CHAPTER III

RESULTS AND DISCUSSION

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

%DD is determined from the ratio of glucosamine and *N*-acetyl glucosamine units ^{1}H NMR was used to determine %DD of chitosan (Figure 3.1). The signal for $-CH_3$ of *N*-acetyl glucosamine units at δ 2.94 ppm and the proton at C-2 of glucosamine units at δ 1.84 ppm were integrated to determine %DD (Table 3.1).

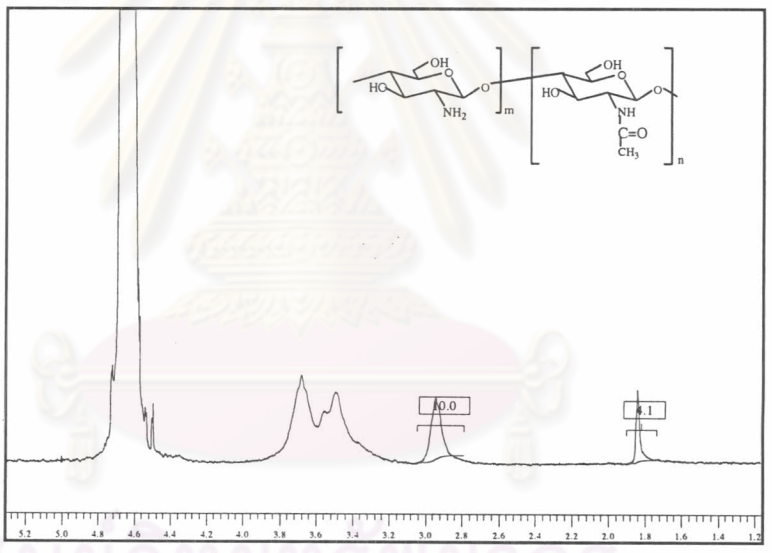


Figure 3.1 ^{1}H NMR spectrum of chitosan from Seafresh Chitosan (Lab) Co., Ltd. (solvent: 1% CD_3COOD in D_2O, 25 °C)

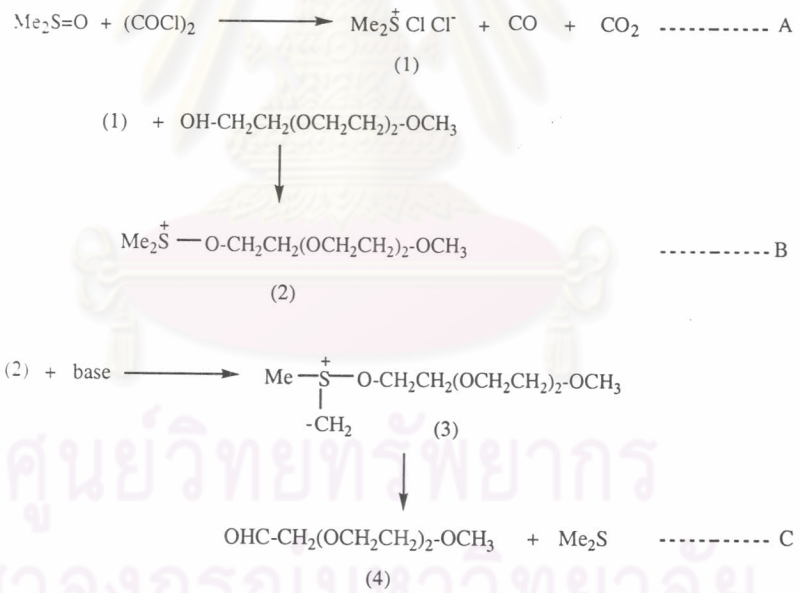
Table 3.1 Information from ^{1}H NMR spectroscopy of chitosan from Seafresh Chitosan (Lab) Co., Ltd.

	δ ppm	Integration	Amount of units in chitosan
-C<u>H</u>NH_2 of GlcN	2.48	10	10
-C<u>H</u>$_3$ of GlcNAc	1.82	4.1	4.1/3

From the data in Table 3.1, %DD could be calculated as follows. The total amount of GlcN and GlcNAc units, in chitosan, are equal to $10+\left(\frac{4.1}{3}\right)= 11.37$ units. If the total repeating units in chitosan are 100%, thus %DD is $\frac{10\times100}{10+\left(\frac{4.1}{3}\right)}$ = 87.95%.

3.2 Synthesis of MTEG-ald and MPEG-ald

3.2.1 Preparation of MTEG-ald



Scheme 3.1 Swern oxidation of MTEG

DMSO-oxalyl chloride 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 active reagent (1) is generated *in situ* at low temperature by the addition of DMSO to $(COCl)_2$ in a solvent such as CH_2Cl_2, ether, or THF (eq A).

