CHAPTER I

INTRODUCTION

1.1 Statement of problem

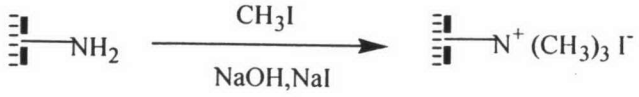
Chitosan, a waste product from food industries, is originated from chitin. Based on its many favorable characteristics, chitosan have attracted considerable attention in the areas such as biotechnology, biomedicine, food ingredients and cosmetics. In the view of biomaterial application, it is well known that whether the biomaterials planted in human's bodies are taken as foreigners by the blood, the key is interaction between the tissues and the material surface, whereas the influence of the bulk property of materials is not so pronounced. The focus of this work is to prepare positively or negatively charged on chitosan films. Since proteins are amphoteric, the numbers of charges on the surface of a protein vary with the pH of the medium. In principle, it is possible to calculate the number of charges on a protein at a particular pH if the pK_a values of all its ionizable groups are known. As a consequence of charge variation on surface-modified chitosan, it is postulated that the charges introduced on the chitosan surface would affect protein adsorption behavior. The outcome of this study should provide fundamental information that can lead to the development of chitosan and perhaps its derivatives for biomedical applications. This is also another way of adding value to the locally abundant polymer.

1.2 Objectives

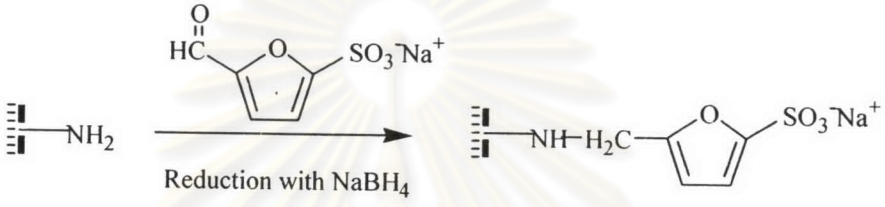
1. To prepare positively and negatively charged chitosan films.
2. To determine the behavior of protein adsorption on the surface-charged chitosan films.

1.3 Scope of the investigation

1. Positively-charged chitosan films were prepared by reaction between chitosan and methyl iodide.



2. Negatively-charged chitosan films were prepared by reaction between chitosan and 5-formyl-2-furansulfonic acid.



3. Characterization of the surface-modified chitosan films was conducted by
   - X-ray photoelectron spectroscopy (XPS) for atomic composition on the films
   - Attenuated total reflection infrared spectroscopy (ATR-IR) for identifying functional group on the films
   - Air-water contact angle measurements for determination of hydrophobicity of the films.
   - Zeta potential measurement for determination of surface charge of the films
4. Determination of protein adsorption on surface-modified chitosan films using proteins having different molecular weight and pI. Proteins used in this study are shown in Table 1.1.

Table 1.1 Molecular weight and pI of proteins used in this study.

Protein	Molecular weight (Da)	*pI*
Albumin	66,500	4.85
Fibrinogen	340,000	5.5
Lysozyme	14,500	11.1
RNase	13,680	9.4

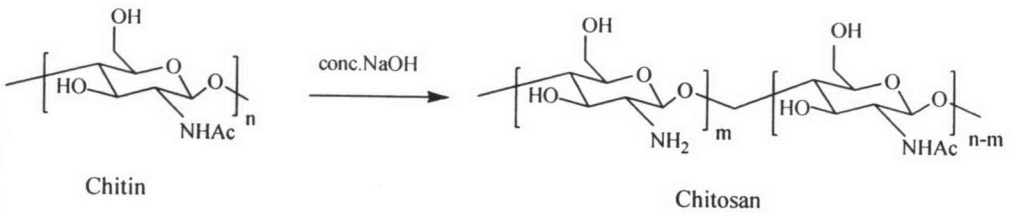
1.4 Theory

1.4.1 Chitosan

Chitin, a homopolymer comprised mainly of 2-acetamido-2-deoxy-D-glucopyranose units, served as a major structural material in form of microfibrils, in various marine invertebrates, insects, fungi, and yeasts [1]. Chitosan, the second most abundant natural polysaccharide, is the *N*-deacetylated derivative of chitin. Chemical structures of chitin and chitosan are shown in Scheme 1.1.

Chitosan is obtained by heating chitin in concentrated NaOH. It is soluble in aqueous inorganic acids such as HCl, HNO_3, and organic acids, preferably acetic acid and formic acid. Various techniques were used for determination of percentage of degree of deacetylation (%DD) such as IR[2], NMR[3], and metachromatic titration[4].

A number of intriguing properties of chitosan have been known for many years. The polymer has been used in the fields of agriculture, industry and medicine. Chitosan is also noted for its application as a film-forming agent in cosmetics [5], a dry-binder for textiles, and a strengthening additive in paper [6]. In medical field, it has been tested extensively as a biomaterial owing to its immunostimulatory activities [7], anticoagulant properties [8], antibacterial and antifungal action [9] and for its action as a promoter of wound healing [10].



