Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 68 (2007) 44–53
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Surface-charged chitosan: Preparation and protein adsorption

Voravee P. Hoven [a,*], Varawut Tangpasuthadol [a], Yaowamand Angkitpaiboon [b], Napanporn Vallapa [b], Suda Kiatkamjornwong [c]

[a] *Organic Synthesis Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University, Phayathai Road, Pathumwan, Bangkok 10330, Thailand*
[b] *Program of Petrochemistry and Polymer Science, Faculty of Science, Chulalongkorn University, Phayathai Road, Pathumwan, Bangkok 10330, Thailand*
[c] *Department of Imaging and Printing Technology, Faculty of Science, Chulalongkorn University, Phayathai Road, Pathumwan, Bangkok 10330, Thailand*

Received 26 April 2006; received in revised form 5 July 2006; accepted 10 July 2006
Available online 28 August 2006

Abstract

Positive and negative charges were introduced to chitosan surfaces via methylation using methyl iodide (MeI) and reductive alkylation using 5-formyl-2-furan sulfonic acid (FFSA). Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS) and zeta potential measurement confirmed the presence of the desired functional groups on the surface-modified chitosan films. The chitosan films having negative charges of *N*-sulfofurfuryl groups on their surface (SFC films) exhibited selective protein adsorption against both negatively charged proteins (albumin and fibrinogen) and positively charged proteins (ribonuclease, lysozyme). Its adsorption can be explained in terms of electrostatic attraction and repulsion. In contrast, the adsorption behavior of chitosan films having positive charges of quaternary ammonium groups on their surface (QAC films) was anomalous. The quantity of the adsorbed protein tended to increase as a function of the swelling ratio of the QAC film regardless of the charge characteristics of the protein.

Keywords: Chitosan; Surface charge; Quaternary ammonium group; Sulfonated group; Protein adsorption

1. Introduction

Protein adsorption on material's surface is generally regarded as a primary event that occurs when the material comes into contact with biological surroundings. To a certain extent, such behavior reflects the performance of the material when it is used as biomedical devices or biomaterials. This type of "biofouling" is detrimental to technologies that require precise manipulation of proteins such as screening of DNA and protein libraries, controlling cell organization, and blood contacting devices. On the other hand, it is beneficial to some applications, for example, wound healing pads which demand plasma protein adsorption and subsequent platelet adhesion and activation. To address biofouling issues, much attention has been directed towards the development of chemical modification strategies that lead to material's surface having desirable biofouling characteristics.

Chitosan is a partially deacetylated form of chitin, a natural substance found abundantly in the exoskeletons of insects, shells of crustaceans, and fungal cell walls. Because of its favorable physicochemical and biological properties such as being biocompatible, non-toxic, and antibacterial, chitosan is considered as an attractive material that can potentially be used in many biomedical-related applications (Fu, Ji, Yuan, & Shen, 2005; Lee et al., 2002; Li, Liu, Liu, Liu, & Yao, 2005; Mi et al., 2001; Wang, Lin, Wang, & Hsieh, 2003; Yamamoto, Kuno, Sugimoto, Takeuchi, & Kawashima, 2005). The repeating units of chitosan are β-(1 → 4)-linked glucosamines that constitute a large number of hydroxy and amino groups. These two functional groups offer several possibilities for derivatiza-

* Corresponding author. Tel.: +66 2218 7626 7; fax: +66 2218 7598.
E-mail address: vipavee.p@chula.ac.th (V.P. Hoven).

0144-8617/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2006.07.008

tion and immobilization of biologically active species (Ho et al., 2005; Lebouc, Dez, Desbrieres, Picton, & Madec, 2005; Liu, Zhang, Cao, Xu, & Yao, 2004).

Researchers have undertaken several routes to modify the surface of chitosan, the key purpose of which is to alter the chemical composition and the surface properties of chitosan to suit specific applications (Amiji, 1997; Chen, Kumar, Harris, Smith, & Payne, 2000; Justi, Favere, Laranjeira, Neves, & Casellato, 2005; Li, Liu, & Fang, 2003; Tsubokawa & Takayama, 2000; Wang, Fang, & Yan, 2001; Wang, Kao, & Hsieh, 2003; Zhu, Chian, Chan-Park, & Lee, 2005; Zhu, Wang, Yuan, & Shen, 2002; Zhu, Zhang, Wu, & Shen, 2002). Recently, we have reported work on chemical modification of the chitosan surface via reactions between the amino groups of chitosan and carboxylic acid derivatives (Tangpasuthadol, Pongchaisirikul, & Hoven, 2003) as well as PEG-functionalized aldehydes (Amornchai, Hoven, & Tangpasuthadol, 2004). The results from model studies suggested that amino groups on the surface of chitosan are reactive enough to react with a number of acid chlorides, acid anhydrides and aldehydes. Evidence from protein adsorption studies has confirmed the assumption that the surface hydrophobicity/hydrophilicity significantly influences protein adsorption (Amornchai et al., 2004; Tangpasuthadol et al., 2003).

Under acidic conditions, chitosan in its original form adopts a positive charge which can attract negatively charged plasma proteins leading to platelet adhesion and activation followed by thrombus formation and blood coagulation (Benesch & Tengvall, 2002; Okamoto et al., 2003). This sequential response should partly be responsible for the success of chitosan in wound healing acceleration (Azad, Sermsintham, Chandkrachang, & Stevens, 2004; Ishihara et al., 2003; Kojima, Okamoto, Miyatake, Kitamura, & Minami, 1998; Kojima et al., 2001). Such thrombogenic properties of chitosan cause serious failure when it is used in blood-contacting applications, however. In light of heparin's success in suppressing plasma protein adsorption, incorporating negative charges of sulfonate or sulfate groups has been proposed as an effective way to reduce the thrombogenic property of chitosan. It was revealed that blood compatibility of a chitosan surface can be improved by complexation-interpenetration methods using sulfonate derivative of poly(ethylene glycol) (Amiji, 1997), heparin and dextran sulfate (Amiji, 1996), and by direct sulfonation using sulfur trioxide–pyridine complex (Lin & Lin, 2001). Under homogeneous conditions, the reaction between the amino groups of chitosan and the sodium salt of 5-formyl-2-furansulfonic acid followed by reduction yielded *N*-sulfofurfuryl chitosan having non-thrombogenic properties (Amiji, 1998). Sulfation of chitosan by chlorosulfonic acid/DMF resulted in sulfated chitosan, which showed strong anticoagulant activity (Vongchan, Sajomsang, Subyen, & Kongtawelert, 2002).