Addition of the alcohol to (1) gives the alkoxysulfonium ion (2) (eq B), which on addition of an amine base is deprotonated to (3). The latter forms the carbonyl product (4) by intramolecular proton abstraction (eq C).

In a typical procedure using TEA as base, DMSO (2.4 equiv) is added to $(COCl)_2$ (1.2 equiv) in CH_2Cl_2 cooled to –78 °C, and then MTEG (1 equiv) is added, followed by TEA (5 equiv). The conversion or degree of oxidation from hydroxy group to aldehyde group was estimated by ^1H NMR as 60 %.

3.2.2 Preparation of MPEG-ald

From Scheme 3.1, using MPEG as primary alcohol. DMSO (8.5 equiv) is added to $(COCl)_2$ (4 equiv) in CH_2Cl_2 cooled to –78 °C, and then MPEG (1 equiv) is added, followed by TEA (15 equiv). The conversion or degree of oxidation from hydroxy group to aldehyde group was estimated by ^1H NMR as 50 %.

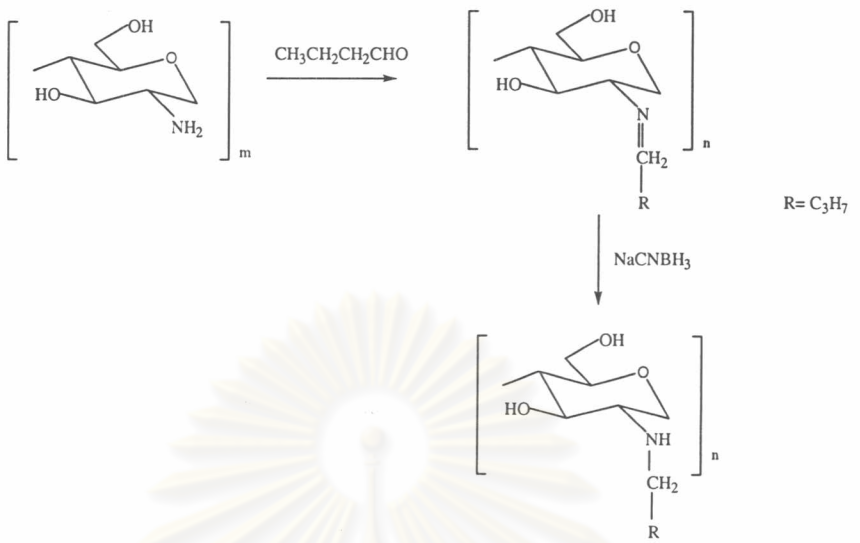
3.3 Reductive alkylation of chitosan

In this section the chemical modification of chitosan was divided into 2 parts. The first part focused on the reaction of chitosan using chitosan solution (1% acetic acid), thus called "homogeneous state". The second part covered the reaction of chitosan films with butyraldehyde and EG-derivatives, thus called "heterogeneous state". The study in homogeneous state was designated as a model study for the modification of chitosan films.

3.3.1 Grafting of chitosan in the homogenous state

3.3.1.1 Reaction with butyraldehyde (homogeneous state)

The reductive alkylation of chitosan with butyraldehyde in presence of an appropriate reducing agent ($NaCNBH_3$) is shown in Scheme 3.2.



Scheme 3.2 Chitosan grafting with butyraldehyde (homogeneous state)

The IR spectrum of unmodified chitosan showed amide-I bond (1650 cm^{-1}) and N-H bending (1598 cm^{-1}) (Figure 3.2). After grafting with butyraldehyde, the signals for C-H deformation (CH_2 (s), CH_3 (as),) at 1465 cm^{-1}, C-H deformation (CH_3 (s)) at 1377 cm^{-1} and C-H stretching at 2876 cm^{-1} increased significantly when compared to signals from other functional groups (Figure 3.3). The increased signals of alkyl unit were believed to be due to the butyl group.

