Surface Modification of Chitosan Films-Grafting Ethylene Glycol Oligomer and Its Effect on Protein Adsorption

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Summary: This work is a part of a series on surface modification of materials made of chitosan. This report focused on grafting monomethoxy ethylene glycol oligomers (mPEG) on the surface of chitosan films. The chemical reactions were performed by immersing the films in organic solvent containing aldehyde derivative of mPEG. The presence of ethylene glycol moieties was determined by attenuated total reflectance-infrared spectroscopy (ATR-IR) and nuclear magnetic resonance (NMR). The hydrophobicity of the modified surface, determined by air-water contact angle, decreased when the ethylene glycol derivatives were grafted on the film. The modified films were also subjected to protein adsorption study in order to assess their uses in biomedical applications. It was found that the presence of ethylene glycol units reduced the adsorption of proteins (albumin and lysozyme) on the films. We therefore have shown that manipulation of the interaction between chitosan and bio-macromolecules is possible by chemically modifying the surface of chitosan.

Keywords: biomaterials; chitosan; poly(ethylene glycol); protein adsorption; surface modification

Introduction

Surface modification of biomaterials has been a main interest for many years, since it is the surface of these materials that first comes into contact with the biological surrounding. A number of techniques have been used to alter the chemical composition and thus, the surface property of the materials. Some of the methods include plasma treatment,[1] blending with other macromolecules,[2,3] and immobilizing small or large molecules on the surface.[4-6] The change of surface property was found to affect adsorption of platelets[2] cells[7-9] and biomacromolecules, such as, proteins,[9-12] on the polymer surface. Chitosan is naturally originated and characterized as non-toxic biomaterials with good biocompatibility. The

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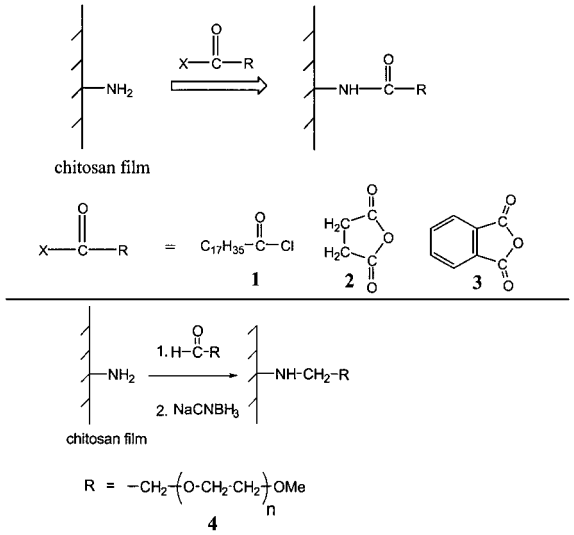
structure of chitosan contains a large number of hydroxy and amino groups , it can thus be modified by various chemical reactions. It can be hypothesized that the reactions of chitosan films with certain molecules having different chain length and flexibility are able to modify the surface properties of the films, and hence, affect the interaction between chitosan and bio-macromolecules. We have reported earlier[13] methods for grafting hydrocarbon molecules onto the chitosan film. Stearoyl chloride, succinic anhydride, and phthalic anhydride were covalently linked to the amino groups of chitosan, i.e. by amide bond (See Scheme 1). It was found that the type of molecules formed on the surface could somewhat affected the amount of BSA (bovine serum albumin) and lysozyme adsorbed on the films.



Scheme 1. Surface modification of chitosan films by the reaction of chitosan and various compounds.

Poly(ethylene glycol) (PEG) has been well recognized as one of a polymeric component that can effectively suppress non-specific protein adsorption. The ability to repel protein is believed to originate from the large excluded volume of the hydrated PEG and its chain mobility. In order to extent the use of chitosan in the biomedical applications in which non-fouling is critically required, monomethoxy poly(ethylene glycol)s [$CH_3O(CH_2CH_2O)_nH$ or mPEG] in the form of aldehyde derivatives were grafted onto the chitosan films. Ethylene glycol oligomers having the molecular weight of 550 (n = 12) and 164 (n = 3) were used in

this study. The films before and after modification were characterized by ATR-IR and NMR for functional group analysis, and by water contact angle measurement for determining surface hydrophilicity. Protein adsorption study was also performed in order to investigate the reduction of protein fouling.

Experimental

Materials

Oxalyl chloride, sodium cyanoborohydride were purchased from Fluka Chemika. Bovine serum albumin, lysozyme, bicinchoninic assay kit, phosphate buffer saline (PBS), and triethylamine (TEA) were purchased from Aldrich Chemical Co. Monomethoxy poly(ethylene glycol) (mPEG) (M_w = 550) and monomethoxy triethylene glycol (mTEG) (M_w = 164) were obtained from Fluka Chemika. Chitosan (M_v = 645,000; 87%DD) was purchased from Seafresh Chitosan (Lab) Co., Ltd., Thailand.