Quaternary ammonium chitosan has attracted considerable interest because of its improved aqueous solubility and antimicrobial activity in a broader pH range in comparison with native chitosan (Jia, Shen, & Xu, 2001; Kim & Choi, 2002; Kim, Choi, Chun, & Choi, 1997). Antimicrobial activity is believed to originate from the ability of cationic species to bind with sialic acid in phospholipids, consequently restraining the movement of microbiological substances (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Oligomeric chitosan can also penetrate into the cells of micro-organisms and prevent the growth of cells by prohibiting the transformation DNA into RNA (Zheng & Zhu, 2003). Derivatives of quaternary ammonioum chitosan are typically synthesized either by direct quaternization of the amino groups of chitosan using alkyl halides under alkaline conditions (Curti, de Britto, & Campana, 2003; Domard, Gey, Rinaudo, & Terrassin, 1987), by the reductive *N*-alkylation reaction of chitosan with aldehydes via Schiff's base intermediates followed quaternization by methyl iodide (Jia et al., 2001; Kim & Choi, 2002; Kim et al., 1997), or by reductive *N*-alkylation reaction of chitosan with quaternary ammonium-type aldehydes (Suzuki, Oda, Shinobu, Saimoto, & Shigemasa, 2000). While the first two methods introduce alkyl groups not only to the amino groups, but also to the hydroxy groups, the last method is more selective to functionalization of amino groups. Reaction of amino groups of chitosan with glycidyl trimethylammonium was also suggested as an alternative to *N*-selective reaction (Lim & Hudson, 2004; Seong, Whang, & Ko, 2000).

Taking advantage of functional group availability for chemical reactions of the chitosan surface and the diversified bioactivity of its charged derivatives, this research aims to tailor protein adsorption of the chitosan surface by chemically introducing charged functionalities specifically to amino groups under heterogeneous conditions. The charged-modified chitosan surfaces are subjected to proteins having different molecular weights and isoelectric points. Besides hydrophobic interaction and hydrogen bonding, it is hypothesized that the extent of protein adsorption should depend also on electrostatic interaction between protein molecules and the modified groups on the chitosan surface. This surface modification should expand the applicability of chitosan in biomedical-related fields.

2. Materials and methods

2.1. Materials

Chitosan with DAC of 88% ($M_V = 645{,}535$ Da) was obtained from Seafresh Chitosan (Lab) Co., Ltd (Thailand). Methanol obtained as commercial grade was distilled over 4A molecular sieves prior to use. Methyl iodide (MeI), 5-formyl-2-furan-sulfonic acid (FFSA), sodium borohydride ($NaBH_4$), sodium hydroxide (NaOH) and sodium iodide (NaI) were purchased from Fluka (Switzerland) and used as received. Bovine serum albumin, fibrinogen, lysozyme, ribonuclease, Bicinchoninic acid assay kit

(QuantiPro™ BCA assay) and phosphate-buffer saline (PBS) were purchased from Sigma Chemical Co (USA).

2.2. Preparation of chitosan film

Chitosan flakes (2 g) were dissolved in 0.1 M aqueous acetic acid solution (100 mL). After stirring for 24 h, the solution was filtered through a sintered glass having medium pore size to remove insoluble substances. The chitosan solution was then cast into a film on a petri dish (2.5 in. in diameter). The solvent was allowed to evaporate in air for 4–5 days. The chitosan film was peeled off from the dish and immersed in 1:1 (v/v) 0.1 M NaOH/methanol and 1:1 (v/v) methanol/water to neutralize the acid used as a solvent. The film was dried under vacuum for 1–2 days. The film thickness was 60–100 μm.

2.3. Preparation of positively charged chitosan film (QAC film)

Anhydrous methanol (10.0 mL) was added into a flask containing chitosan films ($1.5 \times 1.5\ cm^2$) and NaOH (0.16 g, 4.0 mmol). NaI (0.30 g, 2.0 mmol) was added to the flask. Subsequently, MeI (0.31 mL, 2.0 mmol) was added to the mixture. After the reaction was carried out at 40 °C for a desired period of time (1, 2, 4 or 12 h), the same amount of MeI was then added to the mixture. The reaction further proceeded for the same period of time (1, 2, 4 or 12 h). The films were removed from the solution, then rinsed twice with methanol and dried under vacuum for more than 3 days. The overall concentration of MeI and NaOH was also varied from 0.4 M to 0.8 and 1.6 M.

2.4. Preparation of negatively charged chitosan film (SFC film)

FFSA (0.2 g, 0.5 mmol) was dissolved in 10 mL anhydrous methanol and added into a flask containing chitosan films ($1.5 \times 1.5\ cm^2$). The mixture was stirred for 1 h at ambient temperature. $NaBH_4$ (0.04 g, 0.5 mmol) was added into the reaction mixture and the solution was stirred for a desired period of time (5, 11 or 23 h) at room temperature. The films were removed from the solution, then rinsed twice with methanol and dried under vacuum for more than 3 days. The concentration of FFSA and $NaBH_4$ was varied in the range of 0.05–1.0 M.

2.5. Contact angle measurement

Contact angle meter model CAM-PLUS MICRO was used for the determination of water contact angles. A droplet of Milli-Q water is placed on the tested surface by bringing the surface into contact with a droplet suspended from a needle of the syringe. A silhouette image of the droplet is projected on the screen. The angle is promptly measured within 5 s after water drop arrives at the film surface. The rapid measurement is necessary to assure that there is no error caused by water absorption of the film surface.

2.6. Analysis by attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy

All spectra were collected at a resolution of $4\ cm^{-1}$ and for 128 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.

2.7. Analysis by nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were obtained from 1% solution in 1% CF_3COOH/D_2O or D_2O using a 400 MHz Varian mercury-400 spectrometer. Chemical shifts are reported in ppm.

2.8. Analysis by X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectra were obtained on a thermoVGscientific instrument using Mg K_α excitation. In this study, the take off angle at 45° was chosen and the approximate depth of profile is 30 Å.

2.9. Zeta-potential measurement

The zeta-potential of surface-charged chitosan particles was determined using a Zetasizer Nano-ZS (Malvern Instruments, UK). Chitosan particles were prepared according to the published procedure (Qi, Xu, Jiang, Hu, & Zou, 2004). Chitosan particles before and after surface modification were dispersed in deionized distilled water. The analysis was performed at 25 °C using a scattering angle of 173°.

