C)

Table 3.2 Information from ^{1}H NMR spectroscopy of chitosan from Seafresh Chitosan (Lab) Co., Ltd.

	δ ppm	**Integration**	**Amount of units in chitosan**
-C<u>H</u>NH_2 of GlcN	2.78	0.48	0.48
-$C\underline{H}_3$ of GlcNAc	1.68	0.22	0.22/3=0.07

From the data in Table 3.2, %DD of chitosan from Seafresh Chitosan is 87.27%.

3.1.2 Molecular weight determination by solution viscometry method

Results of viscosity measurement by Ubbelohde tube method for chitosan from Seafresh Chitosan (Lab) Co., Ltd. and from Taming Enterprises Co., Ltd. are listed in Table 3.3 and 3.4, respectively. Plots between concentration (C) and η_{sp}/C of both chitosan were shown in Figure 3.3 and 3.4. From the relationship between C and η_{sp}/C, Y intercept is intrinsic viscosity $[\eta]$ of the very dilute solution. $[\eta]$ was used to calculate molecular weight of chitosan from Mark-Houwink equation: $[\eta] = KM_v^a$ ($K = 1.8x10^{-3}$, a = 0.93, temperature = 25 °C).[27]

Table 3.3 Viscosity information of chitosan from Seafresh Chitosan (lab) Co., Ltd.

Concentration (C)	t_i[a] (sec)	$\eta_r = t_i / t_m$[b]	$\eta_{sp} = \eta_r - 1$	η_{sp}/C
0.001	143	1.6250	0.625	625
0.0009	133	1.5113	0.5113	596.5909
0.0008	126	1.4318	0.4318	575.7576
0.0006	119	1.3522	0.3522	563.6364
0.0005	115	1.3068	0.3068	572.7273
0.0004	112	1.2727	0.2727	581.8182

[a] t_i = flow time of chitosan solution

[b] t_m = flow time of solvent = 88 sec.

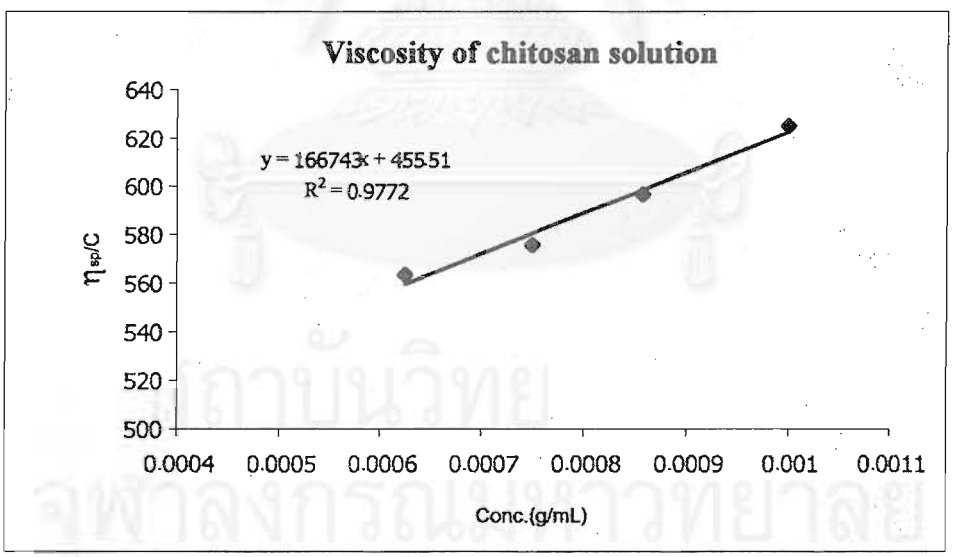


Figure 3.3 A plot between concentration and η_{sp}/C of chitosan obtained from Seafresh Chitosan (lab) Co., Ltd.

For chitosan obtained from Seafresh Chitosan (Lab) Co., Ltd., intrinsic viscosity of solution [η] is equal 455.51. Using Mark-Houwink correlation, viscosity-average molecular weight ($\overline{M}_v$) of chitosan (Seafresh Chitosan (Lab) Co., Ltd.) is 645,535 g/mol.

Table 3.4 Viscosity information of chitosan from Taming Enterprises Co., Ltd.

Concentration (C)	t_i[a] (sec)	$\eta_r = t_i / t_m$[b]	$\eta_{sp} = \eta_r - 1$	η_{sp}/C
0.0020	107	1.2159	0.2159	107.9545
0.0017	104	1.1818	0.1818	106.0606
0.0015	102	1.1590	0.1590	106.0606
0.0013	99	1.1250	0.1250	100.0000
0.0011	97	1.1022	0.1022	95.4545
0.0009	96	1.0909	0.0909	96.9697

[a] t_i = flow time of chitosan solution

[b] t_m = flow time of solvent = 88 sec

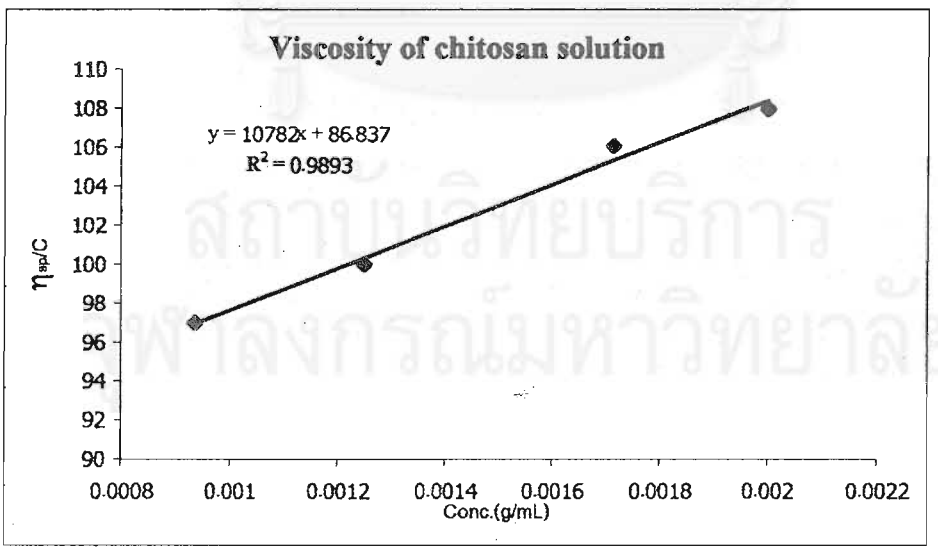


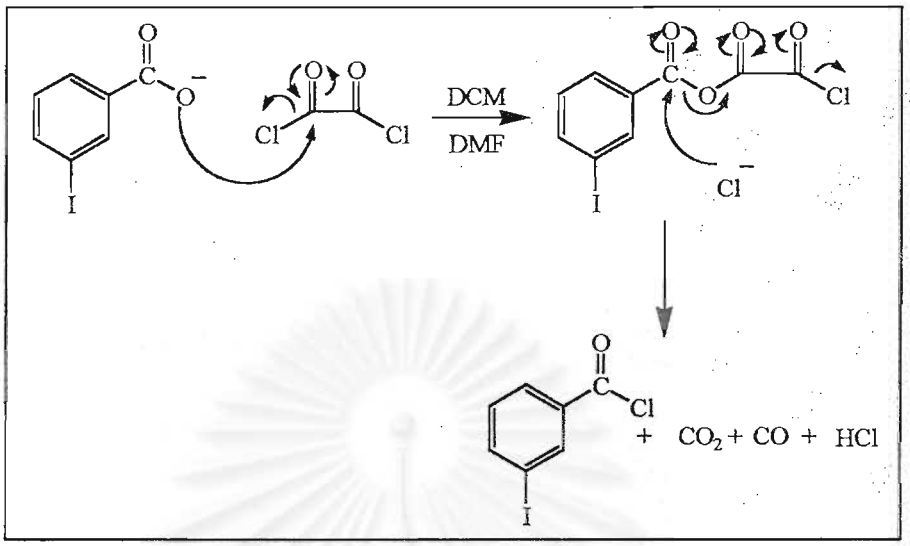
Figure 3.4 A plot between concentration and η_{sp}/C of chitosan obtained from Taming Enterprises Co., Ltd.

From a plot between concentration and η_{sp}/C of chitosan from Taming Enterprises Co., Ltd., intrinsic viscosity of solution [η] is equal to 86.837. Using the same Mark-Houwink correlation, viscosity-average molecular weight ($\overline{M}_v$) of chitosan (Taming Enterprises Co., Ltd.) is 108,630 g/mol.

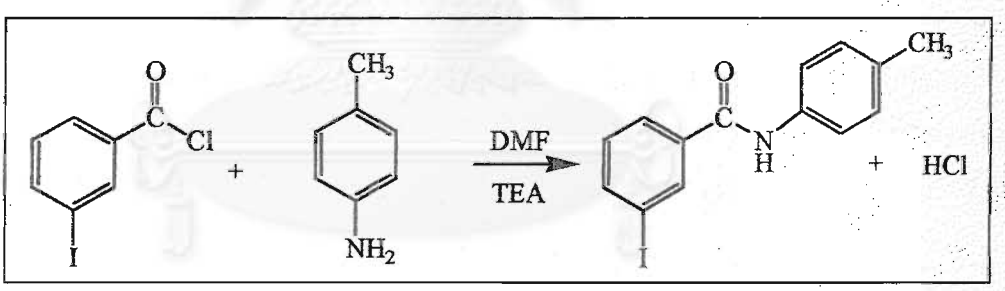
3.2 Reactivity of acylating agents

3.2.1 Reaction between *p*-methyl aniline and *m*-iodobenzoyl chloride

The reactivity of *m*-iodobenzoyl chloride, synthesized from *m*-iodobenzoic acid (Scheme 3.1), was investigated. *p*-Methyl aniline was selected as a model compound to react with *m*-iodobenzoyl chloride (Scheme 3.2). Since the amino group of *p*-methyl aniline is less nucleophilic than the 1°-amino group in the selected chitosan, the success of amide formation of this model study would imply acid chloride is active enough to acylate chitosan. The reaction was carried out in homogeneous fashion. The product obtained was *N*-(*p*-methyl phenyl)-*m*-iodobenzamide, and could be confirmed by ^{1}H NMR spectrum (Figure 3.5). The signal for $C\underline{H}_3$ of *p*-methyl aniline was found at 2.33 ppm. Besides, aromatic protons of *m*-iodobenzoyl and *p*-methyl aniline were found at 7.14-8.17 ppm. The $-NH_2$ of *p*-methyl aniline is less nucleophilic than the 1°-aliphatic amine in chitosan because the lone-pair electrons of nitrogen can delocalize into a benzene ring. The success of *N*-benzylation on *p*-methyl aniline suggested that the reaction between the amino groups of chitosan and benzylating agent was possible.