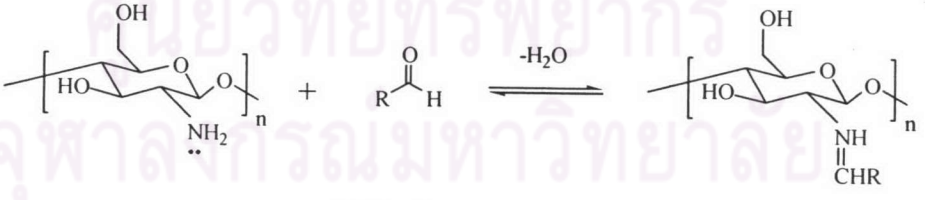
Chitin Chitosan

Scheme 1.1 Structures of chitin and chitosan.

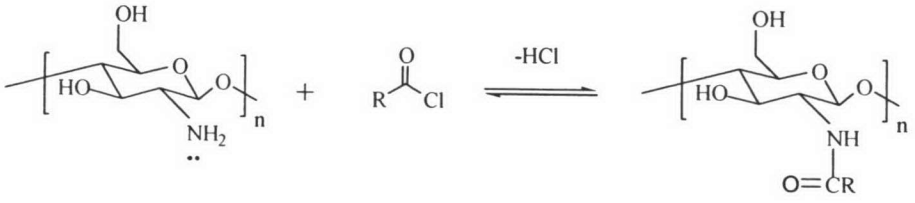
1.4.2 Chemical modification of chitosan

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. The nonbonding pair of electrons on the primary amine group also makes chitosan a potent nucleophile, reacting readily with most aldehydes to form imines. Furthermore, acyl chloride reacts vigorously with chitosan to form the corresponding amide derivatives as shown Scheme 1.2.

Since this research is interested to introduce charged functional, only chemical modification related to those two functional groups are reviewed in the form of quaternary ammonium groups and sulfonate groups. In 1986, Domard, *et al.* reported the conditions for preparation of the *N*-trimethy chitosan or quaternary ammonium chitosan (QAC). The reaction was performed between chitosan and methyl iodide in the presence of sodium hydroxide (Scheme 1.3). The obtained product showed higher hydrophilicity than native chitosan in a broader pH range, especially at the physiological pH value (7.4) [11].



aldehyde imine

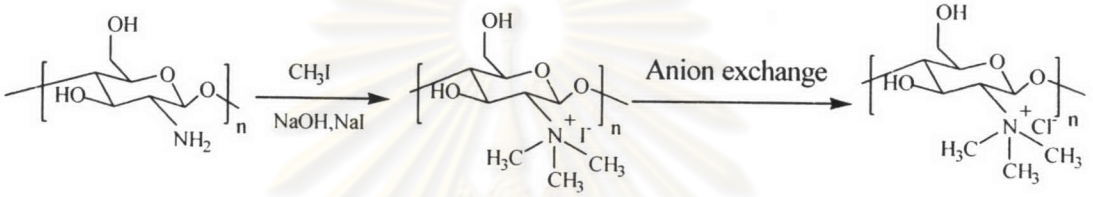


acyl chloride amide

Scheme 1.2 Nucleophilic reaction of chitosan.

To increase the degree of methylation, Sieval, *et al.* studied quaternized conditions, steps and duration of the reaction. Their results showed that higher charged density or degree of quaternization required a two-step reaction [12].

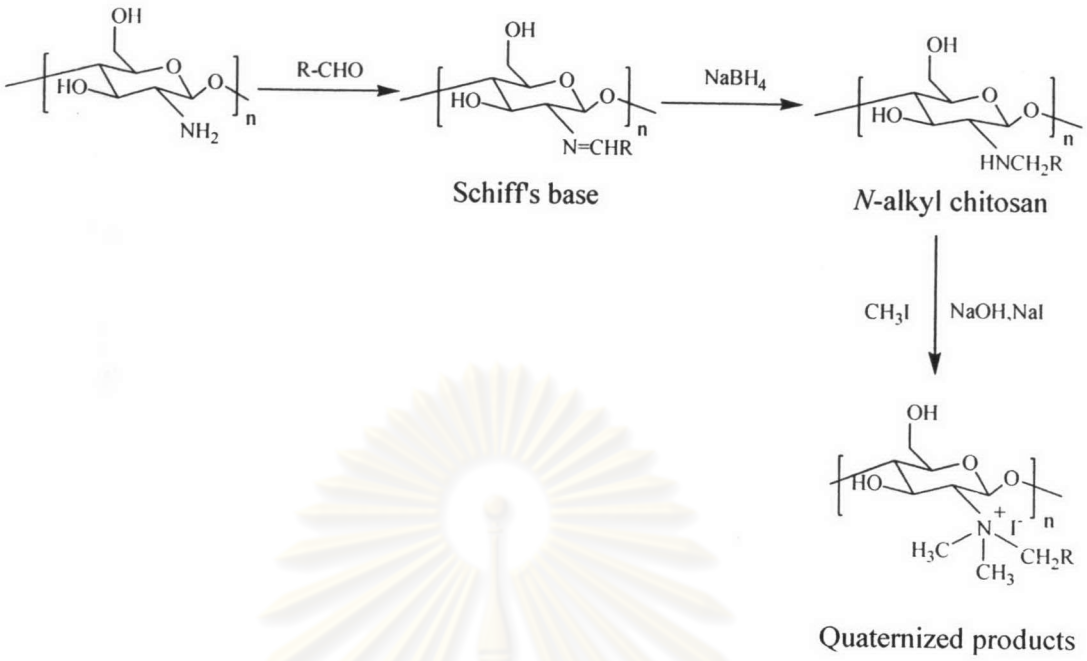
In 2002 Hamman, *et al.* administered quaternary ammonium chitosan (12-59% degree of quaternization) with [^{14}C]-mannitol in the nasal route of rats at a pH 7.40. The optimum degree of quaternization for co-administration was reached at 59%. This can probably explained by steric effects caused by the attached methyl groups and changes in the flexibility of the quaternary ammonium chitosan[13].