2.10. Protein adsorption

Protein solutions were freshly prepared by dissolving albumin, fibrinogen, lysozyme and ribonuclease in PBS at pH 7.4 to give a final concentration of 1 mg/mL. To reach an equilibrium hydration, the film substrate was immersed in the PBS solution overnight prior to adsorption. Each sample was removed from PBS solution and suspended into the wells containing 3 mL protein solution before incubation at 37 °C for 3 h. Then the films were removed and rinsed with 4×10 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 2.0 mL of 1.0 wt% sodium dodecyl sulfate (SDS) and soaked for 1 h at room temperature. To determine the total amount of protein adsorbed on the substrates, Bicinchoninic acid (BCA) protein assay was

utilized. The absorbance of the solution was measured at 562 nm by UV-VIS spectroscopy (Microtiter plate reader; model Sunrise, Tecan Austria GmbH). The amount of adsorbed protein was determined by comparison of the absorbance of the samples with a calibration curve. Three repetitions were performed for all samples.

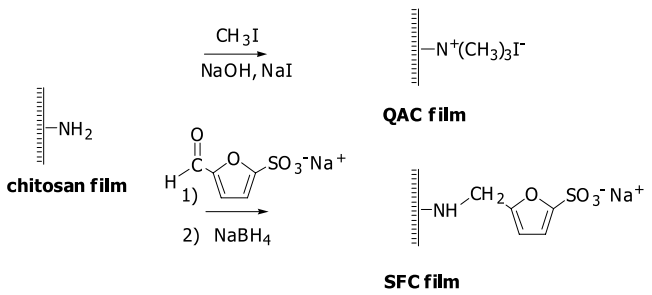
3. Results and discussion

3.1. Preparation and characterization of surface-charged chitosan

The methods for modifying the chitosan film surface are outlined in Scheme 1. The positively charged chitosan surface was prepared by a reaction between the amino group of chitosan and methyl iodide (MeI) to form the quaternary ammonium-functionalized chitosan surface (QAC film). A negatively charged sulfonate group was introduced to the chitosan surface by a reaction between the amino group of chitosan and 5-formyl-2-furan sulfonic acid (FFSA) followed by reduction using $NaBH_4$ to form the sulfonate-functionalized chitosan surface (SFC film).

Contact angle measurement, one of the most convenient surface-sensitive techniques, was first used as a primary tool to monitor the extent of surface modification as a function of reaction time and reagent concentration. As anticipated, the surface hydrophobicity of chitosan films decreased upon the introduction of the charged moiety. Fig. 1 reveals that the longer the reaction proceeded, the more hydrophilic the surface became. Using 0.4 M MeI, the surface quaternization reaches its maximum extent after 8 h. On the other hand, at least 12 h is necessary for the substitution of *N*-sulfofurfuryl groups to attain its highest extent using 0.1 M FFSA. Reagent concentration (MeI or FFSA) also affects the extent of surface modification. According to Fig. 2, a certain concentration is required in order to achieve the highest surface substitution (0.4 M of MeI for quaternization and 0.5 M of FFSA for sulfonation). Although contact angle data do not provide quantitative information on the degree of substitution of charge functionality on the surface, they give a general idea on how to control the extent of surface modification.

ATR-FTIR spectroscopy was used to confirm the transformation of amino groups of chitosan to charged



Scheme 1. Introduction of charged functional groups to the surface of chitosan.

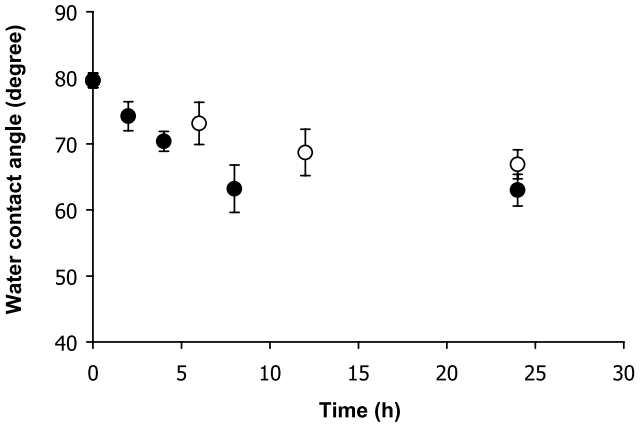


Fig. 1. Water contact angle of QAC films using 0.4 M MeI (●) and SFC films using 0.1 M FFSA (○) as a function of reaction time.

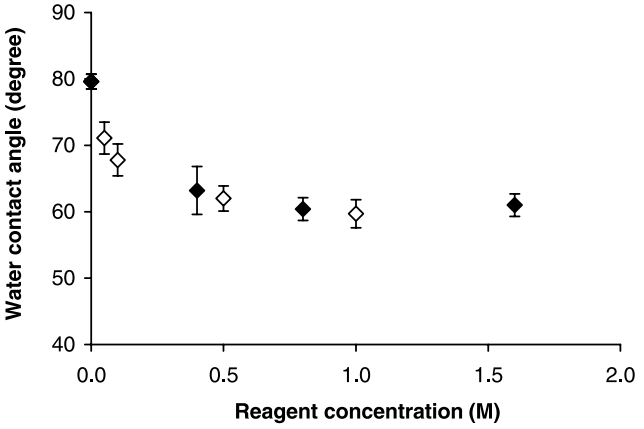


Fig. 2. Water contact angle of QAC films as a function of MeI concentration using a reaction time of 8 h (♦) and water contact angle of SFC films as a function of FFSA concentration using a reaction time of 24 h (◇).

functionalities. Fig. 3 shows ATR-FTIR spectra of chitosan films before and after surface quaternization. Absorption peaks at *ca.* 1650 and 1590 cm^{-1} were assigned to the

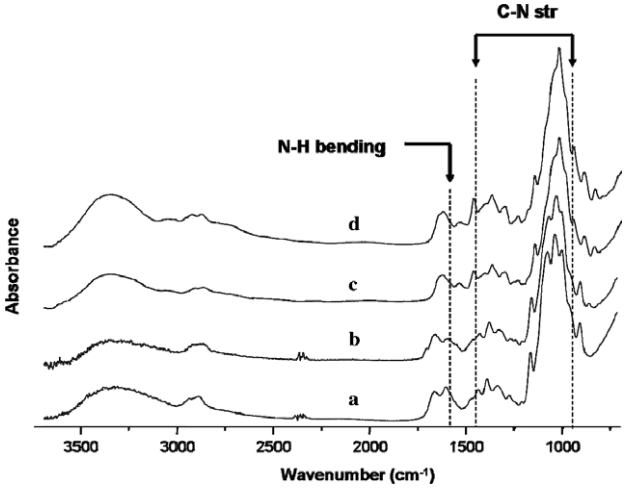


Fig. 3. ATR-FTIR spectra of (a) chitosan, and QAC films prepared by using different concentrations of MeI (M): (b) 0.4, (c) 0.8, and (d) 1.6.

carbonyl stretching (amide I) and N–H bending (amide II) of the glucosamine unit, respectively. Upon increasing the concentration of MeI, the intensity of the characteristic N–H bending peak of chitosan at 1590 cm^{-1} correspondingly decreased, whereas the intensity of the characteristic C–N stretching peaks at 1456 and 970 cm^{-1}, observed in the spectra of all QAC films, proportionally increased. ATR-FTIR spectra of SFC films prepared using different concentrations of FFSA are illustrated in Fig. 4. The presence of a signal of S=O stretching in the range of 1050–1200 cm^{-1} readily verified the success of surface sulfonation. Results from the ATR-FTIR analysis clearly indicated that both quaternization and sulfonation have proceeded to a depth of at least 1–2 μm (estimated depth of ATR-FTIR sensitivity) and the reagent concentration has a significant impact on the extent and/or the depth of surface modification.