Scheme 3.1 Mechanism of synthesis *m*-iodobenzoyl chloride from *m*-iodobenzoic acid



Scheme 3.2 Reaction between *p*-methyl aniline and *m*-iodobenzoyl chloride

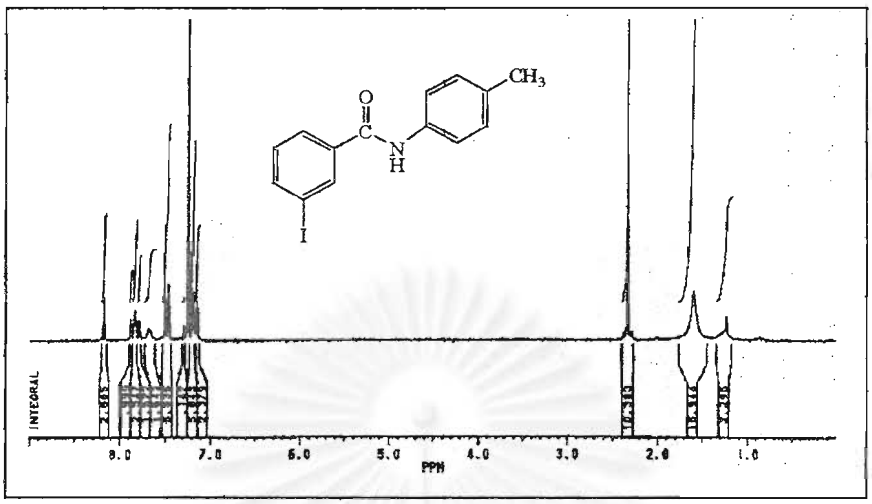
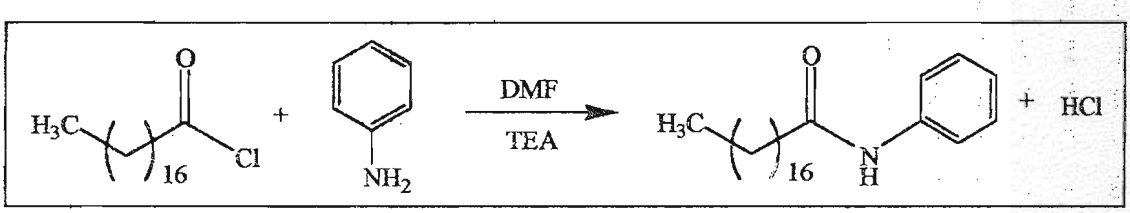


Figure 3.5 ^{1}H NMR spectrum of *N*-(*p*-methyl phenyl)-*m*-iodobenzamide

3.2.2 Reaction between aniline and stearyl chloride

Similar to the reaction described in 3.2.1, stearyl chloride and aniline were used as model compounds to test the reactivity of stearyl chloride toward the amino groups on chitosan (Scheme 3.3). *N*-phenyl stearamide was obtained as confirmed by ^{1}H NMR spectrum (Figure 3.6). From ^{1}H NMR, the signals for aromatic protons of aniline were found at 7.03-7.51 ppm and the protons of stearyl were found at 0.85, 1.23, 1.66, 2.16, and 2.33 ppm.



Scheme 3.3 Equation of reaction between aniline and stearyl chloride

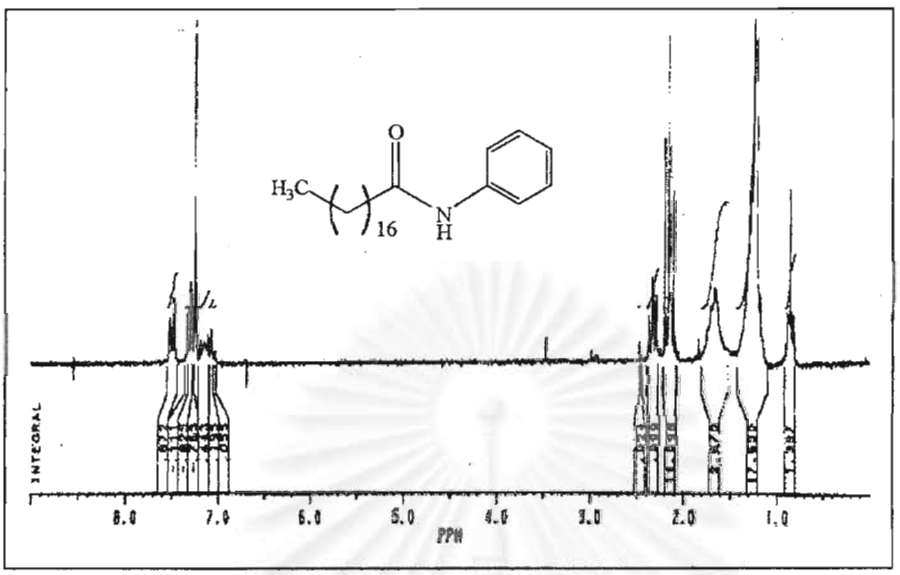
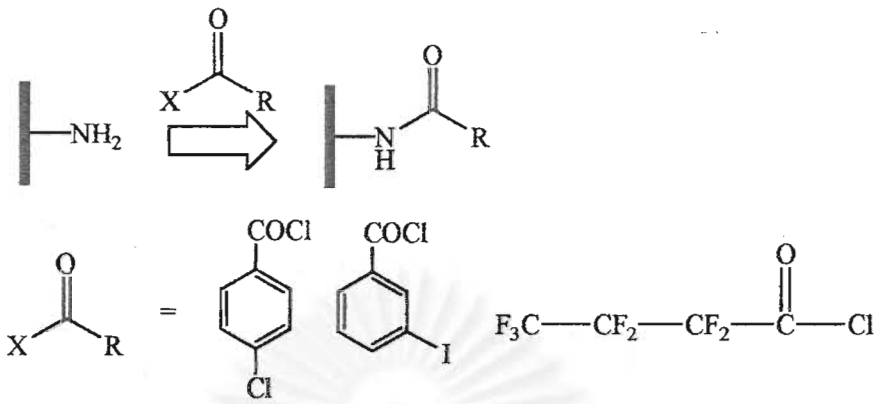


Figure 3.6 ^{1}H NMR spectrum of *N*-phenyl stearamide

3.3 Preliminary study for chemical modification of chitosan films

Since chitosan already has lots of good properties, the goal for this study was to modify only at the surface of chitosan films. The chemical structure of chitosan should, therefore, remain the same in the bulk. Chemical modification was carried out under heterogeneous reaction. Moreover, the modified chitosan films were easily purified by solvent rinsing to remove non-reacted agents and by products.

The focus of this preliminary study is to test the possibility of modifying chitosan surfaces. In this study, three derivatives of carboxylic acid that contain halogen atoms were used to react with chitosan films (Scheme 3.4). Results were analyzed by XPS, air water contact angle, and ATR-IR. XPS was used to measure the amount of halogen atoms on the film surface. %Yield of the reaction was thus calculated by comparing %N (%nitrogen) to %X (% halogen). Two take-off angles, 15° and 75°, were used in order to analyze atomic contents in depths from the outmost surface of 10 Å and 40 Å respectively.



Scheme 3.4 The equation of reaction between amino groups on chitosan films and derivatives of carboxylic acids containing halogen atoms

XPS analysis

XPS spectrum of chitosan film was shown in Figure 3.7. Only signals from C_{1s} (285 eV), N_{1s} (402 eV), O_{1s} (530 eV) of chitosan were identified. The XPS spectra of modified films (Figure 3.8-3.11) showed additional characteristic peaks of halogen atoms. Depending on the derivatives of carboxylic acids used, the signal for F_{1s}, Cl_{2p} and I_{3d} were located at 680, 200, and 620, 640 eV, respectively. The %yield was calculated and shown in Table 3.5. Moreover, the %yield at the depth of 40 Å is lower than one at the depth of 10 Å. It can be described that the reaction occurred to a lesser extent at a deeper layer than at the outer film layer. Besides, the %yields of chitosan molecular weight 100,000 and 645,000 are not significantly different.

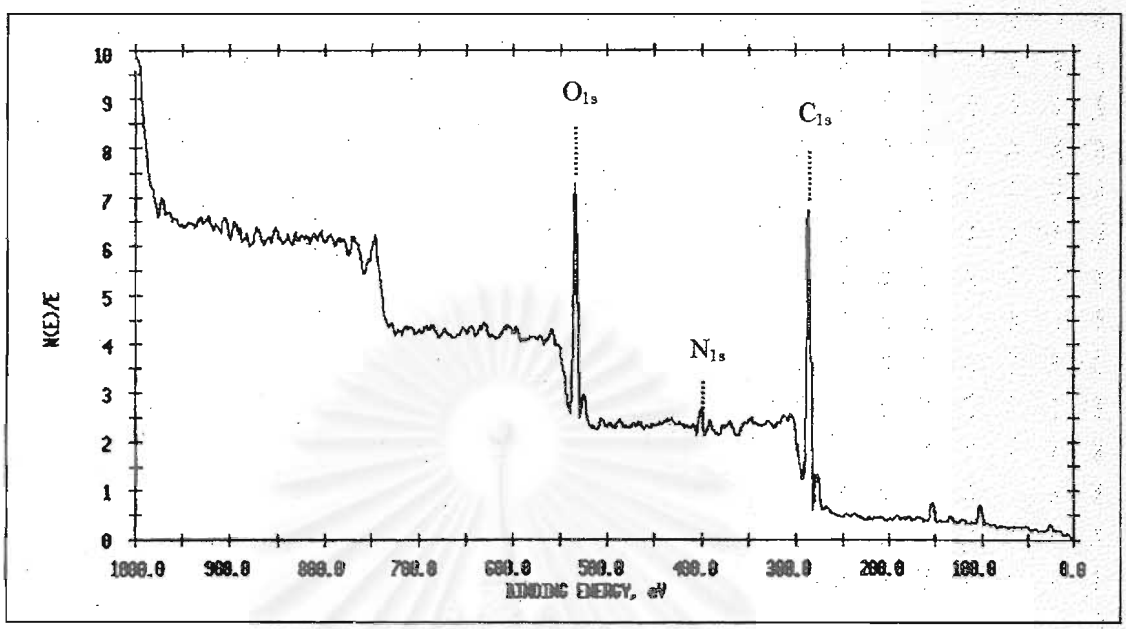


Figure 3.7 XPS spectrum of chitosan film

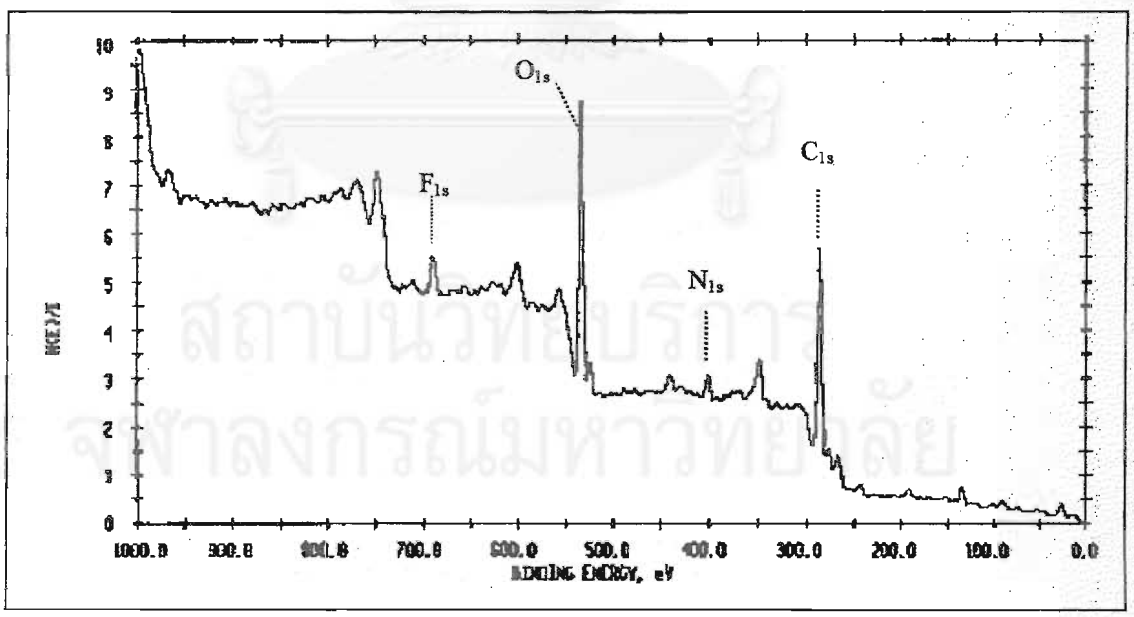


Figure 3.8 XPS spectrum of chitosan film after reacting with heptafluorobutyryl chloride