Scheme 1.3 Synthesis of quaternary ammonium chitosan (QAC).

Other quaternized derivatives were also suggested by Kim, *et al.* They were interested in antimicrobial activity of quaternary ammonium derivatives of *N*-alkylated chitosan. They synthesized *N*-alkyl chitosan by introducing alkyl groups into the amine groups of chitosan *via* Schiff's base intermediates. These compounds were then quaternized by reaction with methyl iodide (Scheme 1.4) [14]. The best reducing agent of Schiff's base for the *N*-alkylation step is sodium cyanoborohydride as reported by Desbrieres, *et al.* [15].

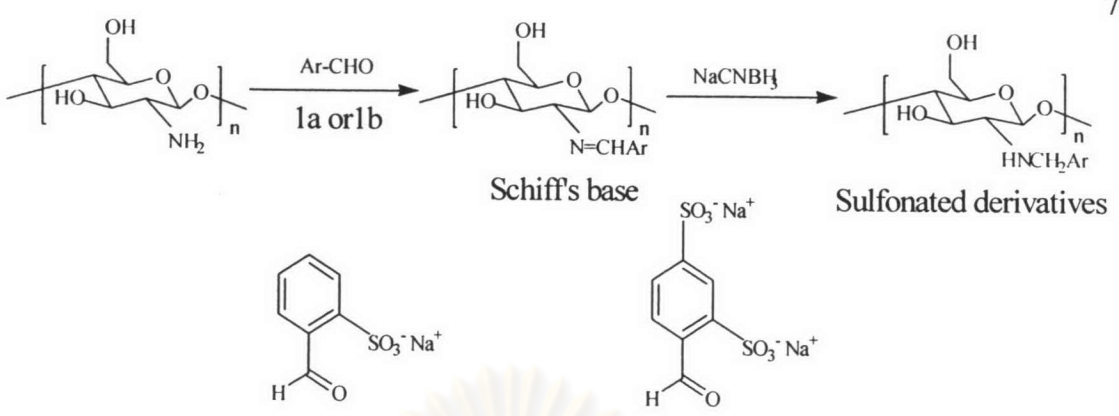
Others have shown that QAC and its *N*-alkylated derivatives could also be used as antistatic materials, especially for textile [16].



Scheme 1.4 Synthesis of quaternary ammonium salt of *N*-alkyl chitosan.

Recently, attempts have also been made to improve blood compatibility of chitosan with physical blends, surface modification and synthesis of blood compatible derivatives. It has been reported that an introduction of sulfonate groups to chitosan can improve blood compatibility. The method involves the chemical modification of chitosan by grafting with negatively charged modifiers containing sulfonic acid. Recent studies have shown that ionomers containing sulfonic acid have favorable blood-contacting responses including anticoagulant, nonthrombogenic, reduced complement activity and anti-calcification properties.

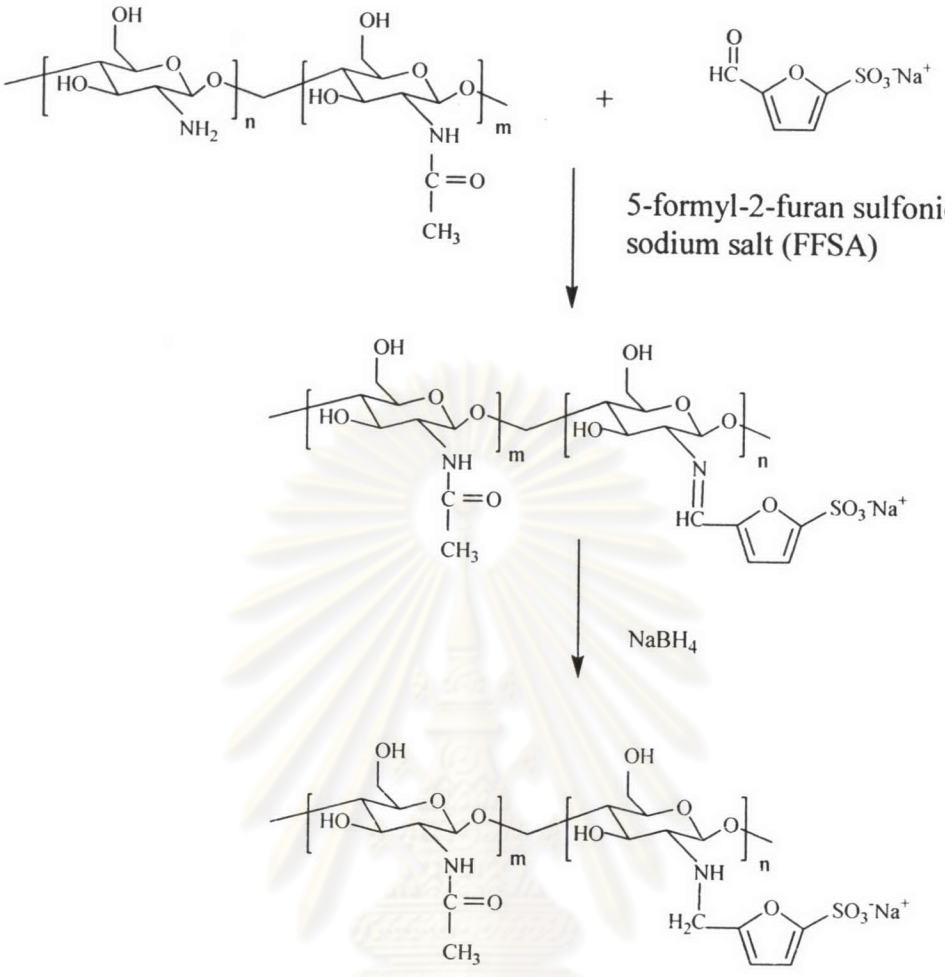
In 1997, Gregorio, *et al.* synthesized *N*-benzyl sulfonated derivatives of chitosan by reactions with 2-formylbenzene sodium sulfonate and 4-formylbenzene sodium disulfonate in the presence of sodium cyanoborohydride. One-dimensional and two-dimensional NMR spectroscope were used for the product characterization [17].