Using methanol, which can significantly swell chitosan films as a solvent, the extent of chemical modification can be quantitatively determined by a bulk technique like ^1H NMR. From the ^1H NMR spectrum of a chitosan film after quaternization (Fig. 5b), the signal at 3.2 ppm was assigned without ambiguity to the protons of three methyl groups of the quaternary ammonium salt. Other signals were assigned after examination of the 2D homonuclear ^1H–^1H correlation (data not shown). The signals at 3.25, 2.90 and 2.65 ppm, assigned to the trimethyl ($-N^+(CH_3)_3$), dimethyl ($-NH^+(CH_3)_2$) and methyl amino groups ($-NH_2^+$ (CH_3)), respectively, are not correlated with any chitosan protons (Fig. 5a). A weak peak at 3.35 ppm was assigned to methyl protons connected to oxygen (from the hydroxyl group at the 6th position of chitosan) as a consequence of *O*-methylation. Similar peak assignments were previously reported by others (Polnok, Borchard, Verhoef, Sarisuta, & Juginger, 2004; Sieval et al., 1998). According to Fig. 5c, signals of two furan protons at 6.3 and 6.6 ppm evidently confirm the attachment of the *N*-sulfofurfuryl moiety. Furthermore, there was a new signal appearing approximately at 4.4 ppm, assigned to the two protons of the methylene group that links the amino group of chitosan to the furan ring. The analysis of the ^1H–^1H homonuclear correlation (data not shown) also supports these assignments.

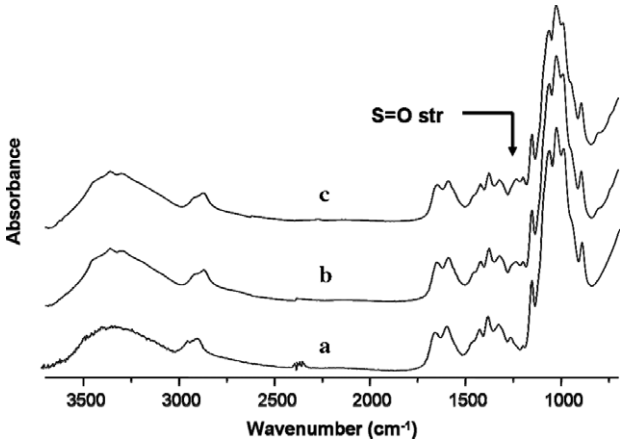


Fig. 4. ATR-FTIR spectra of (a) chitosan, and SFC films prepared by using different concentrations of FFSA (M): (b) 0.1, and (c) 0.5.

Table 1 lists degree of substitution (%DS) calculated from ^1H NMR data of QAC and SFC films. The %DS of the quaternary ammonium group was determined from the relative ratio between the peak integration of 9 protons from 3 methyl groups of the quaternary ammonium moiety and the peak integration of 5 protons of chitosan (δ 3.4–3.8 ppm). The %DS of the sulfonate group was examined from the relative ratio between the peak integration of the 2 protons of the methylene group ($-CH_2-$) that links the amino group of chitosan to the furan ring of FFSA and the peak integration of the proton H_2 of chitosan. Apparently, %DS was elevated as the concentration of the reagent (MeI or FFSA) was raised. This outcome is in good agreement with the results described earlier based upon ATR-FTIRanalysis. Of particular note, these calculated values are %DS of the whole film. Therefore, they should presumably be lower than the actual %DS in the surface region. This implication was also substantiated by the XPS analysis.

X-ray photoelectron spectroscopy was used to confirm the existence of quaternary ammonium groups on QAC films and *N*-sulfurfuryl groups on SFC films. For a chitosan film, (Fig. 6a), only signals from C_{1s} (285 eV), N_{1s} (402 eV), and O_{1s} (530 eV) are observed. After the reaction with MeI (Fig. 6b), there were peaks appearing at ~619 eV and ~630 eV which were assigned to I_{3d} (a counter ion), indicating the presence of quaternary ammonium groups on the film. The absence of the I_{3d} signal on an unmodified chitosan film (as a control surface) after exposure to NaI solution suggested that the appearance of I_{3d} on the surface of the QAC film was truly a consequence of ionic attraction between the quaternary ammonium group and the iodide counter ion, and was not caused by non-specific adsorption of iodide ions from NaI solution (data not shown). Besides signals from C_{1s}, N_{1s} and O_{1s}, the XPS spectrum of SFC film depicted in Fig. 6c reveals two additional peaks: one at 168 eV and the other at 1072 eV. The former peak, assigned to S_{2p} can be used as an indication of surface sulfonation. The latter, recognized as Na_{1s}, indicates the sodium counter ion.

Table 2 outlines XPS atomic compositions of chitosan and selected surface-modified chitosan films having the highest %DS in their series according to ^1H NMR analysis (See Table 1): 5.0% for the QAC film and 7.7% for the SFC film. The %DS of sulfonate groups on the surface of SFC film can be directly calculated from

$$\%DS = \frac{\%X/A}{\%n} \times 100$$

where $\%n$ is the percentage of nitrogen atoms of the amino group on the surface, $\%X$ is the percentage of sulfur from substituted *N*-sulfurfuryl groups on the surface, and A is the number of sulfur atoms in proportion to the