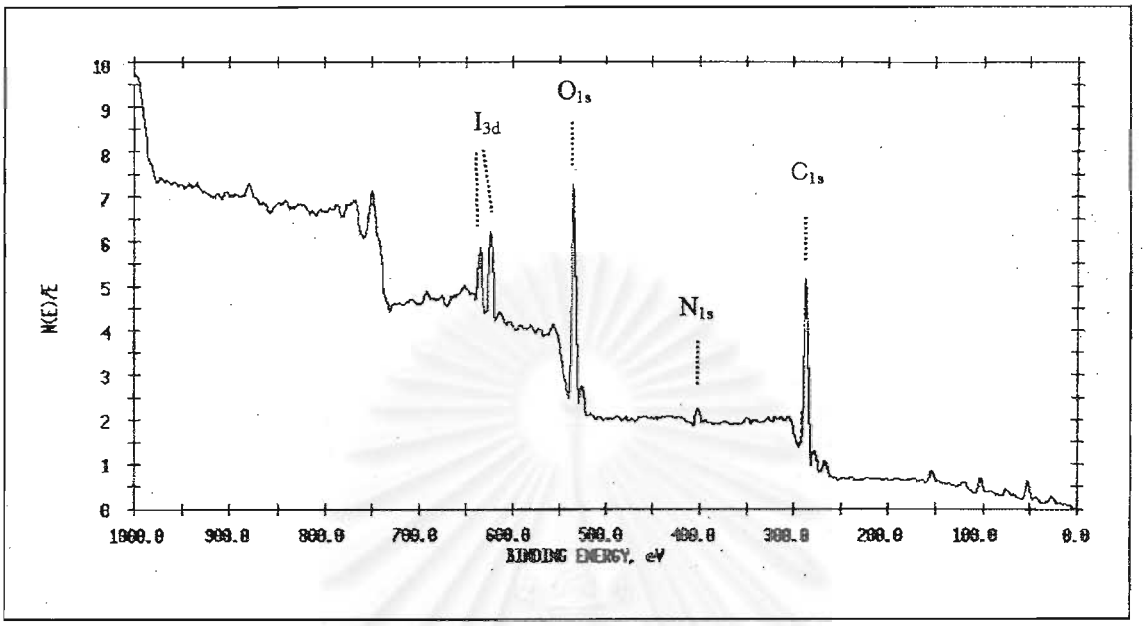


Figure 3.9 XPS spectrum of chitosan film after reacting with *m*-iodobenzoyl chloride

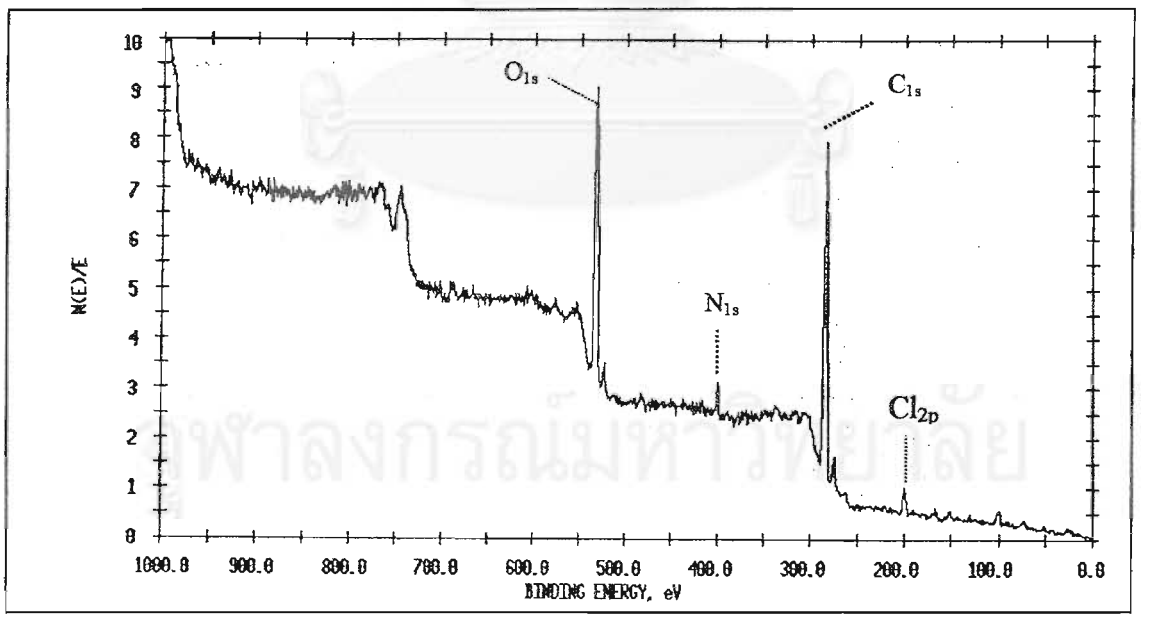


Figure 3.10 XPS spectrum of chitosan film after reacting with *p*-chlorobenzoic acid coupling with PFNB

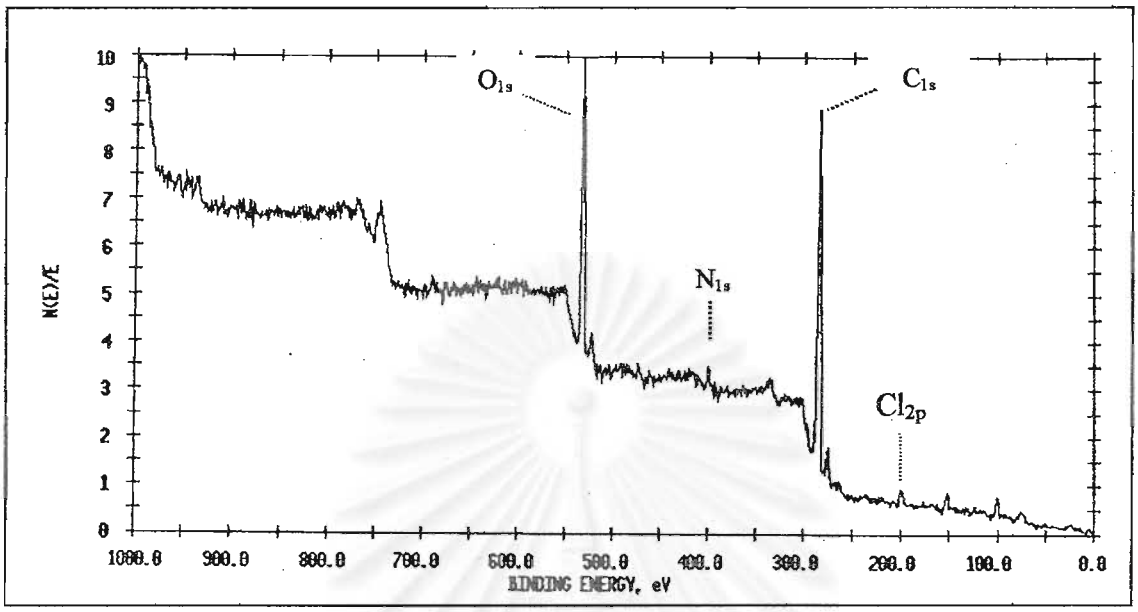


Figure 3.11 XPS spectrum of chitosan film after reacting with *p*-chlorobenzoic acid using DCC as a coupling reagent

Table 3.5 Data from XPS

a) 15° take off, depth ~ 10 Å

Sample	%X			%N	%Yield[a]	Mw of chitosan
	Cl	I	F			
Chi	0	0	0	2.87	0	645 K
Chi-F[b]	0	0	4.19	3.88	18	645 K
Chi-F[b]	0	0	2.86	2.68	18	100 K
Chi-I[b]	0	1.05	0	4.01	32	100 K
Chi-Cl[b]	0.31	0	0	2.36	15	645 K
Chi-Cl[c]	1.64	0	0	3.15	60	645 K
Chi-Cl[d]	1.38	0	0	2.47	64	645 K

Table 3.5 (continued)

b) 75° take off, depth ~ 40 Å

Sample	%X			%N	%Yield[a]	Mw of chitosan
	Cl	I	F			
Chi-F[b]	0	0	4.29	6.04	12	645 K
Chi-F[b]	0	0	3.21	5.28	10	100 K
Chi-Cl[b]	0.50	0	0	5.64	10	645 K
Chi-Cl[c]	2.22	0	0	4.99	51	645 K
Chi-Cl[d]	1.75	0	0	4.38	46	645 K

Note [a] % yield is calculated from $\frac{\%n}{\%N} = \frac{\%DD}{100}$

$$\%yield = \frac{(\%X/a)}{\%n} x100$$

%n is percent mole of the amino groups in chitosan.

%N is percent mole of nitrogen in the chitosan both amino and amide groups.

%X is percent mole of halide atom on chitosan surface.

a is number of moles of halide atom in 1 mole of carboxylic compound.

[b] chitosan reacted with acid chloride derivatives

[c] chitosan reacted with PFNB (active ester)

[d] chitosan reacted with carboxylic acid compounds using DCC as a coupling agent

Air-water contact angle

Data from air-water contact angle is used to determine hydrophobicity of the film surface. The water contact angle of hydrophilic surface is normally smaller than hydrophobic one. Results of air-water contact angle were shown in Table 3.6. The films modified with either *p*-chlorobenzoic acid or *m*-iodobenzoyl chloride were slightly more hydrophobic than the non-modified one. Insignificant differences of contact angles before and after modification may be explained as a result of low reaction yield at the surface (<30% yield). Pictures of water droplets on chitosan surface before and after modification were shown in Figure 3.12.

