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Surface modification of chitosan films. Effects of hydrophobicity on protein adsorption

Varawut Tangpasuthadol,[a,b,*] Noppong Pongchaisirikul,[a] Vipavee P. Hoven[a,b]

[a]*Department of Chemistry, Organic Synthesis Research Unit, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*
[b]*Center for Chitin/Chitosan Biomaterials, Chulalongkorn University, Bangkok 10330, Thailand*

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Abstract

The surface of chitosan films was modified using acid chloride and acid anhydrides. Chemical composition at the film surface was analyzed by attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) and X-ray photoelectron spectroscopy (XPS). ATR-FTIR data verified that the substitution took place at the amino groups of chitosan, thus forming amide linkages, and the modification proceeded to the depth at least 1 μm. Choices of molecules substituted at the amino groups of the glucosamine units did affect the hydrophobicity of the film surface, as indicated by air–water contact angle analysis. The surface became more hydrophobic than that of non-modified film when a stearoyl group ($C_{17}H_{35}CO$–) was attached to the films. The reaction of chitosan films with succinic anhydride or phthalic anhydride, however, produced more hydrophilic films. Selected modified films were subjected to protein adsorption study. The amount of protein adsorbed, determined by bicinchoninic acid (BCA) assay, related to the types of attached molecules. The improved surface hydrophobicity affected by the stearoyl groups promoted protein adsorption. In contrast, 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 hydrophilic surface. While the amount of albumin adsorbed was decreased possibly due to negative charges that gave rise to repulsion between the modified surface and albumin. This study has demonstrated that it is conceivable to fine-tune surface properties which influence its response to bio-macromolecules by heterogeneous chemical modification.

Keywords: Chitosan; Surface modification; Protein adsorption; Amide bond; Hydrophobicity

1. Introduction

Chitosan is a partially deacetylated form of chitin, a substance found naturally in the exoskeletons of insects, shells of crustaceans, and fungal cell walls. The repeating units of chitosan are β-(1→4)-linked glucosamines. Thus chitosan contains a large number of hydroxy and amino groups. These two functional groups provide several possibilities for derivatization or grafting of desirable bioactive groups. Since chitosan is non-toxic and biocompatible with the human physiological system, it has been investigated for use as wound healing pads[1,2] and drug carriers.[3,4]

Modification of the surface of biomaterials have been of major interest for many years, since it is the surface of these materials that first comes into contact with the biological surroundings. A number of surface modification techniques were used in order to alter the chemical composition and thus the surface properties of the materials. Some of the methods are plasma-treatment techniques,[5] blending with other macromolecules,[6,7] and immobilizing small or large molecules on the surface.[8–10] The change in surface properties was found to affect the interaction of the surface with the surroundings. There are reports that adsorption of platelets[6] and biomacromolecules, such as proteins,[11–14] and cells[14–16] on the polymer surface are quantitatively changed, depending on the type of molecules used.

It should be noted that surface modification by chemical reactions can be accomplished by allowing active reagents to react only at the functional group

* Corresponding author. Tel.: +66-2218-7626; fax: +66-2218-7598
E-mail address: varawut.t@chula.ac.th (V. Tangpasuthadol).

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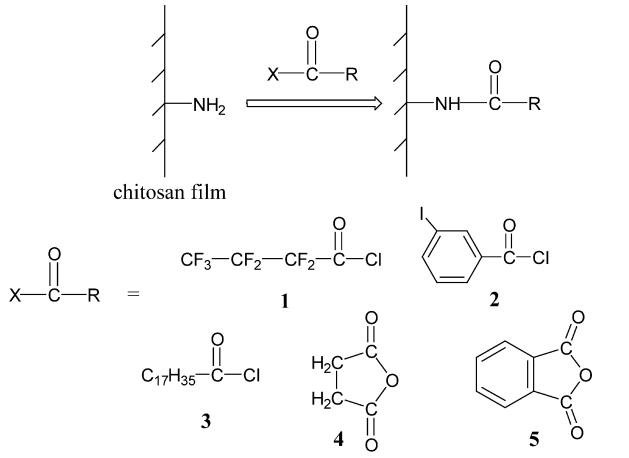


Scheme 1. Attachment of carboxylic acid derivatives on to the surface of chitosan film via amide linkages.