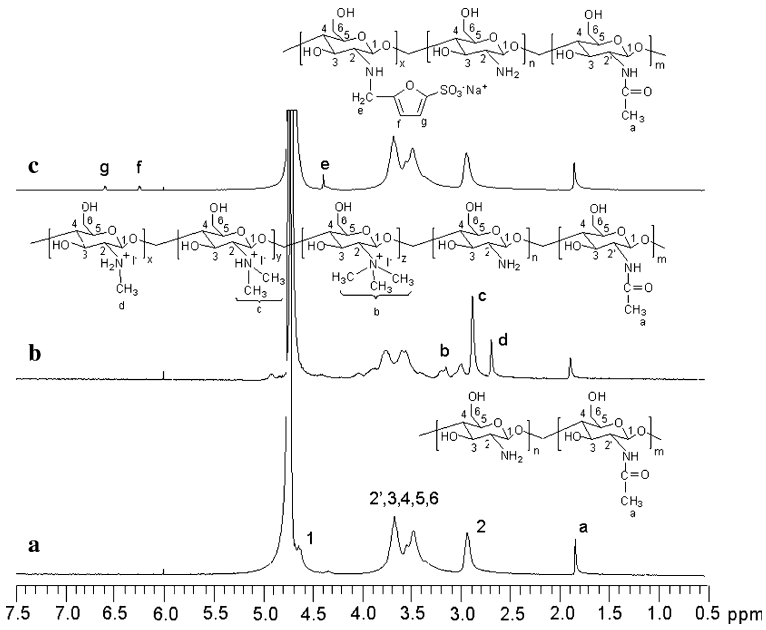


Fig. 5. ^{1}H NMR spectra of (a) chitosan film, (b) QAC film, and (c) SFC film.

Table 1
%DS of quaternary ammonium and *N*-sulfofurfuryl groups on QAC and SFC films, respectively calculated from ^{1}H NMR data

Sample	Concentration of MeI or FFSA (M)	%DS
QAC film	0.4	0.5
	0.8	3.9
	1.6	5.0
SFC film	0.05	3.1
	0.1	4.4
	0.5	7.7

number of nitrogen atoms of the amino group. Note that $\%n$ is equal to $\%N \times (\%DD/100)$, where %N is the percentage of nitrogen (from both amino and amide groups) detected on the surface by XPS, and %DD is equal to 88%.

Using the same equation, %DS of quaternary ammonium groups on the surface of QAC film can also be determined based on the assumption that the quaternary ammonium group bound stoichiometrically with its iodide counter ion. Then $\%X$ and A are alternatively defined as the percentage of iodine bound to substituted quaternary ammonium groups on the surface and the number of iodine atoms in proportion to the number of nitrogen atoms of the amino group, respectively. %DS calculated indirectly from the percentage of the counter ion may be overestimated due to the fact that not only can iodide ion bind to quaternary ammonium groups, but it can also bind to other available positively charged moieties including dimethyl and methyl amino groups. Nonetheless, the result from XPS analysis helps confirming that %DS in the surface region is intrinsically higher than the one deduced from ^{1}H NMR data.

3.2. Protein adsorption

Protein adsorption is known to be a complex phenomenon. Several interactions, namely hydrophobic interaction, hydrogen bonding, and electrostatic interaction, simultaneously take part in controlling its behavior. Although the protein adsorption on a biomaterials surface is a competitive phenomenon in the actual physiological environment which simultaneously involves multi-protein systems, understanding a response of a single protein is preliminarily necessary to gain a basic knowledge about the correlation between physical properties of the protein and its adsorption behavior. The information should be fundamentally important in apprehending the more complex systems of which many proteins exist. In particular, this research intends to find out whether electrostatic interaction can play a dominant role in controlling protein adsorption.

The proteins used in this investigation include albumin (BSA), fibrinogen (FIB), ribonuclease (RNase), and lysozyme (LYZ). All proteins are globular proteins and vary in size and charge under the experimental condition (pH 7.4 buffer). Their physical properties are outlined in Table 3. BSA and FIB were selected as models of negatively charged proteins, whereas RNase and LYZ represent proteins carrying a positive charge. Based on the assumption that the higher the extent of functional group substitution on the chitosan surface, the lower the water contact angle, a set of surface-modified chitosan films having various water contact angles was chosen for protein adsorption studies (See Table 4). It should be underlined that the protein concentration of 1 mg/mL used in adsorption studies is in the plateau region of the adsorption isotherm determined for all four proteins.

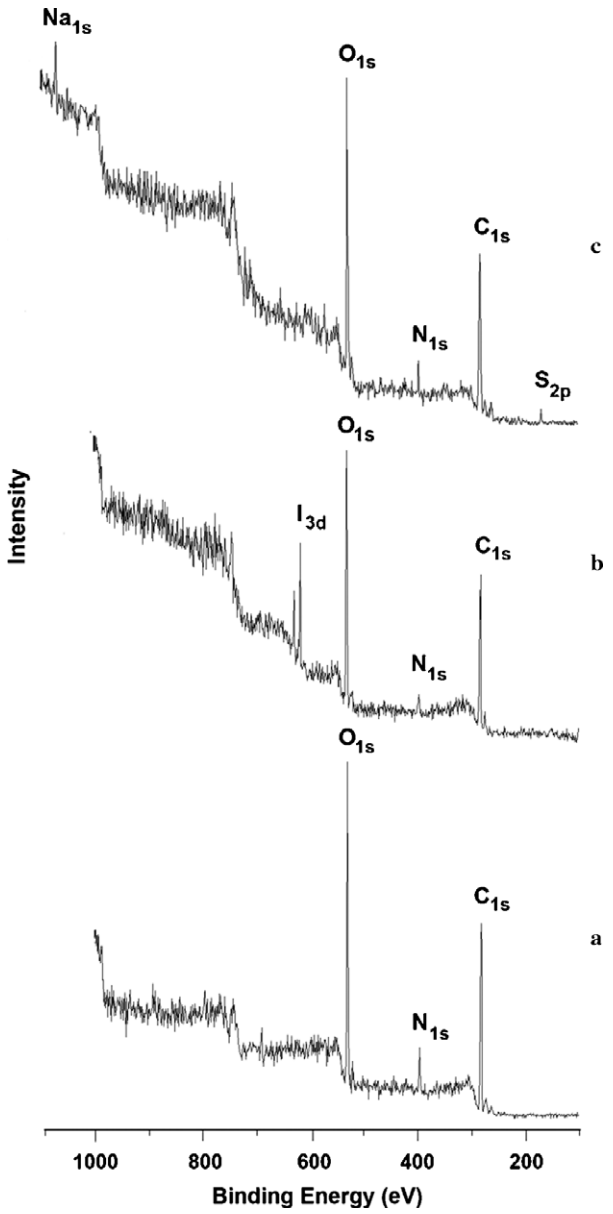


Fig. 6. XPS survey spectra of (a) chitosan film, (b) QAC film, and (c) SFC film.