Table 3.6 Air-water contact angle of chitosan film after reacting with derivatives of carboxylic acid

Reagent	-		*p*-chlorobenzoic acid + PFNB		*m*-iodobenzoyl chloride	
Mw of chitosan	100K	645K	100K	645K	100K	645K
Contact angle (Degree)	89±5.8	93±1.6	-	97±4.5	100±2.0	-

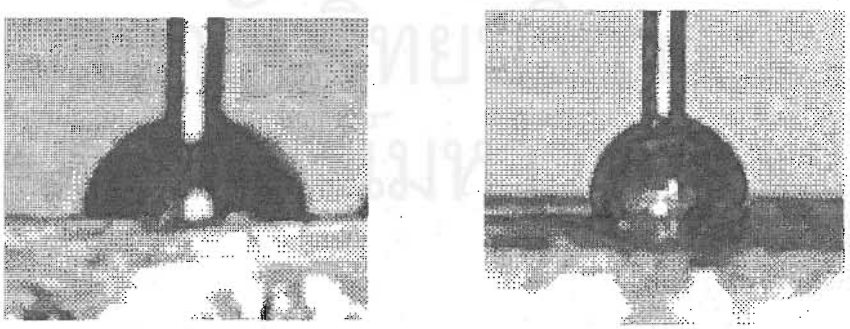


Figure 3.12 Images of water droplets on chitosan surface before and after modification

ATR-IR

ATR-IR was used to characterize the functional groups on the surface of chitosan films (with the sampling depth ~ 1 μm) before and after modification. The spectra of chitosan films before and after modification were shown in Figure 3.13 to 3.16. Absorption peaks at *ca.* 1650, 1590, and 1550 cm^{-1} were assigned to the carbonyl stretching of secondary amides (amide I band), N-H bending vibrations of 2-aminoglucosamine primary amines, and N-H bending vibrations of secondary amides (amide II band), respectively. After chemical modification, the intensity of the signal at 1550 cm^{-1} for N-H bending vibrations of secondary amide II evidently increased (Figure 3.14-3.16).

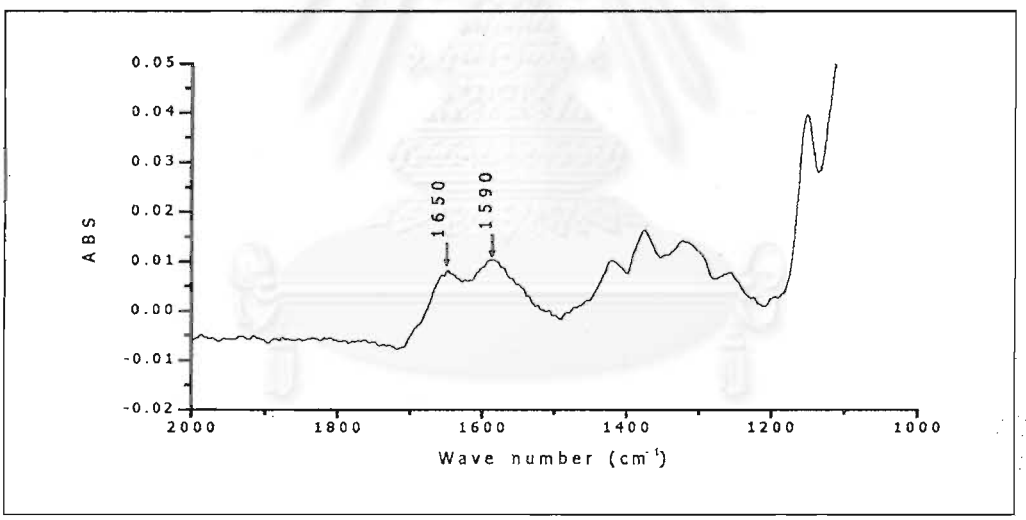


Figure 3.13 ATR-IR spectrum of non-modified chitosan film

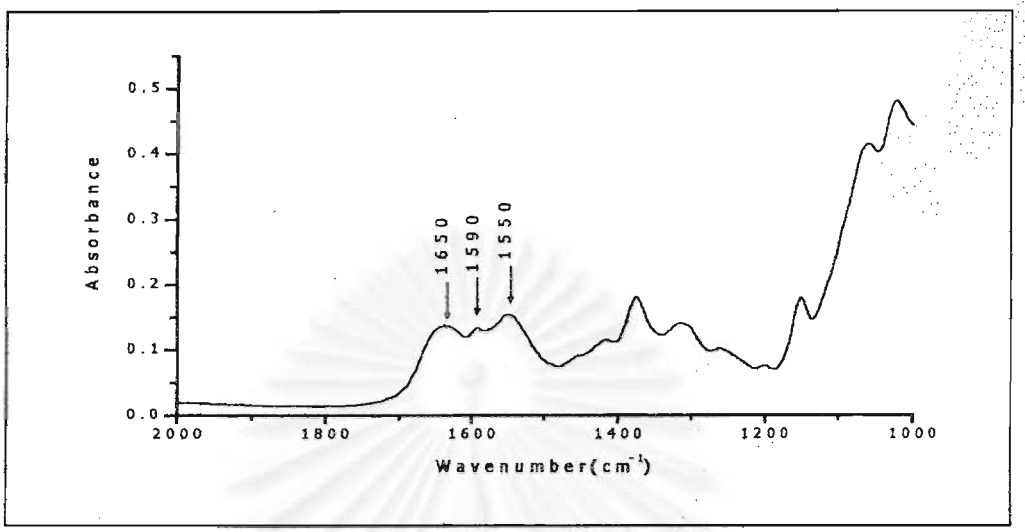


Figure 3.14 ATR-IR spectrum of chitosan after reacting with *p*-chlorobenzoic acid using PFNB as a coupling agent in DMF for 48 h

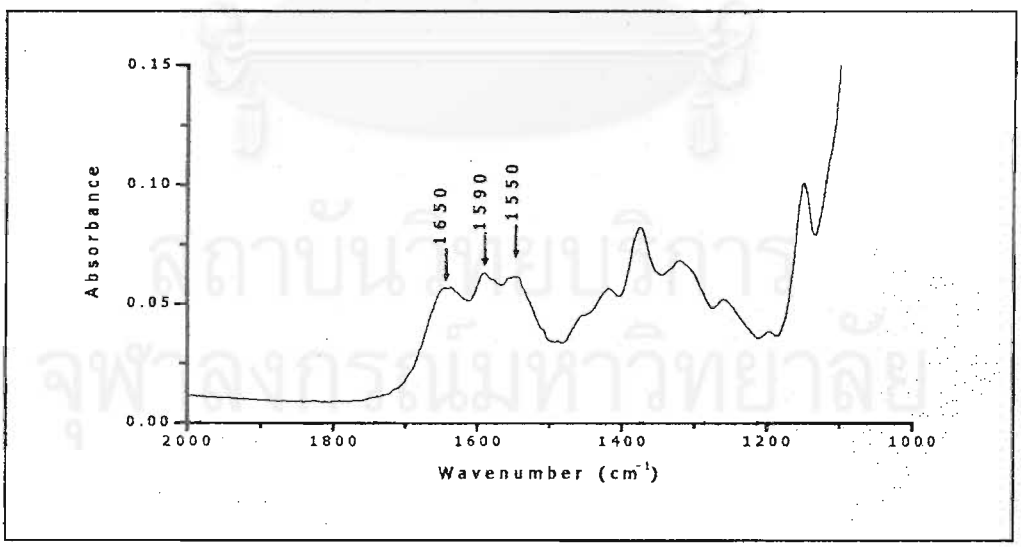


Figure 3.15 ATR-IR spectrum of chitosan after reacting with *p*-chlorobenzoic acid using DCC as a coupling agent in DMF for 48 h

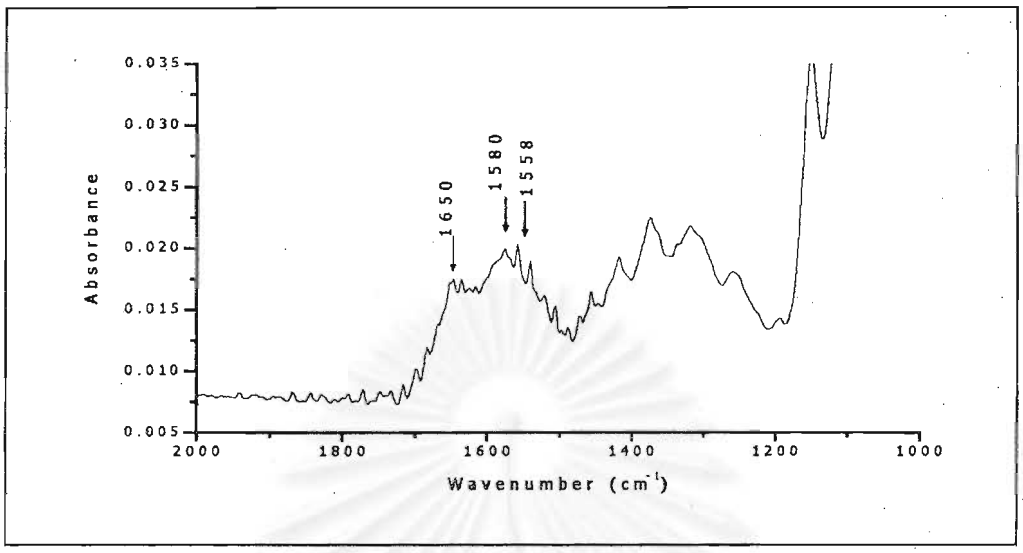


Figure 3.16 ATR-IR spectrum of chitosan after reacting with *m*-iodobenzoyl chloride in DMF for 48 h