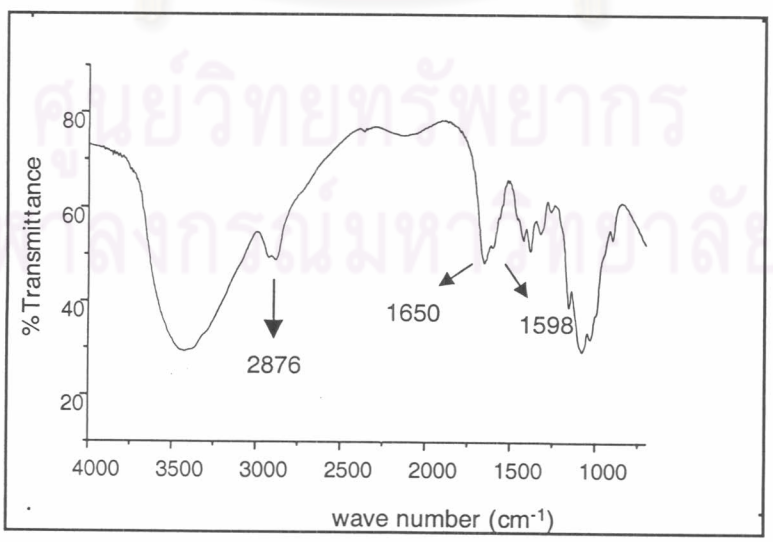


Figure 3.2 FT-IR spectrum of unmodified chitosan

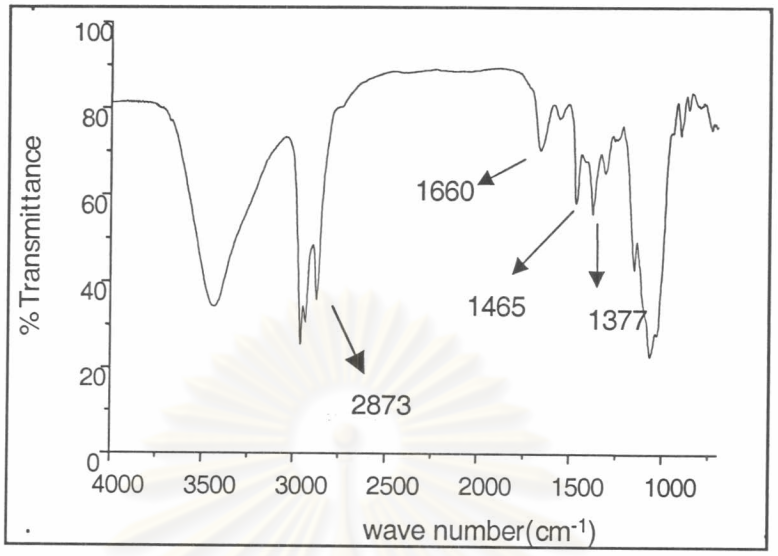


Figure 3.3 FT-IR spectrum of chitosan by grafting with butyraldehyde

From Figure 3.4, the bond between chitosan and butyraldehyde, -NH-C$\underline{H}_2$-CH_2- groups were identified by ^{1}H NMR peak around 3.3-4.4 ppm. From the data in Table 3.2, %degree of substitution (% DS) of *n*-butyl chitosan is 29.82 %.

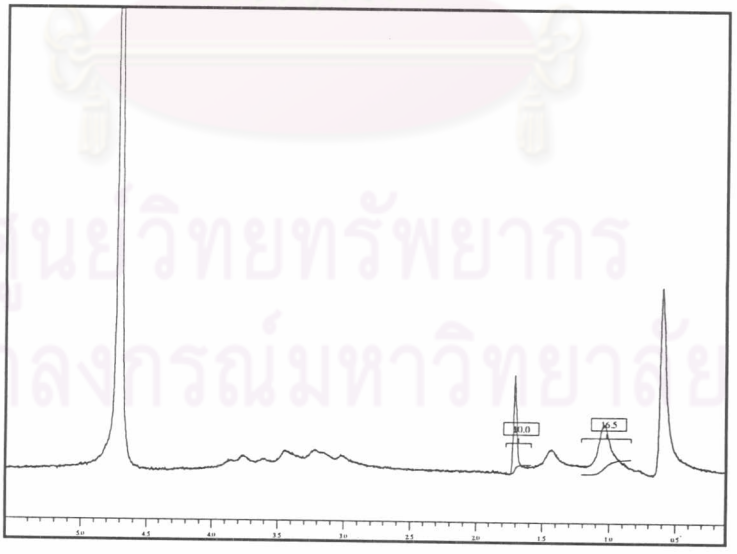


Figure 3.4 ^{1}H NMR spectrum of *n*-butyl chitosan (solvent: 1% CD_3COOD in D_2O, 25 °C)

Table 3.2 Information obtained from ^{1}H NMR spectrum of *n*-butyl chitosan

	δ ppm	Integration
CH_3-$C\underline{H}_2$-CH_2 of butyraldehyde	1.11	16.5/2
-$C\underline{H}_3$ of GlcNAc	1.77	10/3

3.3.1.2 Reaction with MTEG-ald (homogenous state)

Chitosan was reacted with the monoaldehyde derivative of MTEG under homogeneous state in an aqueous acetic acid/methanol solution. On addition of sodium cyanoborohydride, the solution became gels. The resulting mixture was dialyzed against deionized water to give clear solutions, which were then concentrated and washed with acetone and CH_2Cl_2.