1a 2-Formylbenzenesulfonic acid 1b 4-Formyl-1,3-benzenedisulfonic acid

Scheme 1.5 Synthesis of sulfonate derivatives of chitosan [17].

In 1998, Mansoor and coworkers have synthesized an amphoteric derivative of chitosan by reacting the polymer with sodium salt of 5-formyl-2-furansulfonic acids to obtain sulfonated chitosan derivative. *In vitro* blood compatibility of the sulfonated chitosan was evaluated by measuring the number of adherent platelets and the extent of platelet activation. The sulfonated chitosan appeared to possess non-thrombogenic properties and may be suitable for some blood-contacting applications [18].



N-Sulfofurfuryl chitosan

Scheme 1.6 Synthesis of *N*-sulfofurfuryl chitosan [18].

1.4.3 Surface characterization

Surface characterization is a method for analyzing chemical and physical properties of material surface. In this research, surface of chitosan films, before and after modification, were analyzed for functional groups using ATR-IR, atomic composition using XPS, and hydrophilicity using air-water contact angle. Various techniques were used as follows:

1.4.3.1 Attenuated total reflectance infrared spectroscopy (ATR-IR)

The IR beam from the spectrometer is focused onto the beveled edge of an internal reflection element (IRE) where the sample is placed in close contact with the beam is then reflected, generally numerous times, through the IRE crystal, and directed to a detector (Figure 1.1).

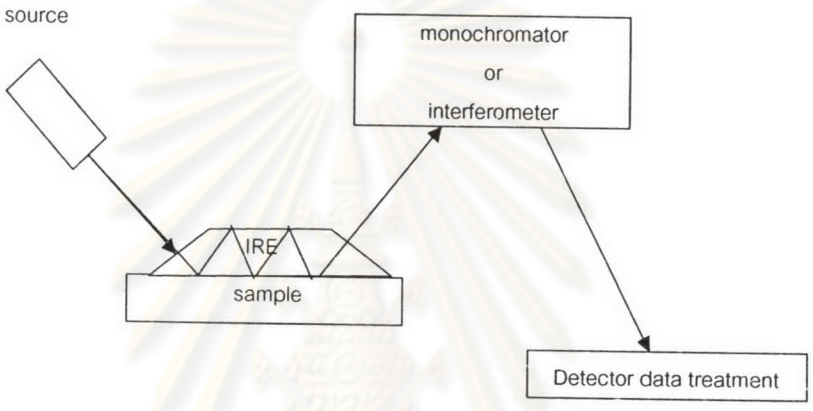


Figure 1.1 Diagram of ATR-IR.

The radiation can penetrate a short distance into the sample, thus interacts with any functionalities existing 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 the following equation:

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

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. In this study, ATR-IR was used for identifying functional groups on the surface of the films. Sampling depth of characterization is 1-1.5 μm.

1.4.3.2 X-ray photoelectron spectroscopy (XPS)

XPS is an abbreviation for X-ray Photoelectron Spectroscopy. Another name is ESCA which is an acronym for Electron Spectroscopy for Chemical Analysis. 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^{-10} -10^{-8} Torr), 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 1.2. 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:

$$E_B = h\nu - E_K - \phi. \qquad \text{.....(2)}$$

where $h\nu$ is the x-ray photon energy, E_K is the electrostatic energy and ϕ is the sample work function. Since h is a well-defined quantity, the electron binding energies can be calculated by measuring the kinetic energies of the electrons that are ejected from the sample, using the above equation. The electron energies are measured using an electrostatic energy analyzer such as a "hemispherical analyzer". The analyzer measures the kinetic energy distribution of the emitted electrons. 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, the result was used to confirm the success of functional groups modifications on the surface of chitosan films.