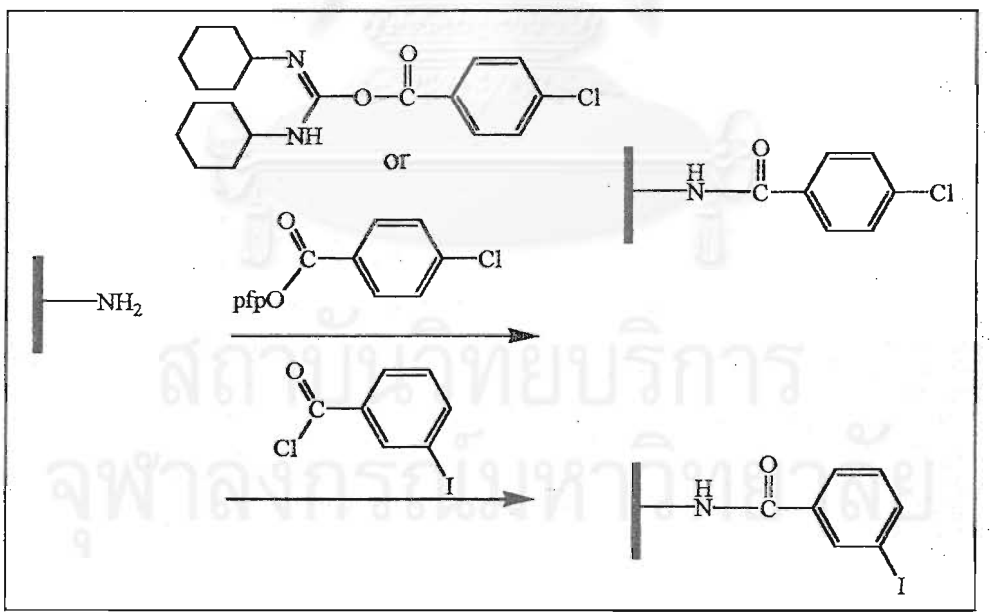
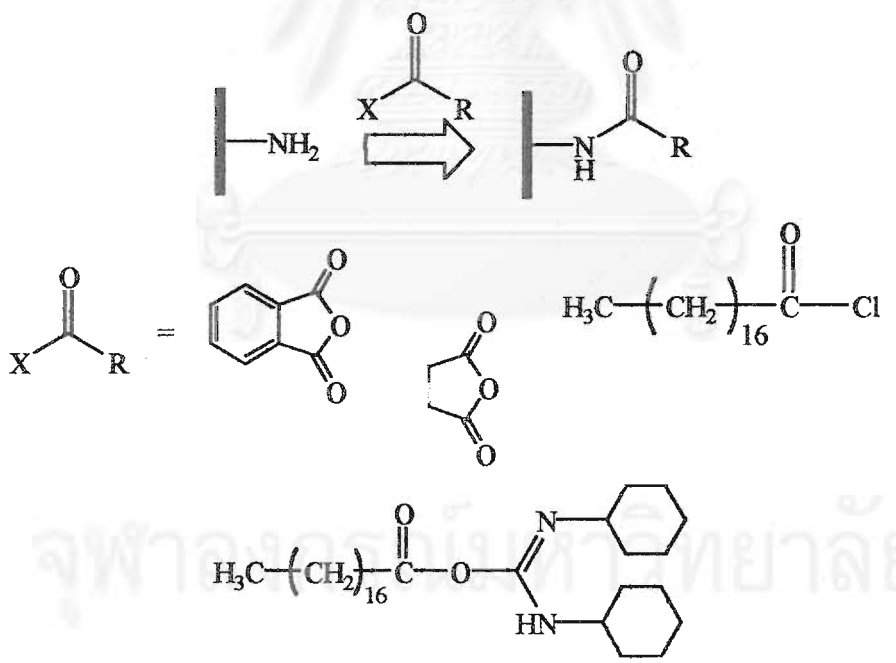


Figure 3.17 The chemical structure of chitosan after reacting with *m*-iodobenzoyl chloride, *p*-chlorobenzoic acid using DCC and PFNB as a coupling agent

It is, therefore, confirmed by XPS, contact angle, and IR that the derivatives of carboxylic acid could be attached on the amino groups of chitosan films via amide bond (Figure 3.17). It was shown in this preliminary study that modifying the surface of chitosan films could be achieved by immersing the films in selected reagents for a certain time.

3.4 Control of surface hydrophobicity

In this study, phthalic anhydride and succinic anhydride were selected to react with the amino groups of chitosan in order to modify the hydrophobicity of the films. In the case of stearic acid, it was changed to a more active compound, stearyl chloride, or by means of using DCC as a coupling agent (Scheme 3.5).



Scheme 3.5 Reaction between amino groups on chitosan films and derivatives of carboxylic acid

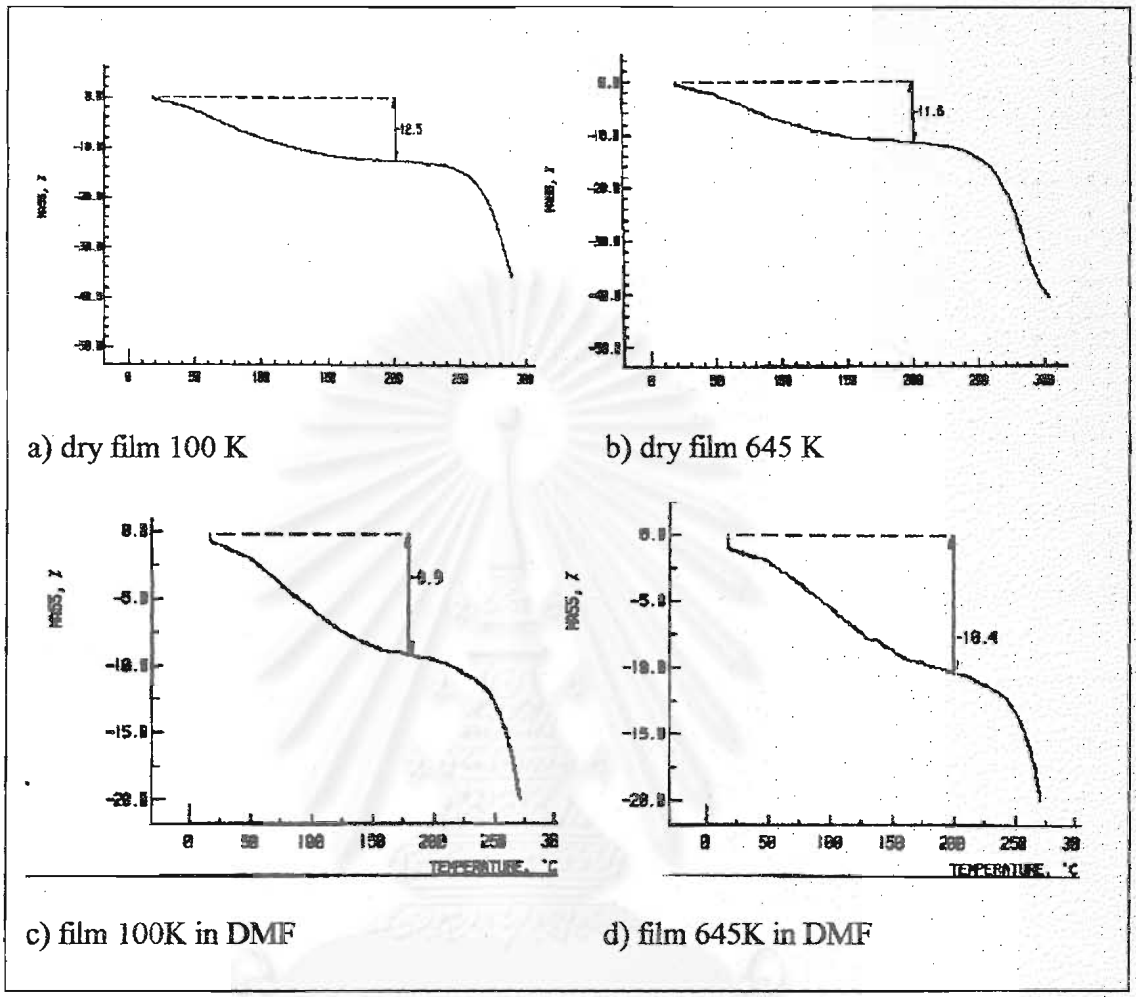
Hydrophobicity modification of chitosan films was studied by attaching derivatives of carboxylic acid on the film surfaces. The hydrophobicity of modified film surface was analyzed by air-water contact angle. Moreover, ATR-IR was used to characterize the functional groups on the film surface. In this study, factors that could effect the change of hydrophobicity were also investigated. They are choices of solvent, reaction temperature, molecular weight of chitosan, and type of carboxylic derivative.

3.4.1 Effect of solvent

The difference of air-water contact angle between chitosan films soaked in MeOH or DMF for 48 h and vacuum dried for 1 day and non-soaked chitosan films are shown in Table 3.7. The air-water contact angle of the film once soaked in DMF is significantly lower than the non-soaked films. It is possible that the drying period of 1 day is not enough to completely remove DMF out of the film. TGA thermogram of the non-soaked films and DMF-soaked films reveals that the solvent content in bulk films were not different and ranged from 8.9% to 12.5% (Figure 3.18). This result has suggested that the left over solvent only existed at the film surface. Hence, air-water contact angle of chitosan films soaked in solvents were used as control film for comparing with modified chitosan films in the next study.

Table 3.7 Air-water contact angle of chitosan films that were soaked in MeOH and DMF for 48 h and vacuum dried for 1 day (This soaking period is identical to the time used for modifying the film surface.)

Solvent	-		MeOH				DMF			
Mw of chitosan	100K	645K	100K		645K		100K		645K	
Temperature (°C)	RT	RT	RT	50	RT	50	RT	80	RT	80
Degree	89±5.8	93±1.6	90±2.4	91±1.4	88±1.2	90±1.5	74±6.5	82±2.3	86±4.9	84±6.6



a) dry film 100 K

b) dry film 645 K

c) film 100K in DMF

d) film 645K in DMF

Figure 3.18 TGA thermogram of chitosan films a) dry film 100 K, b) dry film 645 K, c) film100 K soaked in DMF and vacuum dried for 1 day, d) film 645 K soaked in DMF and vacuum dried for 1 day

3.4.2 Effect of reaction temperature

From data in Table 3.7 and 3.8, when the reaction temperature was varied from RT to 50 and 80 °C, the degree of air-water contact angle was not significantly different. Effect of reaction temperature cannot be concluded from available data. It seems however that the contact angle trend to decrease after reacted with phthalic and succinic anhydride at high temperature.

Table 3.8 Air-water contact angle of chitosan film after reacting with anhydride derivatives for 48 h and vacuum dried for 1 day

Solvent	MeOH				DMF			
Mw of chitosan	100K		645K		100K		645K	
Temperature (°C)	RT	50	RT	50	RT	80	RT	80
Chitosan Phthalic anhydride	77±3.8	71±7.0	83±2.3	75±3.6	51±6.8	53±7.1	52±4.1	52±1.5
Chitosan Succinic anhydride	62±3.8	103±7.2	71±2.4	70±4.7	56±1.5	59±3.1	55±3.9	37±5.3
Chitosan Stearyl chloride	-	-	-	-	101±4.1	-	97.2±2.8	-
Chitosan Stearic + DCC	-	-	-	-	88±0.3	-	87±2.0	-

Note - : not analyzed

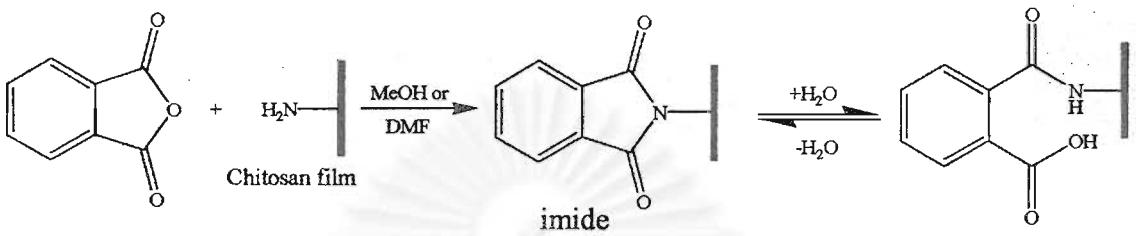
3.4.3 Effect of molecular weight of chitosan

Chitosan with molecular weight of 100,000 and 645,000 were used in this study. It was found that the air-water contact angle of modified films from both batches of chitosan were not significantly different. The molecular weight of chitosan selected in this work seems to have no effect on the controlling of hydrophobicity under heterogeneous reaction.