Preparation of Chitosan Films

Chitosan (2 g) was dissolved in 0.1 M acetic acid (100 mL). After stirring for 24 h, the solution was filtered through a medium pore size sintered glass to remove insoluble substances. The solution was then poured into a Teflon-coated mold. Solvent was allowed to evaporate in air for 4 days. Then the film was washed with 0.1 M NaOH/methanol (1:1) and methanol/water (1:1) to neutralize the acid. The final drying step was carried out under vacuum. Film thickness was between 40 to 100 μm.

Synthesis of mTEG-ald

To the solution of oxalyl chloride (35 mmol) in CH_2Cl_2, DMSO (70 mmol) in CH_2Cl_2 were carefully added under N_2 and cooled in a dry-ice-acetone bath. The solution was stirred for 5 min. Then a solution of mTEG (29.7 mmol) in CH_2Cl_2 was added dropwise. The mixture was stirred for 3 h. TEA (144 mmol) was added dropwise over a period of 20 min. The reaction mixture was left for 30 min at –78 °C and then allowed to reach room temperature. The crude product was concentrated by rotary evaporator to become viscous and colorless liquid. yield: 60% ^{1}H NMR δ 3.1 (3H, s, OCH_3), 3.3-3.4 (8H, t, OCH_2CH_2), 3.9 (2H, s, $C\underline{H}_2CHO$), 9.6 (1H, s, CHO).

Synthesis of mPEG-ald

The same oxidation procedure for mPEG was similar to the one used for synthesizing mTEG-ald. Viscous and colorless liquid was obtained after the crude mixture was concentrated by a rotary evaporator. yield: 50% ^{1}H NMR δ 2.9 (3H, s, OCH_3), 3.1-3.3 (40H, m, OCH_2CH_2), 3.8 (2H, s, $C\underline{H}_2CHO$), 9.6 (1H, s, CHO).

Grafting of mTEG and mPEG on Chitosan Films (4)

A solution of mTEG-aldehyde or mPEG aldehyde (aldehyde eq. = 10 folds of $-NH_2$ units of chitosan) was dissolved in methanol and was added into a flask containing chitosan films. The mixture was stirred for 30 min at rt. Sodium cyanoborohydride (10 eq.) in 5 mL methanol was added to the reaction mixture dropwise for 20 min. The solution was stirred for 3 more days at rt. Chitosan films were later washed in methanol, and dried in vacuo. The second set of experiment was carried out by increasing the ratio of glucosamine unit:-aldehyde:$NaCNBH_3$ from 1:10:10 to 1:30:30.

ATR-FTIR Analysis

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

Air-Water Contact Angle Measurement

A goniometer model CAM-PLUS MICRO was used to measure air-water contact angle in a static mode. All measurements were performed at 22-25 °C. The reported data were averages of 8 measurements.

Protein Adsorption Study

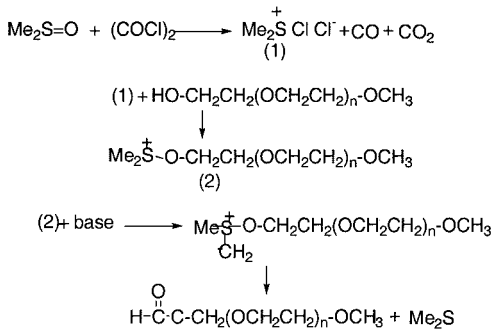
Film substrates were pre-immersed in PBS solution for 2 h. The adsorption experiments were

performed in polyethylene bottles, by immersing the film in the protein solution and incubated at 37 °C. After 3 h, the films were removed and rinsed with 10 mL buffer 4 times. Each film was then immersed in 1.0 wt% sodium dodecyl sulfate (SDS) for 1 h at rt, followed by sonication for 10 min to remove the adsorbed protein. Micro-bicinchoninic acid (BCA) protein assay was utilized to determine the amount of protein, using a UV-spectrometer with microtiter plate reader (model Sunrise; Tecan Austria GmbH) at a wavelength of 562 nm.[14]