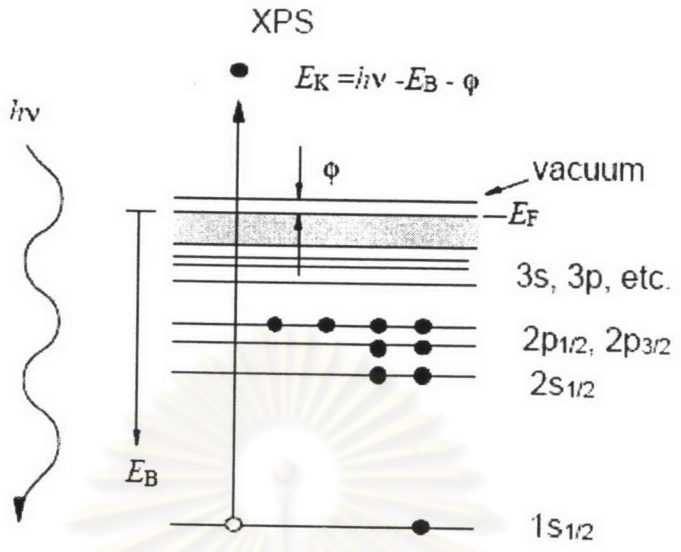


Figure 1.2 Schematic energy diagram of the x-ray photoelectron emission process and of excited ion decay [36].

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 1.3).

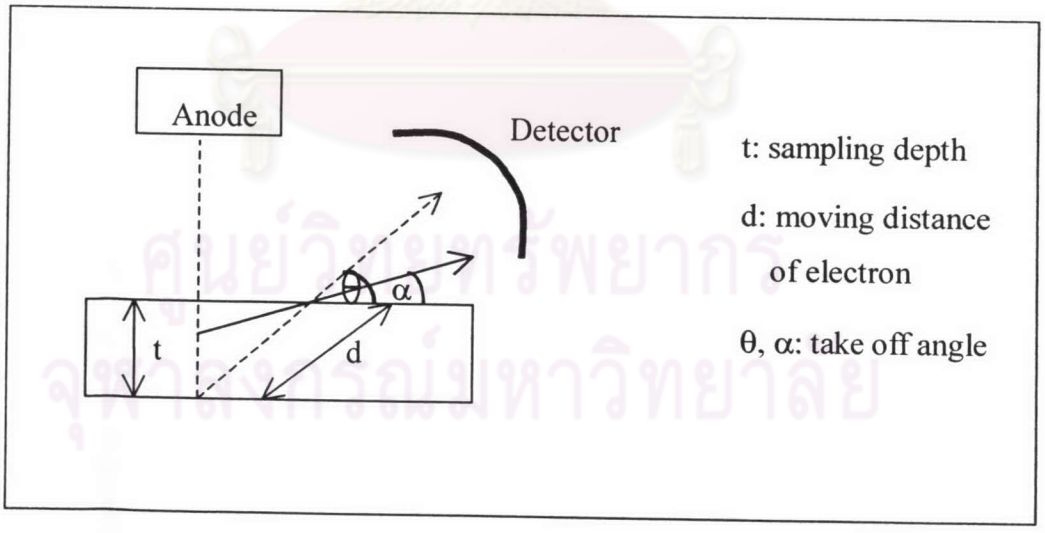


Figure 1.3 Effect of take-off angle to the depth profile.

1.4.3.3 Air-water contact angle measurement

Contact angle measurement is probably the most common method of surface tension measurement of solids. The basis of the measurement of solid surface tension by contact angle is the equilibrium of the three-phase boundary, shown in Figure 1.4, which can be described by Young's equation. As the surface becomes more hydrophobic, θ will be larger if water is used as a probe fluid.

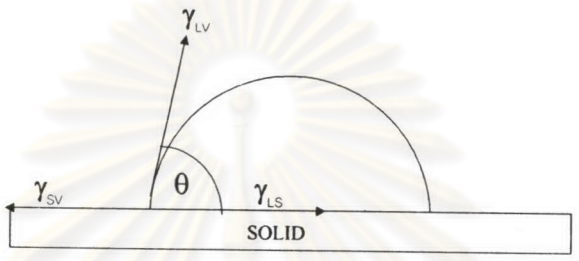


Figure1.4 Equilibrium of the three-phase boundary on solid surface.

Young's Equation: $\gamma_{SV} - \gamma_{LS} = \gamma_{LV} \cos\theta$

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

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

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

Direct interpretations from this equation can be made based on several assumptions that predict only one intrinsic contact angle (θ_0) regardless of how that angle is measured. The assumptions are:

1. The solid surface is rigid and non deformable (surface modulus> 3.5×10^5 dynes/cm^2).
2. The solid surface is highly smooth.
3. The solid surface is chemically homogenous.
4. The solid surface does not interact in any way with the liquid other than the three-phase equilibrium (i.e. swelling).

5. The surface functional groups do not reorganize in response to change in the environment during the measurement.

6. The liquid must not cause extraction or partitioning of material from the solid phase to the liquid phase.

It can be seen from Figure 1.5 that low values of θ indicate that the liquid spreads, or wets well, while high values indicate poor wetting. If the angle θ is less than 90 the liquid is said to wet the solid. If it is greater than 90, it is said to be non-wetting. A zero contact angle represents complete wetting.

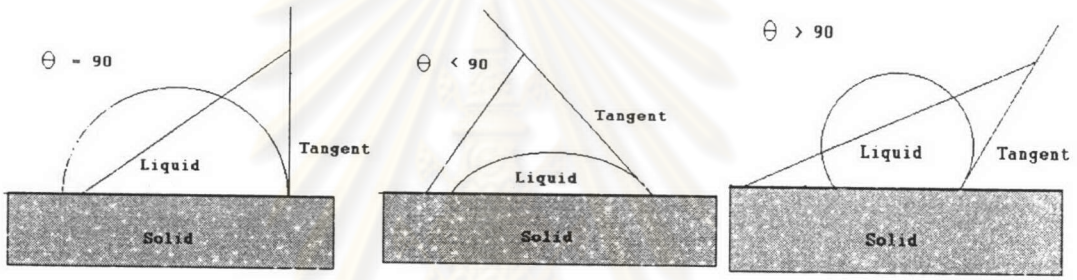


Figure 1.5 A liquid droplet in equilibrium with a horizontal surface surrounded by a gas. The wetting angle θ between the horizontal layer and the droplet interface defines the wettability of the liquid. To the left: A non-wetting fluid with $0 \leq \theta \leq 90$. To the right: A wetting fluid with $90 \leq \theta \leq 180$ [37].