3.4.4 Effect of carboxylic derivatives

The degree of air-water contact angle of the chitosan film was decreased after modified with phthalic or succinic anhydride. This result suggested that anhydride derivatives caused the film surface to become more hydrophilic. This could be due to

the formation of carboxylic acid after imide bond is hydrolyzed when the film surface came into contact with water (Scheme 3.6).



Scheme 3.6 Possible results from the reaction between phthalic anhydride and chitosan

The formation of imide bond was confirmed by ATR-IR. IR spectra of chitosan films modified with phthalic or succinic anhydride in MeOH at RT showed signals of imide bond at 1710 and 1770 cm^{-1} (Figure 3.19, 3.20).

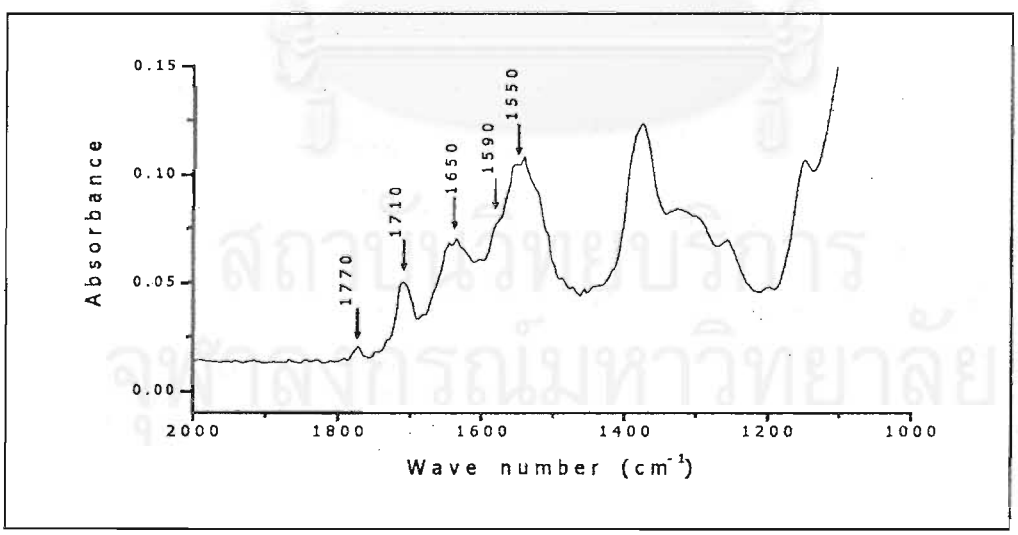


Figure 3.19 ATR-IR spectrum of chitosan film after reacting with phthalic anhydride in MeOH at RT for 48 h

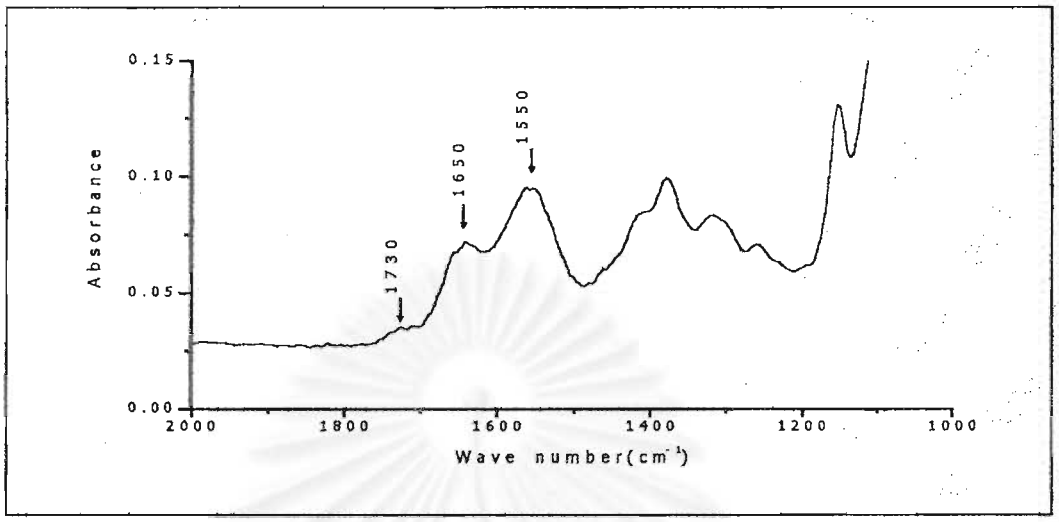


Figure 3.20 ATR-IR spectrum of chitosan after reacting with succinic anhydride in MeOH at RT for 48 h

The air-water contact angle of chitosan surface was, however, increased after modified with stearyl chloride. Stearyl group has a long aliphatic hydrocarbon chain (C17). It, therefore, caused the modified films to become more hydrophobic. The attachment of stearyl group on the amino of chitosan is also confirmed by ATR-IR. IR spectrum of chitosan film after modified with stearyl chloride shows a shoulder peak N-H bending vibration of secondary amide at 1550 cm^{-1} (Figure 3.21). Figure 3.22 is the spectrum of chitosan film after reacted with stearic acid by using DCC as a coupling agent. This spectrum also shows N-H bending vibration of secondary amide at 1550 cm^{-1}. Scheme 3.7 describes the mechanism between stearic acid and amino groups on chitosan films using DCC as a coupling agent. The reaction of stearyl chloride with amino groups on chitosan films was shown in Scheme 3.8.

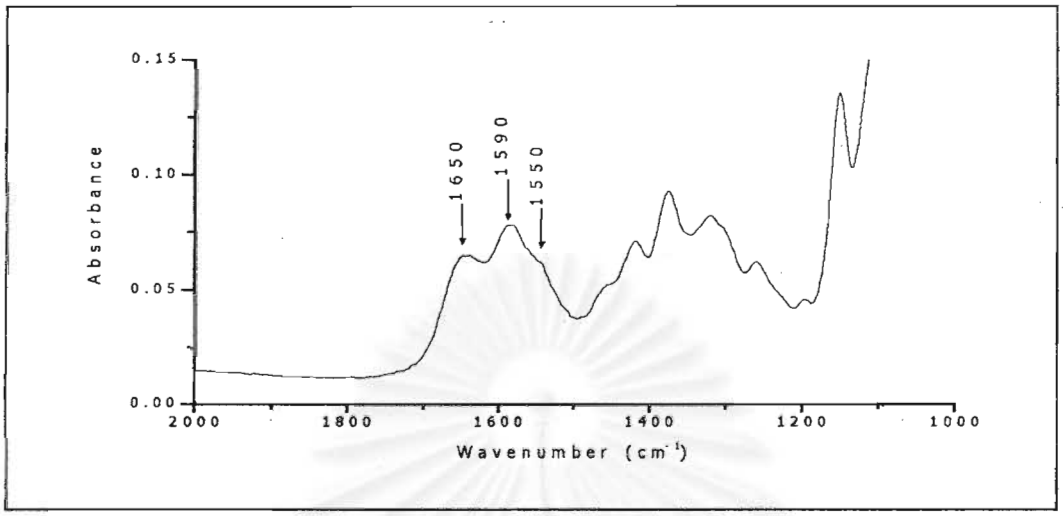


Figure 3.21 ATR-IR spectrum of chitosan film after reacting with stearyl chloride in DMF at RT for 48 h

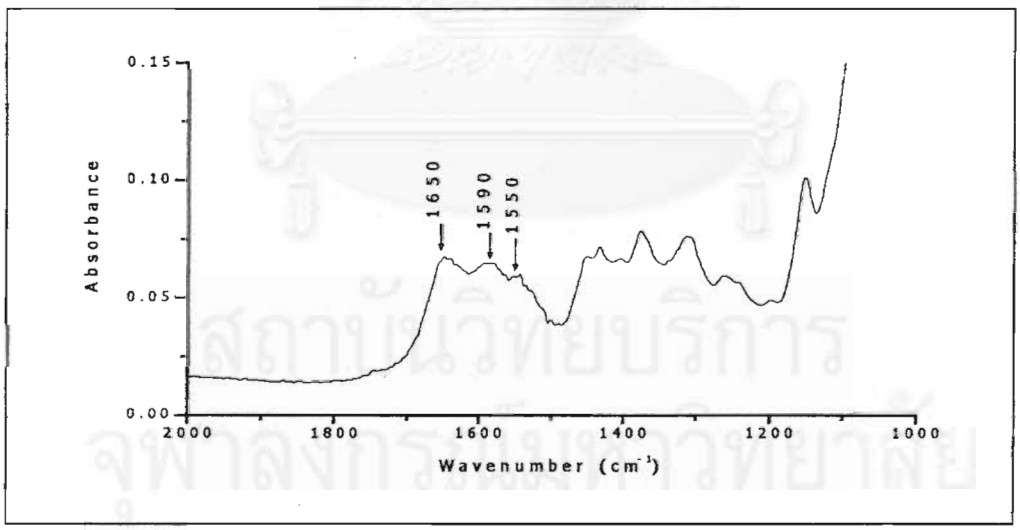
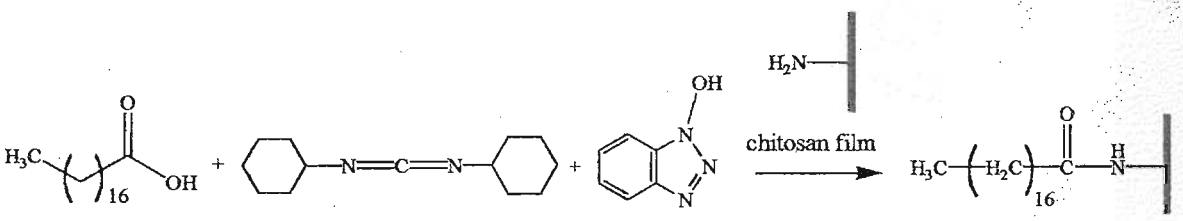
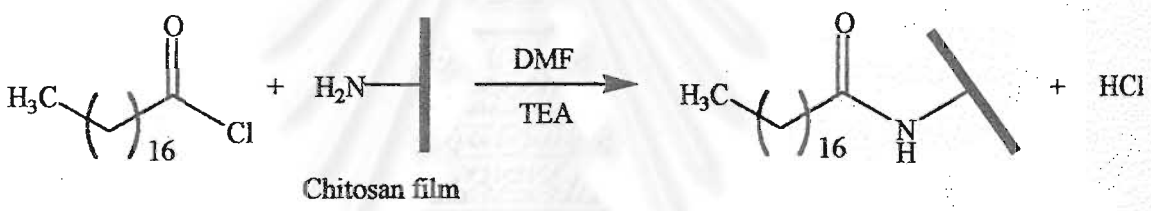


Figure 3.22 ATR-IR spectrum of chitosan film after reacting with stearic acid using DCC as a coupling agent in DMF at RT for 48 h



Scheme 3.7 Reaction between stearic acid and amino groups of chitosan film using DCC as a coupling agent



Scheme 3.8 Reaction between stearyl chloride and amino groups of chitosan film

3.5 Protein adsorption study

Albumin and lysozyme were the two proteins chosen for adsorption on the surface of chitosan film. Lysozyme is an antimicrobial protein that has a heterogeneous exterior with hydrophobic patch and an uneven charge distribution (Figure 3.23).[28] Bovine serum albumin (BSA) is one of the more abundant blood proteins. Its biological functions are transport and maintenance of colloid osmotic pressure.[29] Both proteins are model globular proteins and known to be resistant to denaturation. However, they vary in size and charge as well as conformational stability under the experimental condition. Bicinchoninic acid assay was used to measure the amount of protein.[30] Only a selected number of modified films that had different contact angles were selected for this study.