Table 2
XPS atomic composition of chitosan, QAC, and SFC films

Sample	Atomic composition (%)						%DS on the surface
	C	O	N	S	Na	I	
Chitosan	69.8	25.3	4.9	–	–	–	–
QAC	63.0	28.6	6.8	–	–	1.6	26.7
SFC	62.2	29.9	4.9	0.9	2.1	–	20.9

Surface-charged chitosan particles prepared by conditions equivalent to those used for the films were subjected to zeta potential measurements in order to determine the relative charge characteristics and density of surface-charged chitosan. Zeta potential values of chitosan particles both before and after surface modification are also illustrated in Table 4. As anticipated, all surface-quaternized chitosan particles (equivalent to QAC films) possess positive zeta potential whereas all surface-sulfonated chitosan particles (equivalent to SFC films) exhibit negative zeta potential as opposed to +13.1 mV of chitosan particles. The magnitude of the zeta potential, which should correspond to the charge density, expectedly conforms to the extent of surface modification as expressed in terms of the contact angle. The higher the magnitude of the zeta potential, the lower the contact angle.

Table 3
Physical properties of proteins used for adsorption studies

Property	Albumin	Fibrinogen	Lysozyme	Ribonuclease
Mass (Da)	69,000	340,000	14,600	13,680
Isoelectric point (pH units)	4.8	5.5	11.1	9.4

Table 4
Water contact angles of SFC and QAC films used for protein adsorption studies and zeta potential values of the corresponding surface-modified chitosan particles

Sample	Symbol	Water contact angle (degrees)	Zeta potential of surface-modified chitosan particles (eV)
Chitosan film	Chi	79.6 ± 1.1	+13.10
SFC film	S1	71.1 ± 2.4	−12.85
	S2	67.8 ± 2.4	−29.57
	S3	62.0 ± 1.9	−44.06
QAC film	Q1	74.2 ± 2.2	+18.50
	Q2	63.2 ± 3.6	+25.53
	Q3	61.0 ± 1.7	+31.25

With respect to chitosan films, the adsorbed amounts of all proteins on SFC films are shown in Fig. 7. Since the SFC film bears the same charge as BSA and FIB, protein adsorption was varied as a function of the strength of ionic repulsion. Increasing the zeta potential value, which should correspond to the charge density of sulfonate groups on the surface, caused suppression of both BSA and FIB adsorption. This outcome is in accord with the results previously reported on crosslinked *N*-sulfofurfuryl chitosan membranes (Liu, Zhang, Cheng, Cao, & Yao, 2004).

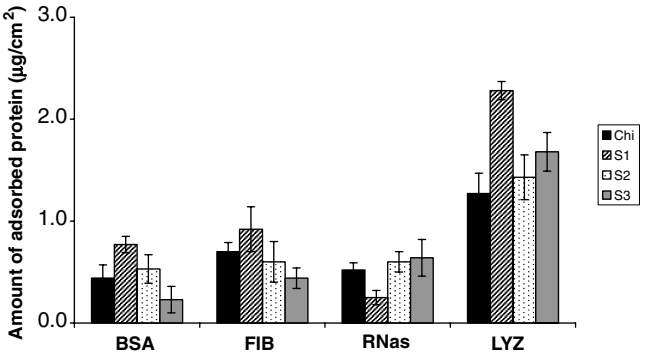


Fig. 7. Amount of adsorbed proteins on SFC films in comparison with chitosan films.

In comparison with BSA and FIB adsorption, the trend of RNase adsorption on the SFC film is reversed as a consequence of ionic attraction between the negative charge on the SFC film and the positive charge of RNase. The adsorption was promoted as a function of charge density. Considering only S2 and S3, LYZ adsorption follows the same tendency as RNase adsorption. However, S1 is capable of adsorbing a significantly larger quantity of LYZ despite its lowest charge density in the series. Taking the chitosan film as a control, the responses of S1 to other 3 proteins (BSA, FIB, and RNase) were also peculiar and did not follow the predicted behavior. S1 should adsorb a smaller quantity of BSA and FIB and a greater quantity of RNase than chitosan. Obviously, neither was the case. It is suspected that such a slight negative charge of S1 may not be sufficient for the electrostatic interaction to take a leading role in controlling protein adsorption. This unpredicted feature occurred with S2 in some cases as well. It seems that prediction with reasonable confidence can only be made in the case of S3, for which the zeta potential is the highest.

As opposed to SFC films, QAC films apparently exhibited an unusual trend of protein adsorption (Fig. 8). The adsorbed amount per unit surface area on QAC films was relatively higher than that on SFC films for all proteins, especially LYZ. A combination of its relatively low molecular weight and its enzymatic character towards chitosan is believed to be responsible for the larger quantity of adsorbed LYZ. The fact that the amount of protein adsorbed on the QAC films was inversely proportional to the water contact angle and independent of the protein charge suggests that protein adsorption was no longer primarily governed by either hydrophobic/hydrophilic interaction or ionic interaction.

Originally, it was speculated that the absorption of water soluble proteins may simultaneously took place and perhaps overshadowed the adsorption process if the films possessed high swelling ability. In fact, such a diffusion of proteins into highly swollen polymer hydrogels has been previously observed (Burczak, Fujisato, Hatada, & Ikada, 1994; Kato, Sano, & Ikada, 1995). Nonetheless, that did not seem to be a reasonable explanation considering the fact that the equilibrium swelling ratios of chitosan, Q1, Q2, and Q3 of 63%, 59%, 70%, and 153%, respectively are not so much different from those of SFC films which are in the range of 65–113%. As characterized by DSC analysis (data not shown), there are 2 states of freezing water (bound water and free water) detected in the swollen chitosan, SFC and QAC films. The same feature was also recognized in other systems of chitosan hydrogels (Qu, Wirsen, & Albertsson, 2000; Ratto, Hatakeyama, & Blumstein, 1995). We found no concrete evidence that can distinguish QAC films from SFC or virgin chitosan films in terms of the swelling ratio and the state of water. It is possible that QAC films may exhibit a unique environment that allows proteins to trap within their modified layers. So far, the existing evidences are not adequate to prove such an assumption. Detailed investigation on the conformation of the adsorbed proteins and perhaps model studies on the interactions between the proteins and these particular surface-charged chitosan films are yet to be explored before an unambiguous conclusion can be drawn.

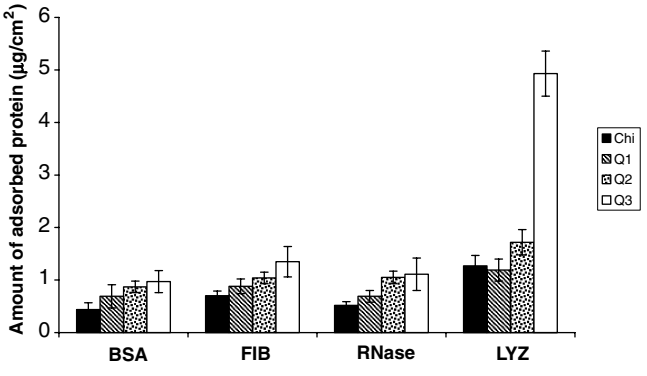


Fig. 8. Amount of adsorbed proteins on QAC films in comparison with chitosan films.