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

1.4.3.4 Zeta potential (ζ -potential)

Zeta potential is defined as the potential at the surface of shear, because any relative movement of the surface with respect to the solution will cause some of the counter ions to be shared off resulting in only partial compensation of the surface charge. One of the most effective methods is to apply an electric field to the suspension and to measure how fast the particles move as a result. That process is called *electrophoresis.* It is that potential which is measured, when one measures the velocity of the particles in a D.C electric field. Laser light with a 632.8 nm wavelength from a 10 mW He-Ne laser was used as the incident beam. The measurements were performed at the scattering angle of 5° with an electric field of 32.5-34.4 V/cm. Because of the Doppler effect, the frequency of the scattered laser light is different from the frequency of the incident laser beam. This frequency shift is related to the particle velocity. The relationship between the frequency shift and electrophoretic mobility is expressed by the equation.

$$u = (v_d\lambda)[2En\sin(\theta/2)] \qquad \text{.....(3)}$$

where v_d is the Doppler frequency, u is the electrophoretic mobility, E is the electrical field, n is the refractive index, λ is the wavelength of the incident laser beam, and θ is the scattering angle. From the obtained electrophoretic mobility, the ζ potential was calculated using the Smoluchowski equation as

$$\zeta = 4\pi\eta u/\varepsilon \qquad \text{.....(4)}$$

where u is the electrophoretic mobility, and η and ε are the solution viscosity and electrical permittivity of the solution, respectively. All measurements were performed at 25±1 °C. The experiments were carried out five times for each experimental condition, and the mean value (± standard deviation) of each experimental point is indicated.

The net charge at the surface of material in contact with a polar medium is governed by three processes via ionization/dissociation of surface chemical groups, adsorption of ionic species and dissolution of ions from the material into solution.

The electric field pulls the surface charged in one direction but it will also be pulling the counter ions in the opposite direction. Some of the counter ions will move with the surface charges so the measured charge will be a net charge taking that effect into account. The electrostatic potential near the particle surface is shown in Figure 1.6. It changes very quickly (and linearly) from its value at the surface through the first layer of counter ions and then changes more or less exponentially through the diffuse layer. The junction between the bound charges and the diffuse layer is again marked by the broken line. That interface separates the bound charge from the diffuse charge around the surface when an external field is applied and. It is called the *surface of shear* or the *slip surface*.

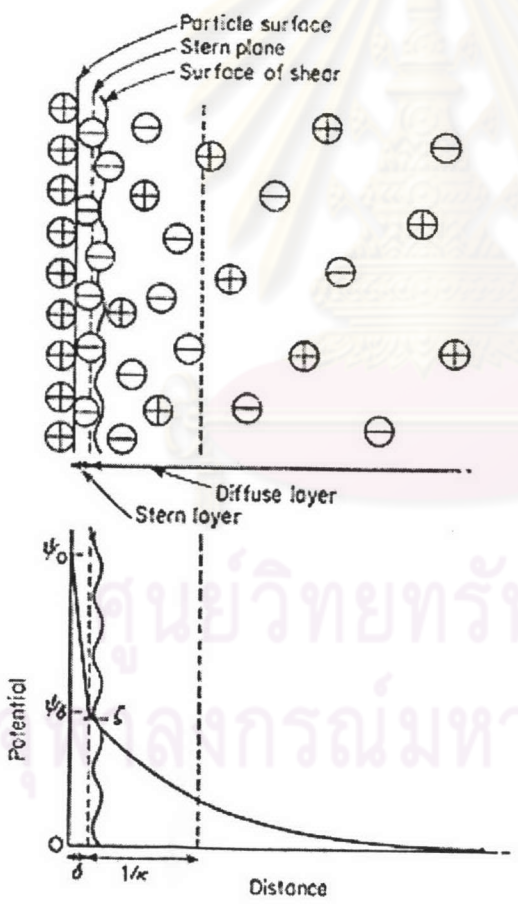


Figure 1.6 Stern model of the electrochemical double layer [38].

1.4.4 Protein adsorption on polymer surface

The study of protein adsorption has attracted considerable attention in the last few decades [19, 20]. The adhesion of proteins to surfaces is particularly important in such fields like bioengineering and bioscience. The interaction forces between protein molecules and surface can be classified as follows: (1) Van der Waals, (2) hydrophobic interactions, (3) hydrogen bonding, and (4) ionic interactions.

(1) *Van der Waals interaction.* This interaction force is operative over small distances, only when water has been excluded and the two nonpolar groups come close to each other. Lewin's calculation showed that the Van der Waals interaction is negligible compared with the forces involved in the entropy increases, i.e., hydrophobic interaction.

(2) *Hydrophobic interaction.* It has been known that hydrophobic interaction has a major role in protein adsorption phenomena. The adsorption of proteins on the low charge surface occurs by this interaction force.