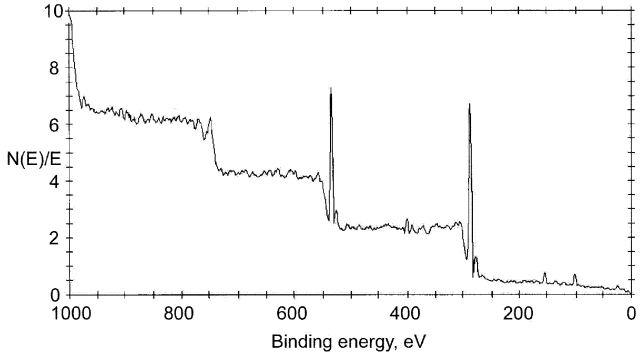


Fig. 1. XPS spectrum of a chitosan film (before chemical modification).

located on the polymer surface. The reaction is performed by immersing polymer materials in appropriate reagents. Since chitosan only dissolves in acidic water, such as HCl, acetic acid and formic acid, this left us with choices of polar organic solvents, such as MeOH and DMF to use for the surface reaction.

In this report, chitosan films were allowed to react with various types of carboxylic acid derivatives (Scheme 1). It was postulated that surface hydrophobicity of the films could be changed depending on the polarity of the derivatives used. A model study using halogen-containing acid chloride derivatives was performed in order to assess the reactivity of the chitosan amino groups. The amount of halogen atoms on the modified surface was analyzed by X-ray photoelectron spectroscopy (XPS). Changes of surface hydrophobicity were determined by air–water contact angle measurements. Lastly, to investigate the uses of modified chitosan films in biomedical applications, a study of protein adsorption on the modified surface was also pursued.

2. Results and discussion

2.1. Reactivity of chitosan surface toward acid chloride derivatives

In order to investigate the reactivity of chitosan surface toward chemical reactions, chitosan films were treated with heptafluorobutyryl chloride (**1**) and *m*-iodobenzoyl chloride (**2**). An XPS spectrum of a chitosan film is shown in Fig. 1. Only signals from C_{1s} (285 eV), N_{1s} (402 eV), O_{1s} (530 eV) of chitosan are visible. For the modified films, the signal for F_{1s} and I_{3d} are located at 680 eV for the chitosan film reacted with **1** (Chi-F_7), and 620 and 640 eV for the chitosan film reacted with **2** (Chi-I), respectively (Figs. 2 and 3). The amount of halogen atoms on the modified chitosan surface shown in Table 1 suggests that the acid chloride is linked to the chitosan. The small extent of reaction at the surface may be attributed to two possible reasons: (1) only limited amount of amino groups were available to react at the surface; (2) the amino groups were not sufficiently reactive in the amidation reaction performed under such experimental conditions.

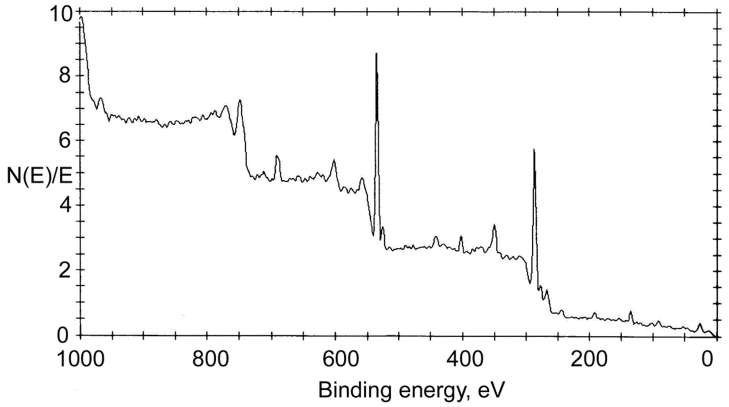


Fig. 2. XPS spectrum of the chitosan film after reacting with heptafluorobutyryl chloride.