Results and Discussion

Synthesis of Ethylene Glycol Aldehyde Derivatives

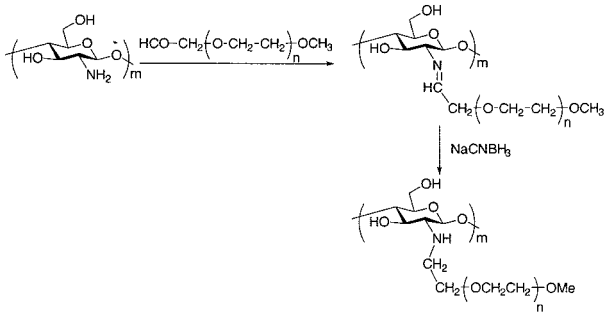
DMSO-oxalyl chloride (called *Swern Oxidation*) is the most widely used DMSO-based reagents for the oxidation of primary and secondary alcohols to aldehydes and ketone, respectively. It usually gives excellent yields with short times and minimal formation of byproduct. The conversion of the terminal hydroxyl group to an aldehyde was determined by the presence of aldehyde proton signal at 9.6 ppm using ^{1}H-NMR spectroscopy. The yields were found to be 60% for mTEG and 50/% for mPEG. The presence of trace amount of water in the ethylene glycol oligomers is believed to retard the oxidation. It should be noted here that the oxidized product could not be removed from the reaction mixture. Therefore, both ethylene glycol reactant and the aldehyde product were present during the grafting step.



Scheme 2. Swern oxidation of mTEG to mTEG-aldehyde.

Grafting of Ethylene Glycol on the Chitosan Films

The reductive alkylation of chitosan with mTEG-ald or mPEG-ald in the presence of $NaCNBH_3$, a reducing agent, is shown in Scheme 3.



Scheme 3. Grafting of monomethoxy ethylene glycol aldehyde on chitosan.

ATR-IR was used to characterize the functional groups on the surface of chitosan films (with the sampling depth of ~ 1 μm) before and after modification. In Fig. 1A, the non-modified chitosan film showed signals at 1650 and 1590 cm^{-1} for the C-O stretching (amide) and N-H bending (amine) respectively. Analysis of the modified films (Fig. 1B and 1C) revealed a slightly more intense signal of C-H deformation at 1450 cm^{-1} than the non-modified sample. The slight increase of the 1450 signal could be due to the presence of ethylene glycol segments attached to the film surface. However, the differences between the non-modified and modified films are not conclusive. This could be due to the fact that the chemical structure of chitosan is very similar to the ethylene glycol units.

In order to obtain more evidences of the grafting, we analyzed the modified chitosan by ^{1}H-NMR (Fig. 2). The signals at 2.4 and 3.1 ppm indicated $-CH_2-$ unit of ethylene glycol that was immediately next to –NH- and $-NH_2^+-$ of chitosan, respectively. An appearance of these signals suggests that the modification proceeds to a significant depth from the surface so that the attachment can be demonstrated by NMR, which is a bulk characterization technique.

Surface Hydrophilicity

Air–water contact angle measurements were used to determine the hydrophilicity of the film surface. In general, the water contact angle of a hydrophobic surface is higher than that of the hydrophilic surface. From Table 1, the hydrophilicity increased as expected after the chitosan films reacted with the hydrophilic mTEG-ald and mPEG-ald. It seems that increasing the molecular weight and mole equivalent of ethylene glycol units do not affect the hydrophilicity of the films.

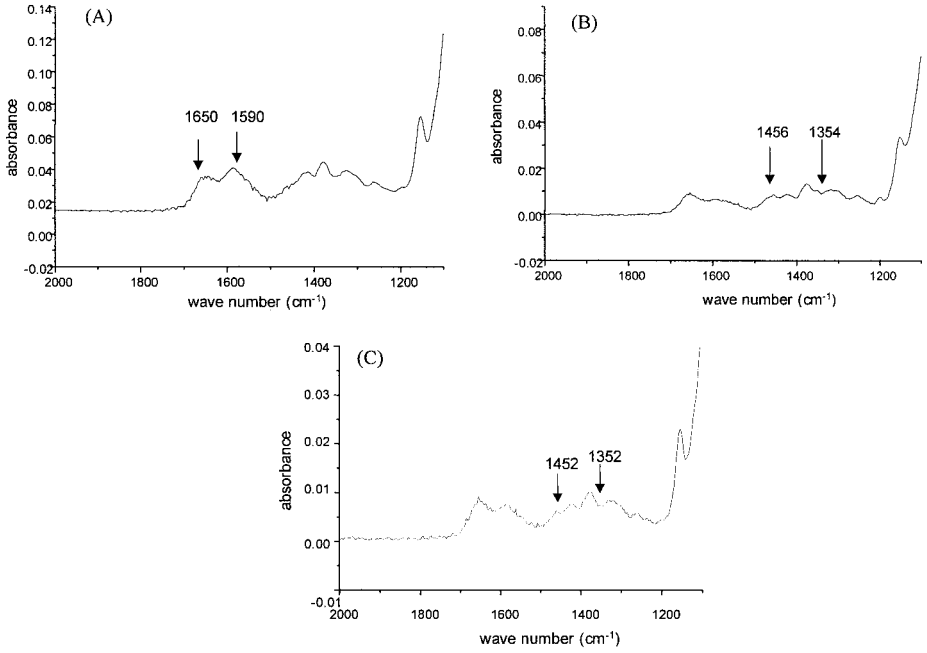


Figure 1. ATR-IR spectra of (A) unmodified chitosan film, (B) mTEG-grafted chitosan film, and (C) mPEG-grafted chitosan film.