(3) *Hydrogen bonding.* Hydrogen bonds are frequently formed between hydroxyl-carbonyl or amide-carbonyl radicals, and hydroxyl-hydroxyl or amide-hydroxyl bonds are also formed in protein adsorption.

(4) *Ionic interaction.* Ionic bonds are formed between negative and positive surface charge of protein molecules.

Most studies of the protein adsorption behavior onto polymer surfaces were carried out under physiological conditions for biomedical use. In 1994, Yuri, *et al.* prepared densely packed charged virus sandwiched in multilayer films of charged polyelectrolyte by successive adsorption of poly(styrene sulfonate) (PSS) and poly(allylamine hydrochloride) (PAH). It was demonstrated that the surface created by particle deposition was smooth. Very precise data on the thicknesses of sublayers were derived from suitable model fitting [21]. In the same year, they prepared multilayer films by means of alternate adsorption of positively charged globular protein (myoglobin or lysozyme) and anionic poly 4-(styrene sulfonate). Regular growth alternate adsorption cycles was analyzed by UV spectroscopy and quartz crystal microbalance (QCM) [22].

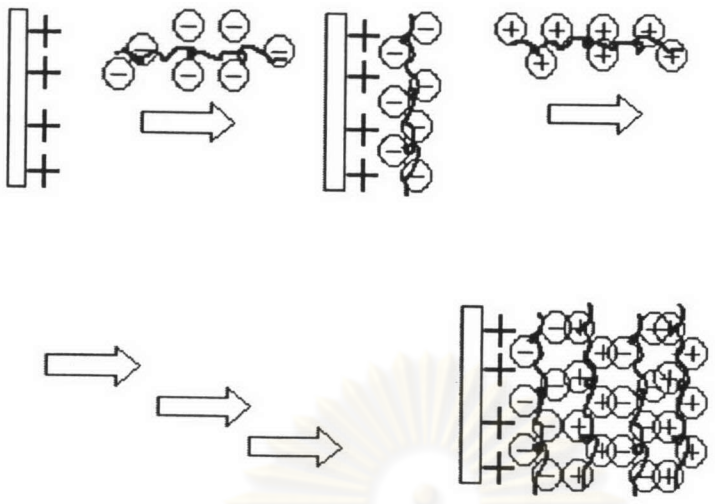


Figure 1.7 Schematic illustration of alternate layer-by-layer adsorption of polyanion and polycation onto a positively charged substrate[21].

In 1995 Koichi, *et al.* reported the amount of proteins with different isoelectric point that adsorbed onto polymer surfaces with grafted polymer chains. The chemical structures of monomers used for grafting are shown in Scheme 1.7. The results could be explained in terms of the electrostatic attraction and repulsion between the charged surfaces and the charged protein molecules (Figure 1.8). It is through the steric hindrance effect that surfaces having non-ionic grafted polymer chains tends to inhibit protein adsorption [23].

1. Anionic monomers

Acrylic acid	CH_2=CH-COOH
Methacryloxyethyl phosphate	CH_2=C(CH_3)COO-CH_2CH_2-OPO_3H_2

2. Cationic monomer

Dimethylaminoethyl methacrylate	CH_2=C(CH_3)COO-CH_2CH_2-N(CH_3)$_2$

3. Nonionic monomers

Acrylamide	CH_2=CH-CO-NH_2
Methoxy diethylene glycol methacrylate	CH_2=C(CH_3)CO-(O-CH_2CH_2)$_2$-OCH_3

Scheme 1.7 Chemical structure of ionic and non-ionic monomers used for grafting[23].

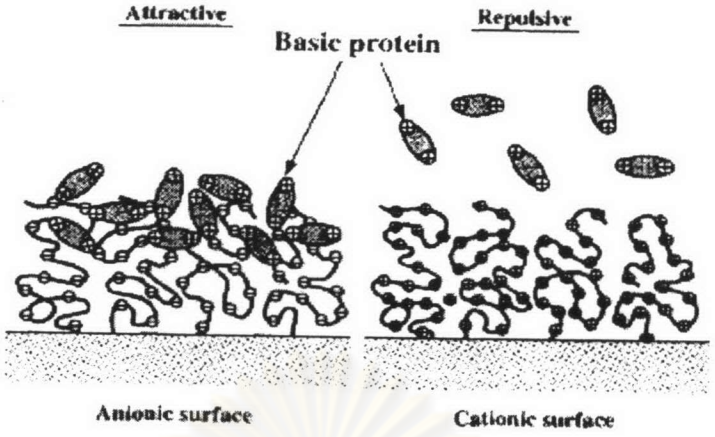
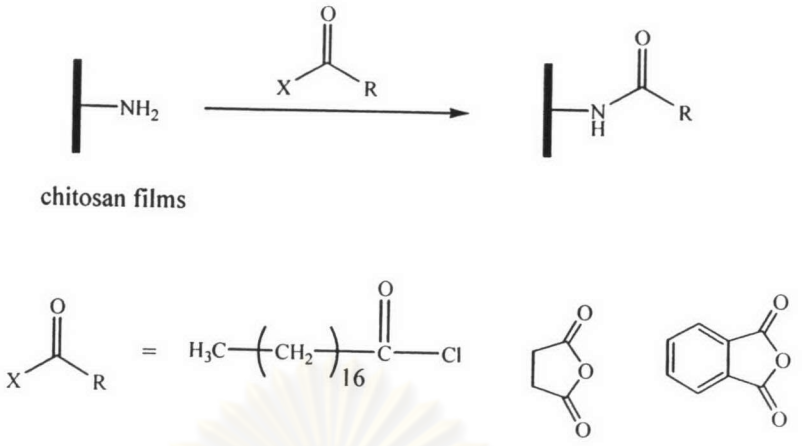


Figure 1.8 Schematic representation of the attraction and repulsion of proteins at the interfaces of polymer substrate [23].