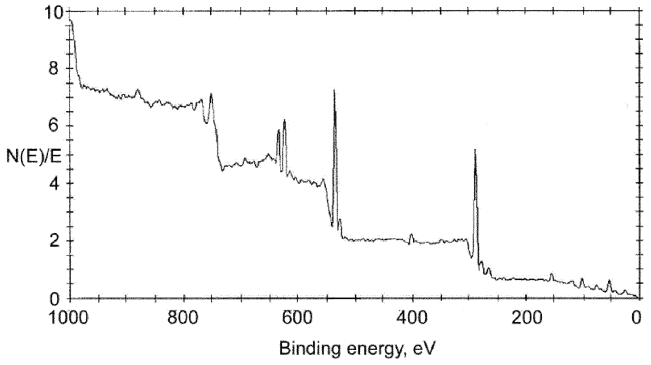


Fig. 3. XPS spectrum of the chitosan film after reacting with *m*-iodobenzoyl chloride.

Table 1
Percentage of atomic compositions by XPS at 15° takeoff angle (depth ~10 Å) of chitosan films before and after chemical modification

Sample	X (%)			*N* (%)	Yield (%) [a]
	F	Cl	I		
Chitosan [b]	0	0	0	2.9	0
Chi-F_7 [b]	4.2	0	0	3.9	18
Chi-I [c]	0	0	1.1	4.0	32

[a] Yield (%) was calculated by comparing to the theoretical amount of amino group in chitosan.
[b] Molecular weight of chitosan = 645,000 Da.
[c] Molecular weight of chitosan = 108,000 Da.

2.2. Hydrophobicity of surface-modified chitosan

The reactions of chitosan films with stearoyl chloride (**3**), succinic anhydride (**4**), or phthalic anhydride (**5**) produced films with various hydrophobicity, as analyzed by air–water contact angle measurement (Table 2). The nonpolar stearoyl group, $C_{17}H_{35}C(O)$–, increases the hydrophobicity of the film surface (the angle was increased from 89 ± 6 to $101 \pm 4°$). Whereas, both anhydride derivatives **3** and **4** cause the film to become more hydrophilic (the angle decreased to 56 ± 1 and $51 \pm 7°$, respectively). Similar trends were also observed for both chitosans having molecular weight of 108,000 and 645,000.

Changes of air–water contact angles for the modified chitosan films are due to the attachment of the selected molecules, as shown by IR spectra (Fig. 4). For the

Table 2
Air–water contact angles of chitosan films before and after chemical modification

Sample	Angle (°)
Chitosan [a]	89 ± 6
N-Stearoylchitosan [a]	101 ± 4
N-Succinylchitosan [a]	56 ± 2
N-Phthalylchitosan [a]	51 ± 7
Chitosan [b]	93 ± 2
N-Stearoylchitosan [b]	97 ± 3
N-Succinylchitosan [b]	55 ± 4
N-Phthalylchitosan [b]	52 ± 4

[a] Molecular weight = 108,000 Da.
[b] Molecular weight = 645,00 Da.

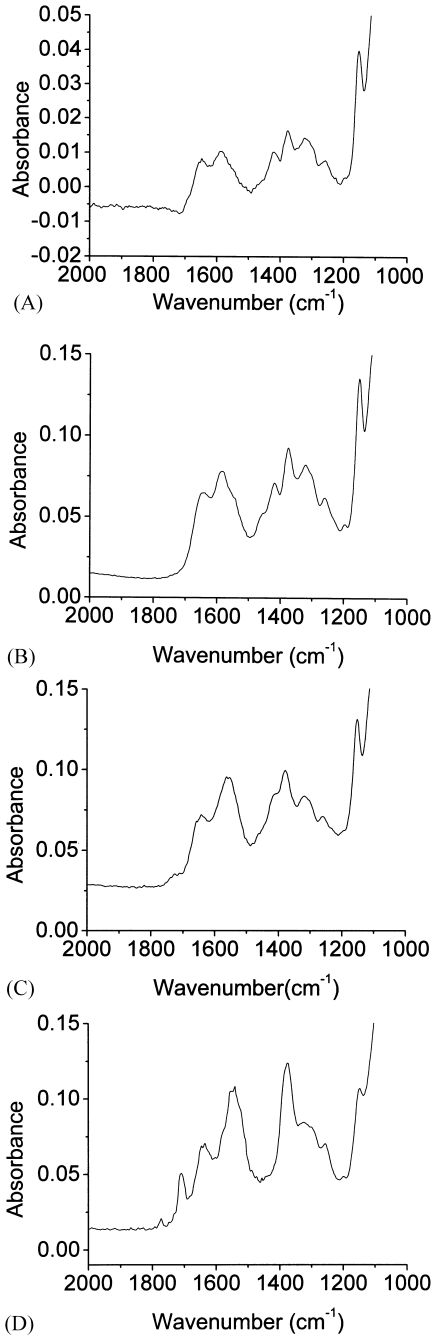


Fig. 4. ATR-FTIR spectra of chitosan films: (A) before reaction; (B) reacted with **3**; (C) reacted with **4**; and (D) reacted with **5**.