4. Conclusions

It has been demonstrated that positive or negative charges can be successfully introduced to the surface of chitosan films using heterogeneous chemical reactions. The extent of surface modification can be tailored by varying the reaction time and reagent concentration. The presence of *N*-sulfofurfuryl and quaternary ammonium groups has a remarkable impact on how the chitosan film responds to charged proteins in terms of adsorbed quantity and selectivity. It is interesting that the surface-modified chitosan films having similar wettability as judged by the contact angle analysis but distinguishable charge characteristics essentially possess different responses to proteins. The ability to sustain their charges in a broader pH range should make these surface-charged chitosan films more versatile for applications than the native chitosan films for which the charge is altered as a function of environmental pH.

Acknowledgements

This research is supported financially by a Research Team Promotion Grant from the Thailand Research Fund (RTA4780004) and a graduate research grant for Yaowamand Angkitpaiboon from Chulalongkorn University. The authors are indebted to Associate Professor Sanong Ekgasit of Chulalongkorn University for use of an ATR-FTIR facility. Appreciation is also extended to Department of Materials Science, Faculty of Science, Chulalongkorn University and Thailand's National Synchrotron Research Center for providing contact angle and XPS facilities, respectively.

References

Amiji, M. M. (1996). Surface modification of chitosan membranes by complexation-interpenetration of anionic polysaccharides for improved blood compatibility in hemodialysis. *Journal of Biomaterials Science-Polymer Edition, 8*(4), 281–298.

Amiji, M. M. (1997). Synthesis of anionic poly(ethylene glycol) derivative for chitosan surface modification in blood-contacting applications. *Carbohydrate Polymers, 32*(3–4), 193–199.

Amiji, M. M. (1998). Platelet adhesion and activation on an amphoteric chitosan derivative bearing sulfonate group. *Colloids and Surfaces B: Biointerface, 10*(5), 263–271.

Amornchai, W., Hoven, V. P., & Tangpasuthadol, V. (2004). Surface modification of chitosan films – grafting ethylene glycol oligomer and its effect on protein adsorption. *Macromolecular Symposia, 216*, 99–108.

Azad, A. K., Sermsintham, N., Chandkrachang, S., & Stevens, W. F. (2004). Chitosan membrane as a wound-healing dressing: characterization and clinical application. *Journal of Biomedical Materials Research Part B – Applied Biomaterials, 69B*(2), 216–222.

Benesch, J., & Tengvall, P. (2002). Blood protein adsorption onto chitosan. *Biomaterials, 23*(12), 2561–2568.

Burczak, K., Fujisato, T., Hatada, M., & Ikada, Y. (1994). Protein permeation through poly(vinyl alcohol) hydrogel membranes. *Biomaterials, 15*(3), 231–238.

Chen, T. H., Kumar, G., Harris, M. T., Smith, P. J., & Payne, G. F. (2000). Enzymatic grafting of hexyloxyphenol onto chitosan to alter surface and rheological properties. *Biotechnology and Bioengineering, 70*(5), 564–573.

Curti, E., de Britto, D., & Campana, S. P. (2003). Methylation of chitosan with iodomethane: effect of reaction conditions on chemoselectivity and degree of substitution. *Macromolecular Bioscience, 3*(10), 571–576.

Domard, A., Gey, C., Rinaudo, M., & Terrassin, C. (1987). ^{13}C and ^{1}H n.m.r. spectroscopy of chitosan and *N*-trimethyl chloride derivatives. *International Journal of Biological Macromolecules, 9*(4), 233–237.

Fu, J. H., Ji, J., Yuan, W. Y., & Shen, J. C. (2005). Construction of anti-adhesive and antibacterial multilayer films via layer-by-layer assembly of heparin and chitosan. *Biomaterials, 26*(33), 6684–6692.

Ho, M. H., Wang, D. M., Hsieh, H. J., Liu, H. C., Hsien, T. Y., Lai, J. Y., et al. (2005). Preparation and characterization of RGD-immobilized chitosan scaffolds. *Biomaterials, 26*(16), 3197–3206.

Ishihara, M., Nakanishi, K., Ono, K., Sato, M., Kikuchi, M., Saito, Y., et al. (2003). Photocrosslinkable chitosan as a dressing for wound occlusion and accelerator in healing process. *Biomaterials, 23*(3), 833–840.

Jia, Z. S., Shen, D. F., & Xu, W. L. (2001). Synthesis and antibacterial activities of quaternary ammonium salt of chitosan. *Carbohydrate Research, 333*(1), 1–6.

Justi, K. C., Favere, V. T., Laranjeira, M. C. M., Neves, A., & Casellato, A. (2005). Synthesis and characterization of modified chitosan through immobilization of complexing agents. *Macromolecular Symposia, 229*, 203–207.

Kato, K., Sano, S., & Ikada, Y. (1995). Protein adsorption onto ionic surfaces. *Colloids and Surfaces B: Biointerfaces, 4*(4), 221–230.

Kim, C. H., Choi, J. W., Chun, H. J., & Choi, K. S. (1997). Synthesis of chitosan derivatives with quaternary ammonium salt and their antibacterial activity. *Polymer Bulletin, 38*(4), 387–393.

Kim, C. H., & Choi, K. S. (2002). Synthesis and antibacterial activity of quaternized chitosan derivatives having different methylene spacers. *Journal of Industrial and Engineering Chemistry, 8*(1), 71–76.

Kojima, K., Okamoto, Y., Miyatake, K., Kitamura, Y., & Minami, S. (1998). Collagen typing of granulation tissue induced by chitin and chitosan. *Carbohydrate Polymers, 37*(2), 109–113.

Kojima, K., Okamoto, Y., Miyatake, K., Tamai, Y., Shigemasa, Y., & Minami, S. (2001). Optimum dose of chitin and chitosan for organization of non-woven fabric in subcutaneous tissue. *Carbohydrate Polymers, 46*(3), 235–239.

Lebouc, F., Dez, I., Desbrieres, J., Picton, L., & Madec, P. J. (2005). Different ways for grafting ester derivatives of poly(ethylene glycol) onto chitosan: related characteristics and potential properties. *Polymer, 46*(3), 639–651.

Lee, J. Y., Nam, S. H., Im, S. Y., Park, Y. J., Lee, Y. M., Seol, Y. J., et al. (2002). Enhanced bone formation by controlled growth factor delivery from chitosan-based biomaterials. *Journal of Controlled Release, 78*(1–3), 187–197.