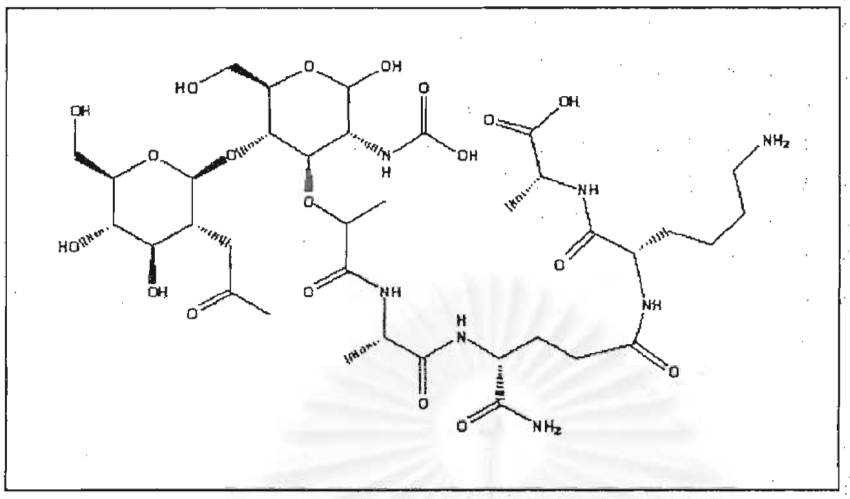


Figure 3.23 Structure of Lysozyme

The color intensity of protein solution after adding BCA working solution depends on concentration of protein solution (Figure 3.24). The calibration curve showing the correlation between the absorbance at 562 nm and protein concentration is shown in Figure 3.25.

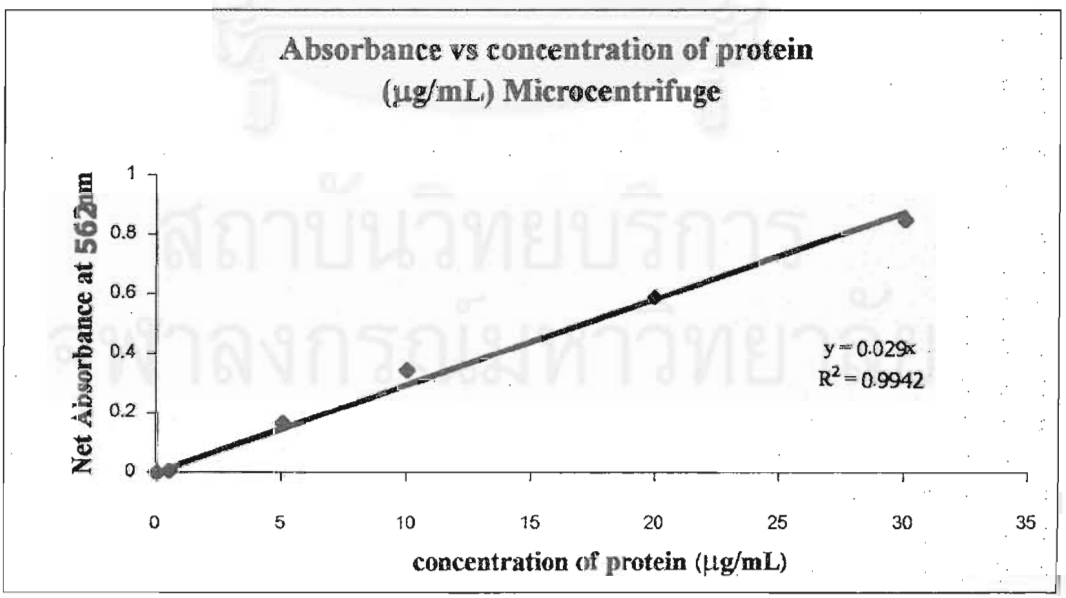


Figure 3.24 Standard curve between absorbance at 562 nm and protein concentration

Figure 3.25 Color intensity of protein solution, 16 h after adding BCA working solution, at different amounts of proteins

The amount of lysozyme and albumin adsorbed on the surface of chitosan films are shown in Table 3.9. The data shows that lysozyme and albumin can be adsorbed on the surface of the unmodified chitosan films. Because the structures of lysozyme and albumin contain both hydrophobic and hydrophilic parts. The structure of chitosan also contains hydroxyl and amino groups. Therefore, driving force for protein adsorption on the surface of chitosan is possibly H-bonding, polar-polar interaction, and van der Waals.

These data also revealed that lysozyme could be adsorbed more on the films modified with anhydrides derivatives; succinic and phthalic anhydride, than on the non-modified films. An isoelectric point (pI) of lysozyme is 11, therefore it contains larger amount of -OH, $-NH_2$ than -COOH (Figure 3.23). H-bonding between the amino groups on lysozyme and the carboxyl, amino, and hydroxyl groups on the modified chitosan could be the reason for the increased adsorption of lysozyme. Moreover, at pH 7.4, lysozyme is positively charged and the surface of modified films is negatively charged. Another driving force for lysozyme adsorption could be electrostatic interaction.

On the other hand, the amount of albumin adsorbed on the chitosan films modified with anhydride derivatives was lower than the adsorbed amount on the non-modified films. This observation could be explained as follows. The pI of albumin is

4.8. It is a -COOH rich protein. At pH 7.4 the carboxylic groups on albumin as well as on the modified chitosan were deprotonated. The repulsion between the negatively charged surface of modified chitosan films and albumin is a possible explanation for the observed low adsorption value.

In the case of chitosan films modified with stearyl group, adsorptivity of albumin and lysozyme on films surface are better than the non-modified film. The structures of albumin and lysozyme contain mostly hydrophobic parts. The surface of chitosan that has stearyl group on the surface is also highly hydrophobic. Therefore, the driving force for the adsorption of both proteins is only van der Waals interaction.

Table 3.9 Amount of lysozyme and albumin adsorption on the surface of modified chitosan films

Mw of chitosan	*Samples*	*Air-water contact angle (degree)*	*Amount of lysozyme ($\mu g/cm^2$)*	*Amount of BSA ($\mu g/cm^2$)*
100,000	Chitosan	89±5.8	4.65±0.82	2.97±0.42
	Chi-stearyl chloride	101±4.1	7.87±4.52	4.86±1.16
	Chi–stearic-DCC (DMF)	88±0.3	7.33±4.87	7.23±1.42
	Chi-Succ (MeOH-50°C)	103±7.2	10.75±2.69	2.33±0.80
645,000	Chitosan	93±1.6	4.58±0.93	8.22±2.97
	Chi-Succ (MeOH-RT)	71±2.4	7.46±0.93	3.91±1.32
	Chi-Succ (MeOH-50°C)	70±4.7	10.69±1.17	4.47±1.08
	Chi-Succ (DMF-RT)	55±3.9	7.30±0.82	1.63±0.20
	Chi-Succ (DMF-80°C)	37±5.3	4.82±1.23	1.64±0.52
	Chi-Pht (DMF-RT)	52±4.1	4.73±0.64	2.58±0.63

CHAPTER IV

CONCLUSIONS

The surface of chitosan film can be modified by reaction between the amino groups on chitosan and derivatives of carboxylic acid under heterogeneous condition. In preliminary study, acid chloride derivatives containing halogen atoms were used to react with amino groups on the chitosan film. Halogen atoms were found on the surface of modified chitosan films by XPS analysis. The hydrophobicity of the chitosan films can be altered by attaching certain derivatives of carboxylic acids on the film surface. ATR-IR spectra of chitosan films that were modified with phthalic and succinic anhydride showed C=O str. of imide at 1710, 1770 cm^{-1}. In protein adsorption study, lysozyme and albumin were chosen as model proteins that differed in their isoelectric points (pI). The adsorptivity of both proteins on the film surface increased after the films were modified with stearic acid. The driving force for the adsorption is van der Waals interaction. In the case of the chitosan films modified with anhydride derivatives, the driving force for lysozyme adsorption could be H-bonding and electrostatic interaction. But the adsorptivity of albumin was decreased possibly due to the repulsion between negative charges on the modified chitosan surface and albumin.

In the future, the condition of the reaction should be optimized for improving the reaction efficiency. The duration time of protein adsorption should be optimized to obtain a maximum adsorption. Furthermore, the derivatives of carboxylic acid compounds that were attached on the surface of chitosan films should be varied to study the relationship between the functional groups and the surface properties.

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APPENDICES

APPENDIX A

Protein adsorption assay

Table a. Protein used in this study

Protein	Source	M_w(kD)[1]	pI[2]	Shape
Lysozyme	Chicken egg	14	11	Ellipsoid
Albumin	Bovine serum	69	4.8	Ellipsoid

1. Molecular weight of protein
2. Isoelectric point of protein

Bicinchoninic acid assay

Bicinchoninic acid assay is method for measurement the amount of proteins. The standard reagents were used in this method e.g. reagent A, reagent B and reagent C. Reagent A consists of an aqueous solution of Na_2 tartrate, Na_2CO_3, NaHCO3 in 0.2 M NaOH, pH 11.25. Reagent B is 4% (W/V) bicinchoninic acid solution, pH 8.5. Reagent C is 4% $CuSO_4 \cdot 5H_2O$ in deionized water.

The principle of the bicinchoninic assay relies on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to protein present. It has been shown that the peptide bond is able to reduce Cu^{2+} to Cu^{1+}. BCA forms a purple-blue complex with Cu^{1+} in alkaline environments, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins.[30] Figure a shows complexation between bicinchoninic acid and Cu^{1+}.