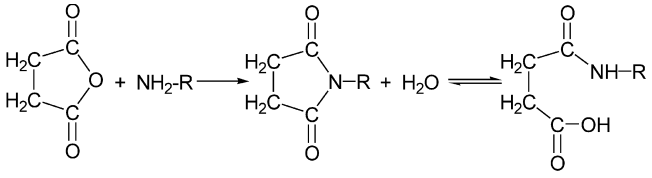
reaction with **3**, the IR spectrum shows a new signal at 1550 cm^{-1} for amide N–H bending that belongs to an *N*-stearoyl unit (Fig. 4(B)). No signals for O-acylation in the region of 1720–1770 cm^{-1} could be observed, suggesting that only the amino group of chitosan was acylated. The films of lower hydrophobicity reacted with anhydrides **4** and **5**, which could result in the formation of a polar amide bond and a carboxylic acid, which are hydrolysis products of the intermediate imide (Scheme 2). The evidence that the imide bond is present in Fig. 4(C), which show signals for carbonyl stretching vibration at 1730 cm^{-1}, and in Fig. 4(D) at 1710 and 1770 cm^{-1}. In addition, these results also suggest that the modified layer is thick enough to be detected by the ATR-FTIR technique with a sampling depth of about 1–2 μm.

2.3. Protein adsorption study

Bovine serum albumin (BSA) and lysozyme, two globular proteins varying in size and charge, were selected for the protein adsorption study. Bicinchoninic acid (BCA) assay was used to determine the amount of adsorbed protein on the selected modified films that possessed different contact angle values. The amounts of proteins adsorbed on the treated films are shown in Table 3.

Both proteins adsorbed slightly more to the films that were reacted with the stearoyl functional group **3**. This suggests that a hydrophobic surface enhances protein adsorption. A similar finding was reported from the study on protein adsorptivity on a different hydrophobic surface based on polypyrrole.[17]

The hydrophilic surface, resulting from the reaction with anhydride derivatives **4** and **5** tended to reduce the adsorption of BSA, but increase the adsorption of lysozyme. As mentioned earlier, the newly formed imide bonds could be hydrolyzed in the presence of water during the protein adsorption study, resulting in formation of amide and carboxylic acid groups. At pH 7.4, the carboxylic acid was converted to a negatively charged carboxylate ion. Therefore, BSA, a carboxylic acid-rich protein, was significantly less adsorbed onto the carboxylic acid-rich chitosan surface possibly due to charge–charge repulsion. On the other hand, lysozyme could be adsorbed more on the surface reacted with anhydride derivatives than on the non-modified films. Lysozyme with a pI value of 11 contains a large number of –OH and $–NH_2$ groups. Therefore, it is positively charged at pH 7.4. Hydrogen-bonding and charge–charge attraction could be responsible for the increased adsorption of lysozyme. The result from this study seems to fit well with the report on the study of charge interaction of protein and polyelectrolyte films reported earlier by Ladam and co-workers.[13]



Scheme 2. A chemical reaction between an amine and anhydride.

3. Experimental

3.1. Materials

Chitosans were purchased from Seafresh Chitosan (Lab) Co., Ltd., Thailand ($M_v = 645{,}000$; 87%DD), and Taming Enterprises Co., Ltd, Thailand ($M_v = 108{,}000$; 83%DD). *N*,*N*-Dimethylformamide (DMF) was distilled over CaH_2 under reduced pressure. Dichloromethane was distilled over CaH_2. Methanol was distilled over 4A molecular sieves. Phthalic anhydride, succinic anhydride, heptafluorobutyryl chloride, *m*-iodobenzoic acid, and stearic acid were purchased from Fluka and used as received. BSA, lysozyme, BCA assay kit, phosphate buffer saline (PBS), and triethylamine (TEA) were purchased from Sigma Chemical Co.