The relationship of hydrophobic and electrostatic interactions was examined through the adsorption isotherms of bovine serum albumin (BSA) onto sulfonated microspheres, in comparison with carboxylated microspheres. Increasing the amount of hydrophilic methacrylic acid or sodium styrene sulfonated on the surface of the microspheres resulted in reinforcing hydrogen bonding or electrostatic interaction, respectively apart from that the general hydrophobic interaction [24].

Surface modification using hydrophobic polymers has also been shown to affect protein adsorption. The surface of chitosan films was modified using acid chloride and acid anhydride (Scheme 1.8). The improved surface hydrophobicity by the stearoyl groups promoted protein adsorption. In addition, selective adsorption behavior was observed in the case of the chitosan films modified with anhydride derivatives. Lysozyme adsorption was enhanced by H-bonding and charge attraction with the negative charge dissociated from the anhydride grafted surface. While the amount of albumin adsorbed was decreased possibly due to the repulsion between the modified surface and albumin [25].



Scheme 1.8 Attachment of carboxylic acid derivatives onto the surface of chitosan film *via* amide linkages [25].

The adsorption of proteins, albumin and lysozyme (LSZ) on hydrogel made of acrylic acid (AA) and 2-hydroxyethylmethacrylate (HEMA) were investigated as function of concentration of protein. Residual proteins absorbency was measured by UV-Vis (M350 Double Beam) at 280 nm, which is the wavelength of the protein UV-Vis absorption. The obtained results indicated that the amount of albumin deposited on the AA surface of hydrogel was higher than that on the surface of HEMA hydrogel. This effect can be related to the negative surface of AA hydrogel and its electrostatic interaction with protein [26]. In the same year, it has been reported that the amount of adsorbed immunogamma globulin (IgG), BSA, myoglobin (MGB) and LSZ on synthetic rodlike calcium hydroxyapatite particle (CaHaps) with various sizes increased with increased mean particle length of CaHaps except for the case of BSA. This result was caused by the specific electrostatic interaction between negatively charged carboxylic acid groups of BSA and positively charged calcium rich sites localized on CaHap surface [27].

In this work, protein adsorption on the modified chitosan surfaces was studied using albumin (bovine serum albumin or BSA), lysozyme (chicken egg white), fibrinogen (bovine serum fibrinogen or FIB) and RNase (bovine pancreas). Albumin is the most abundant carrier protein found in blood. It is a globular protein with molecular weight of 66.5 kDa and a *pI* of 4.85 and has approximated dimensions of 8 nm x 3.8 nm. Its biological functions are transport and maintenance of colloid osmotic pressure. Lysozyme is a small protein having molecular weight of 14.5 kDa and *pI* of 11.10. It is often used as a model protein in studying electrostatic interactions. It is

also an anti-microbial protein that has a hydrophobic patch and an uneven charge distribution. Fibrinogen is the largest protein studied having molecular weight of 340 kDa and *pI* of 5.50. The structure of FIB consists of a central domain attached to two distal elongated domains with approximated overall dimensions of 45 nm x 10 nm. FIB is an adhesive protein which is found to interact well with hydrophilic, charged and hydrophobic surfaces [28]. RNase form crystals under many different conditions and these crystals are very stable. It has molecular weight of 13.6 kDa and *pI* of 9.4.

Adsorptivity of proteins on the chitosan surface before and after modification was compared. These results could be used to design a surface that can control the amount of protein adsorption.

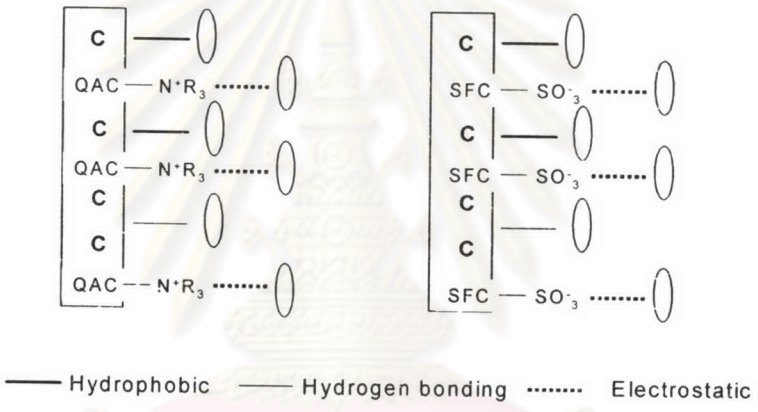


Figure 1.9 Three fundamental interaction forces involved in the protein adsorption.

The illustration of the above three interaction forces is summarized in Figure 1.9. Chitosan has hydrophobic affinity to protein which mainly occurs in protein adsorption, while it primarily occurs by hydrogen bonding or electrostatic interaction when the surface is modified with hydrophilic group. It is hypothesized that the extent of adsorption depends on either electrostatic attraction or repulsion between protein molecules and the modified groups on the chitosan surface.