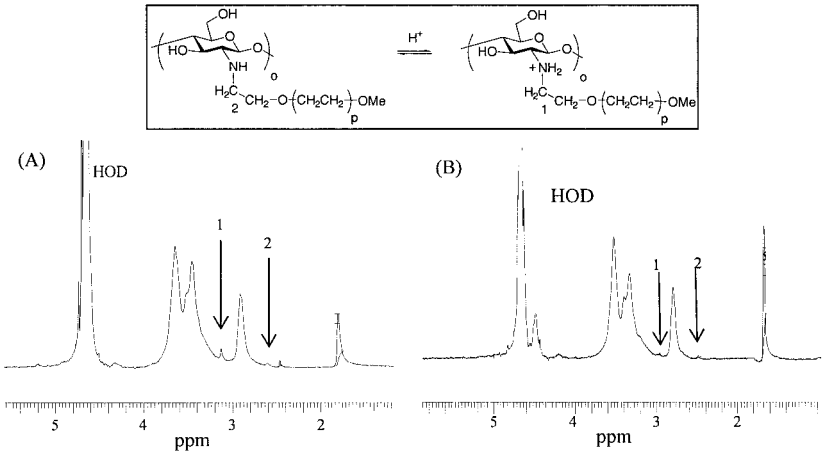


Figure 2. ^{1}H NMR spectra of modified chitosan films after grafting with mPEG-ald (A) and mTEG-ald (B) (solvent: 1% CD_3COOD in D_2O, 25 °C).

Table 1. Air-water contact angle of chitosan films and modified chitosan films (8 repetitions).

Film samples	Solvent	Contact angle (degree)
Chitosan	-	78.4 ±3.2
Chitosan:mTEG-ald:$NaCNBH_3$		
1:10:10	MeOH	58.4 ±2.9
1:30:30	MeOH	64.1 ± 2.6
Chitosan:mPEG-ald:$NaCNBH_3$		
1:10:10	MeOH	59.3 ± 2.5
1:30:30	MeOH	60.7 ± 1.4

Protein Adsorption Study

Albumin and lysozyme were the two proteins chosen for adsorption study on the chitosan films. Both proteins are model globular proteins that vary in size and charge as well as conformational stability under the experimental condition (pH 7.4 buffer). Bicinchoninic acid assay was used to measure the amount of protein.

In Table 2, it was found that PEG molecule was able to reduce the amount of protein adsorbed on the films. This finding agreed well with previous reports,[15, 16] which indicated that the flexible ethylene glycol units could hinder the approaching protein molecules.

Table 2. The amount of proteins adsorbed on the unmodified and modified chitosan films.

Film samples	Amount of Albumin ($\mu g/cm^2$)			Amount of Lysozyme ($\mu g/cm^2$)		
	Set I	*Set II*	Average	*Set I*	*Set II*	Average
Chitosan	*1.60*	*1.53*	1.57	*2.81*	*2.19*	2.86
Chitosan-g-mTEG	*0.36*	*0.42*	0.39	*1.28*	*0.34*	0.81
Chitosan-g-mPEG	*0.84*	*0.71*	0.78	*2.34*	*1.65*	2.00

In conjunction with our previous study on surface-modified chitosan,[13] we have found that choices of chemical compounds attached to chitosan could affect its response to protein adsorption as follow

- Hydrophobic (N-stearoyl chitosan) surface enhances protein adsorption.
- Imide bond on chitosan surface could be hydrolyzed to produce carboxylic groups which could interact differently to proteins having different isoelectric points.
- Ethylene glycol oligomer units grafted on chitosan surface could reduce protein adsorption on the film.

Conclusion

In this study, chitosan films were modified by immersing the solvent-cast films in a solution containing aldehyde derivatives of monomethoxy ethylene glycol oligomers. The presence of ethylene glycol on the films were monitored by ATR-IR and ^{1}H-NMR. The PEG-grafted films became more hydrophilic than the original chitosan. Protein fouling on the film also decreased when ethylene glycol oligomers were attached. We have proved here that the surface of chitosan materials can be modified by attaching a certain chemical group in order to manipulate its response to bio-macromolecules, such as proteins that are commonly found in blood of human and animals.

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