3.2. Preparation of chitosan films

Chitosan (2 g) was dissolved in 0.1 M HOAc (100 mL). After stirring for 24 h, the solution was filtered through

Table 3
The amount of BSA and lysozyme adsorbed on chitosan films before and after chemical modification

Sample	Contact angle (°)	Amount of lysozyme (μg/cm^2)	Amount of BSA (μg/cm^2)
Chitosan [a]	89 ± 6	4.65 ± 0.82	3.04 ± 1.83
N-Stearoylchitosan [a]	101 ± 4	7.87 ± 4.52	4.86 ± 1.16
Chitosan [b]	93 ± 2	4.58 ± 0.93	4.66 ± 1.55
N-Phthalylchitosan [b]	52 ± 4	4.73 ± 0.64	2.58 ± 0.63
N-Succinylchitosan [b]	55 ± 4	7.3 ± 0.82	1.63 ± 0.20

[a] Molecular weight = 108,000 Da.
[b] Molecular weight = 645,00 Da.

a medium-pore-size sintered glass funnel to remove insoluble substances. The solution was then poured into a Teflon-coated plate (8 × 8 inch in size). Solvent was allowed to evaporate in air for 4 days. Then the film was peeled off and immersed in 1:1 0.1 M NaOH–MeOH and 1:1 MeOH–water to neutralize the acid. The final drying step was carried out under vacuum. Film thickness was 40–100 μm.

3.3. Reaction with heptafluorobutyryl chloride (1)

DMF (10 mL) was added into a Schlenk flask containing chitosan films. Triethylamine (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 (rt) under nitrogen atmosphere for 48 h. Then the films were rinsed twice with DMF and MeOH. The final drying step was done in vacuum at rt.

3.4. Reaction with *m*-iodobenzoyl chloride (2)

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 cannula. The solution was stirred at rt under nitrogen atmosphere for 48 h. The films were rinsed twice with DMF and MeOH, and then dried under vacuum before characterization.

3.5. Reaction with stearoyl chloride (3)

The procedure of this reaction was the same as the reaction of chitosan films and *m*-iodobenzoyl chloride, except stearoyl chloride (**3**) was used instead of *m*-iodobenzoyl chloride (**2**).

3.6. Reaction with acid anhydride (4 or 5)

Anhydrous DMF or MeOH (10 mL) was added into a Schlenk flask containing chitosan films and acid anhydride (20 equiv of $-NH_2$ groups). The reaction was stirred at rt, 50 °C for MeOH and 80 °C for DMF under a nitrogen atmosphere. The reaction was allowed to proceed for 48 h. The films were rinsed twice with DMF or MeOH to remove unreacted anhydride. The films were dried under vacuum before characterization.

3.7. XPS analysis

X-ray photoelectron spectra (XPS) were obtained on a Perkin–Elmer Physical Electrons 5100 using Mg K_α excitation (15 kV, 400 W). 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 angles at 15 and 75° were chosen, and the approximate depth profile are 10 and 40 Å, respectively.

3.8. ATR-FTIR analysis

All IR spectra were collected at a resolution of 4/cm and 16 scans using a 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.

3.9. Air–water contact angle analysis

A drop of deionized water was put onto the film surface by a syringe (Gilmont) with a 24-gauge flat-tipped needle. Images of water droplets on film surface were recorded by a digital camera (Sony, Model F707). The images were processed with Adobe Photoshop 6.0 software to obtain contact angle data.

3.10. Protein adsorption on the modified chitosan surface

Film substrates were immersed in PBS solution at pH 7.4 for 2 h prior to adsorption. Protein solutions were freshly prepared by dissolving BSA or lysozyme in PBS at pH 7.4 to give a final concentration of 0.1 mg/mL. Film substrates were incubated in polyethylene bottles, containing 3 mL protein solution at 37 °C. After 3 h, the films were removed and rinsed with 4 × 10 mL PBS solution, followed by sonication in 2 mL of 1 wt% sodium dodecylsulfate (SDS) to remove reversibly adsorbed protein. The amount of protein adsorbed on the substrates was determined by the micro-BCA protein assay.[18] The absorbance of the solution was measured at 562 nm by UV–Vis spectroscopy. Three repetitions were performed for all samples.

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