Li, J., Liu, Q., Liu, Y. J., Liu, S. C., & Yao, S. Z. (2005). DNA biosensor based on chitosan film doped with carbon nanotubes. *Analytical Biochemistry, 346*(1), 107–114.

Li, Y. P., Liu, L., & Fang, Y. E. (2003). Plasma-induced grafting of hydroxyethyl methacrylate (HEMA) onto chitosan membranes by a swelling method. *Polymer International, 52*(2), 285–290.

Lim, S. H., & Hudson, S. M. (2004). Synthesis and antimicrobial activity of a water-soluble chitosan derivative with a fiber-reactive group. *Carbohydrate Research, 339*(2), 313–319.

Lin, C. W., & Lin, J. C. (2001). Surface characterization and platelet compatibility evaluation of surface-sulfonated chitosan membrane. *Journal of Biomaterials Science-Polymer Edition, 12*(5), 543–557.

Liu, W. G., Zhang, J. R., Cao, Z. Q., Xu, F. Y., & Yao, K. D. (2004). A chitosan-arginine conjugate as a novel anticoagulation biomaterial. *Journal of Materials Science-Materials in Medicine, 15*(11), 1199–1203.

Liu, W. G., Zhang, J. R., Cheng, N., Cao, Z. Q., & Yao, K. D. (2004). Anticoagulation activity of crosslinked *N*-sulfofurfuryl chitosan membranes. *Journal of Applied Polymer Science, 94*(1), 53–56.

Mi, F. L., Shyu, S. S., Wu, Y. B., Lee, S. T., Shyong, J. Y., & Huang, R. N. (2001). Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing. *Biomaterials, 22*(2), 165–173.

Okamoto, Y., Yano, R., Miyatake, K., Tomohiro, I., Shigemasa, Y., & Minami, S. (2003). Effects of chitin and chitosan on blood coagulation. *Carbohydrate Polymers, 53*(3), 337–342.

Polnok, A., Borchard, G., Verhoef, J. C., Sarisuta, N., & Juginger, H. E. (2004). Influence of methylation process on the degree of quaternization of *N*-trimethyl chitosan chloride. *European Journal of Pharmaceutics and Biopharmaceutics, 57*(1), 77–83.

Qi, L., Xu, Z., Jiang, X., Hu, C., & Zou, X. (2004). Preparation and antibacterial activity of chitosan nanoparticles. *Carbohydrate Research, 339*(16), 2693–2700.

Qu, X., Wirsen, A., & Albertsson, A.-C. (2000). Novel pH-sensitive chitosan hydrogels: swelling behavior and states of water. *Polymer, 41*(12), 4589–4598.

Rabea, E. I., Badawy, M. E.-T., Stevens, C. V., Smagghe, G., & Steurbaut, W. (2003). Chitosan as antimicrobial agent: applications and mode of action. *Biomacromolecules, 4*(6), 1457–1465.

Ratto, J., Hatakeyama, T., & Blumstein, R. B. (1995). Differential scanning calorimetry investigation of phase transition in water/chitosan systems. *Polymer, 36*(15), 2915–2919.

Seong, H. S., Whang, H. S., & Ko, S. W. (2000). Synthesis of a quaternary ammonium derivative of chito-oligosaccharide as antimicrobial agent for cellulosic fibers. *Journal of Applied Polymer Science, 76*(14), 2009–2015.

Sieval, A. B., Thanou, M., Kotze, A. F., Verhoef, J. C., Brussee, J., & Junginger, H. E. (1998). Preparation and NMR characterization of highly substituted *N*-trimethyl chitosan chloride. *Carbohydrate Polymers, 36*(2–3), 157–165.

Suzuki, K., Oda, D., Shinobu, T., Saimoto, H., & Shigemasa, Y. (2000). New selectively *N*-substituted quaternary ammonium chitosan derivatives. *Polymer Journal, 32*(4), 334–338.

Tangpasuthadol, V., Pongchaisirikul, N., & Hoven, V. P. (2003). Surface modification of chitosan films, effect of surface hydrophobicity on protein adsorption. *Carbohydrate Research, 338*(9), 937–942.

Tsubokawa, N., & Takayama, T. (2000). Surface modification of chitosan powder by grafting of 'dendrimer-like' hyperbranched polymer onto the surface. *Reactive and Functional Polymers, 43*(3), 341–350.

Vongchan, P., Sajomsang, W., Subyen, D., & Kongtawelert, P. (2002). Anticoagulant activity of sulfated chitosan. *Carbohydrate Research, 337*(13), 1239–1242.

Wang, H. T., Fang, Y. E., & Yan, Y. S. (2001). Surface modification of chitosan membranes by alkane vapor plasma. *Journal of Materials Chemistry, 11*(5), 1374–1377.

Wang, Y. C., Kao, S. H., & Hsieh, H. J. (2003). A chemical surface modification of chitosan by glycoconjugates to enhance the cell-biomaterial interaction. *Biomacromolecules, 4*(2), 224–231.

Wang, Y. C., Lin, M. C., Wang, D. M., & Hsieh, H. J. (2003). Fabrication of a novel porous PGA-chitosan hybrid matrix for tissue engineering. *Biomaterials, 24*(6), 1047–1057.

Yamamoto, H., Kuno, Y., Sugimoto, S., Takeuchi, H., & Kawashima, Y. (2005). Surface-modified PLGA nanosphere with chitosan improved pulmonary delivery of calcitonin by mucoadhesion and opening of the intercellular tight junctions. *Journal of Controlled Release, 102*(2), 373–381.

Zheng, L.-Y., & Zhu, J.-F. (2003). Study on antimicrobial activity of chitosan with different molecular weights. *Carbohydrate Polymers, 54*(4), 527–530.

Zhu, A. P., Wang, S. Q., Yuan, Y. L., & Shen, J. (2002). Cell adhesion behavior of chitosan surface modified by bonding 2-methacryloyloxy-ethyl phosphorylcholine. *Journal of Biomaterials Science-Polymer Edition, 13*(5), 501–510.

Zhu, A. P., Zhang, M., Wu, J., & Shen, J. (2002). Covalent immobilization of chitosan/heparin complex with a photosensitive hetero-bifunctional crosslinking reagent on PLA. *Biomaterials, 23*(23), 4657–4665.

Zhu, X., Chian, K. S., Chan-Park, M. B. E., & Lee, S. T. (2005). Effect of argon-plasma treatment on proliferation of human-skin-derived fibroblast on chitosan membrane in vitro. *Journal of Biomedical Materials Research Part A, 73A*(3), 264–274.