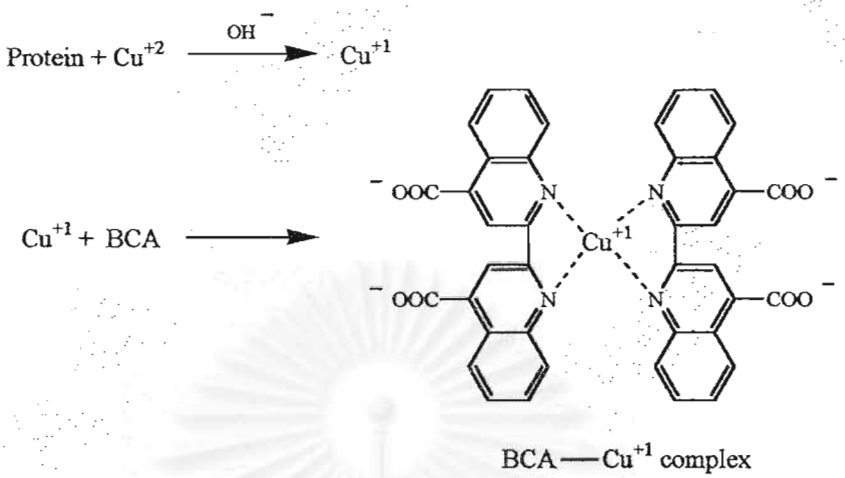

Figure a. Formation of purple complex with BCA and cuprous ion generated from the biuret reaction.

APPENDIX B

ATR-IR spectrum of chitosan before and after neuterization

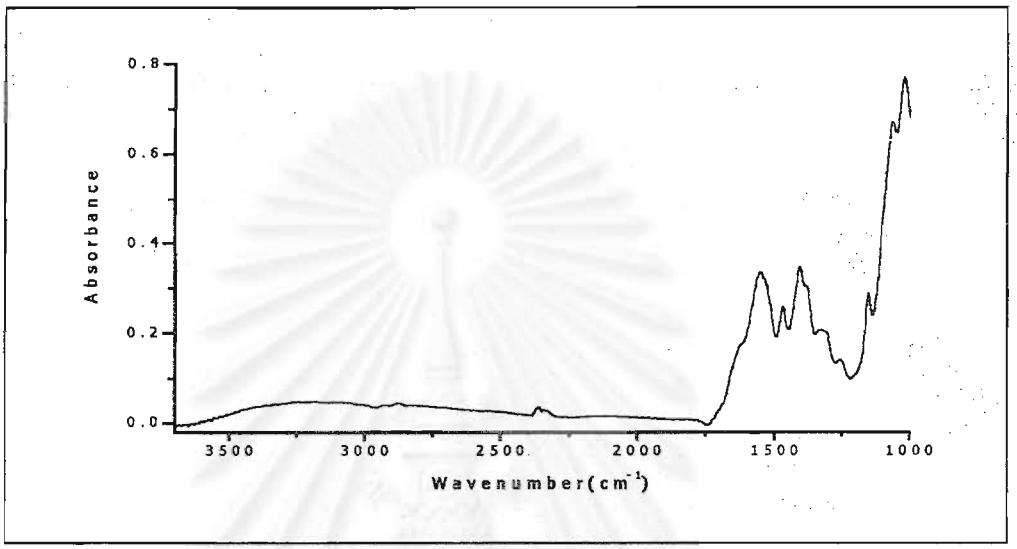

Figure b-1. ATR-IR spectrum of chitosan before neutralization

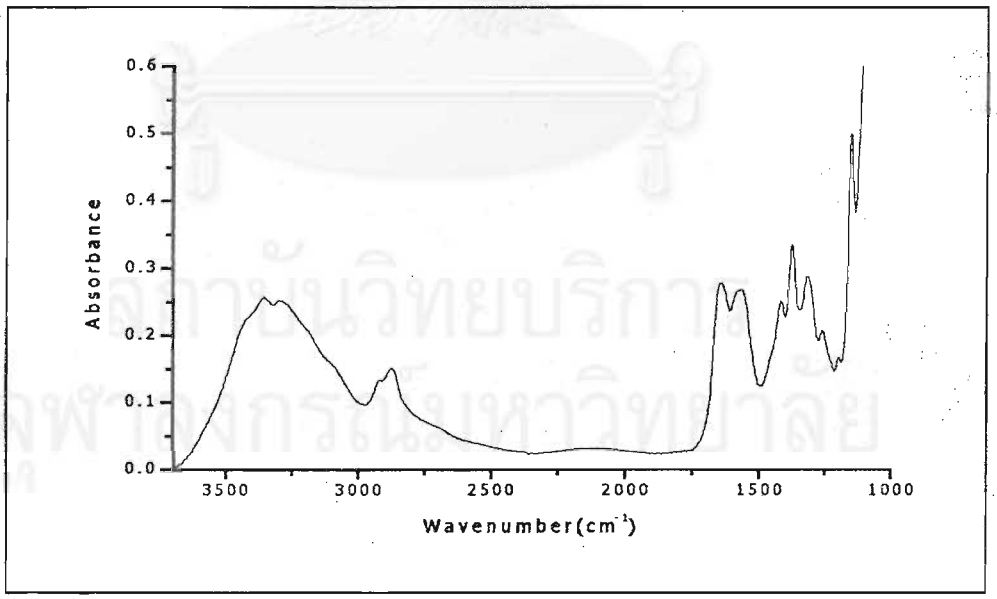

Figure b-2. ATR-IR spectrum of chitosan after neutralization

APPENDIX C

A. ATR-IR spectrum of chitosan after modified with succinic anhydride

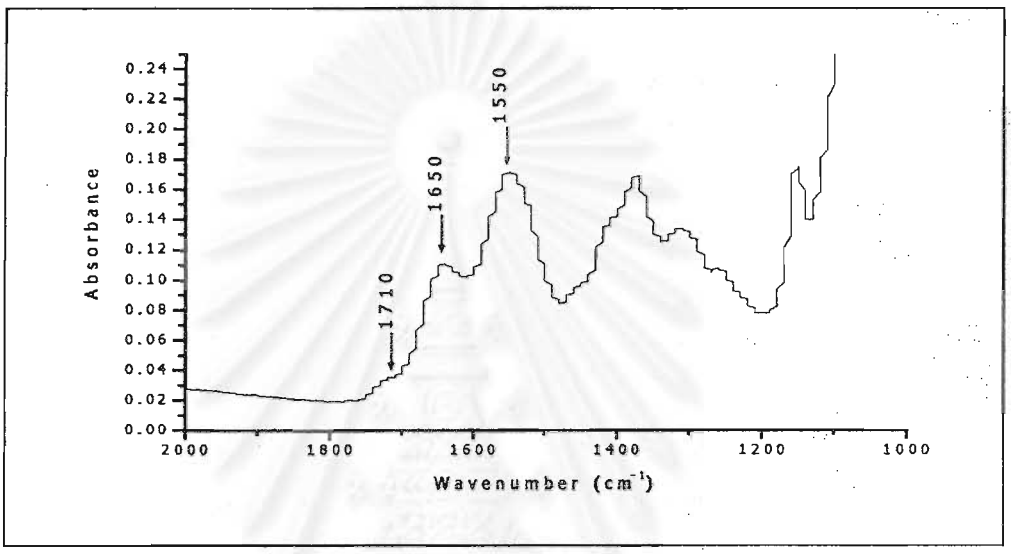

Figure c-1. ATR-IR spectrum of chitosan after reacting with succinic anhydride in MeOH at 50 °C for 48 h

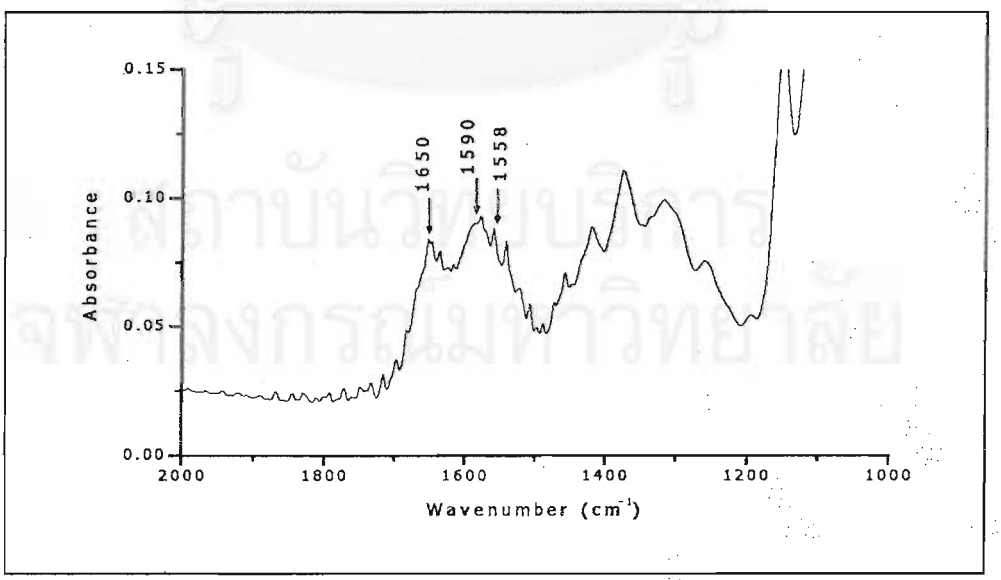

Figure c-2 ATR-IR spectrum of chitosan after reacting with succinic anhydride in DMF at RT for 48 h

B. ATR-IR spectrum of chitosan after modified with phthalic anhydride

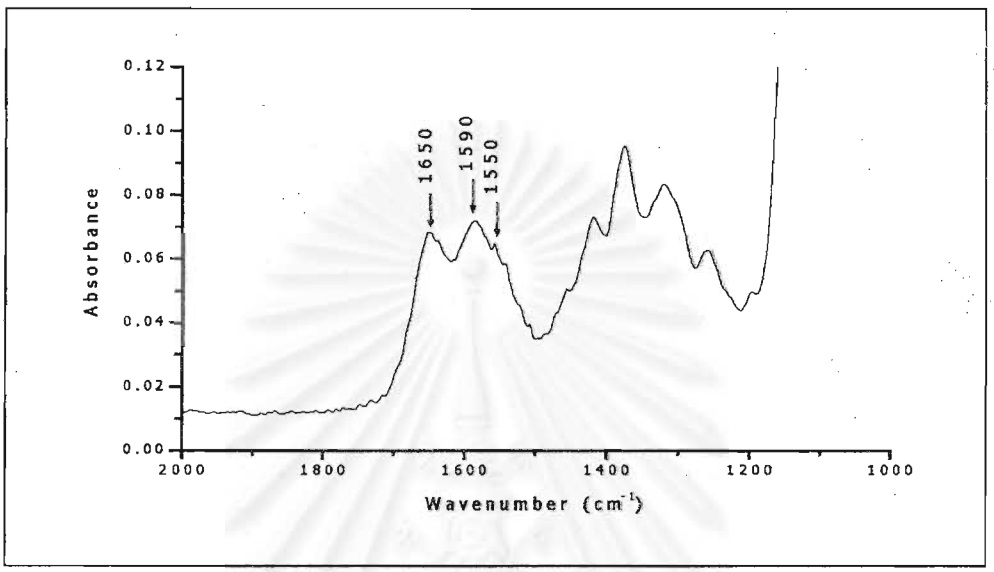

Figure c-3. ATR-IR spectrum of chitosan after reacting with phthalic anhydride in DMF at RT for 48 h

VITA

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