The IR spectrum showed new peaks at 1458 cm^{-1} for C-H deformation ($CH_{2\,(s)}$, $CH_{3\,(as)}$), and at 1353 cm^{-1} for C-H deformation ($CH_{3\,(s)}$). In addition, the signal for C-H stretching at 2883 cm^{-1} increased when compared to other peaks.

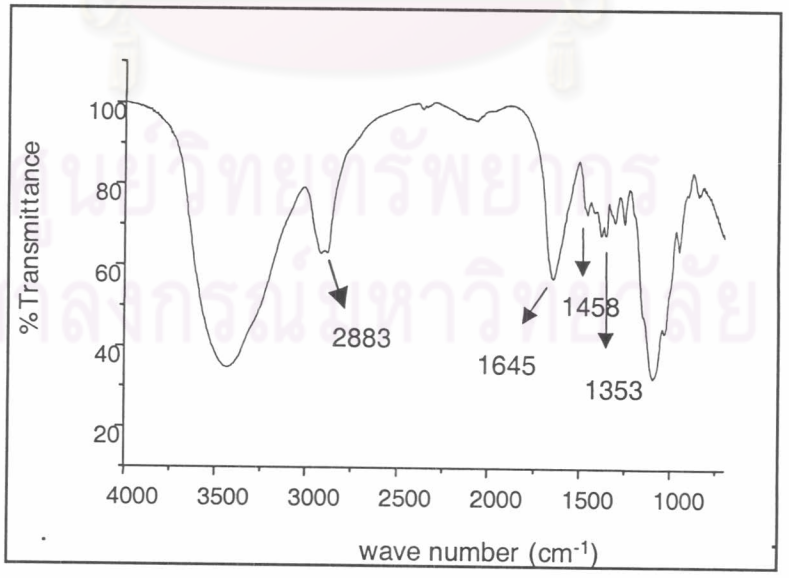


Figure 3.5 FT-IR spectrum of chitosan by grafting with MTEG-ald

From Figure 3.6, the bond between chitosan and MTEG-ald, -NH-C<u>H</u>$_2$-CH$_2$-O- groups were identified by ^{1}H NMR peak at 2.45 ppm.[27] From the data in Table 3.3, %degree of substitution (%DS) of chitosan-g-MTEG is 5.65 %.

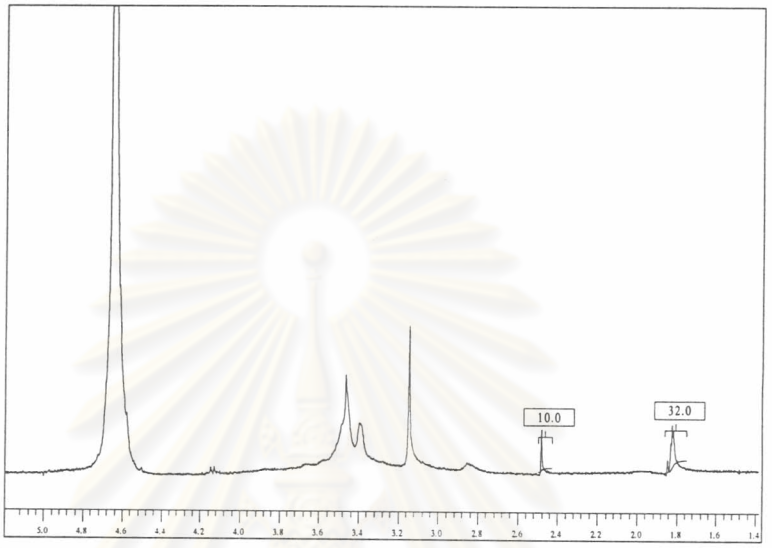


Figure 3.6 ^{1}H NMR spectrum of chitosan-g-MTEG (solvent: 1% CD_3COOD in D_2O, 25 °C)

Table 3.3 Information obtained from ^{1}H NMR spectrum of chitosan-g-MTEG

	δ ppm	**Integration**
-C<u>H</u>$_2$NH- of GlcN	2.48	10/2
-C<u>H</u>$_3$ of GlcNAc	1.82	32/3

3.3.1.3 Reaction with MPEG-ald (homogeneous state)

The IR spectrum showed new peaks at 1464 cm^{-1} for C-H deformation (CH_2 (s), CH_3 (as)) and at 1351 cm^{-1} for C-H deformation (CH_3 (s)). In addition, the signal for C-H stretching at 2878 cm^{-1} when compared to other peaks.

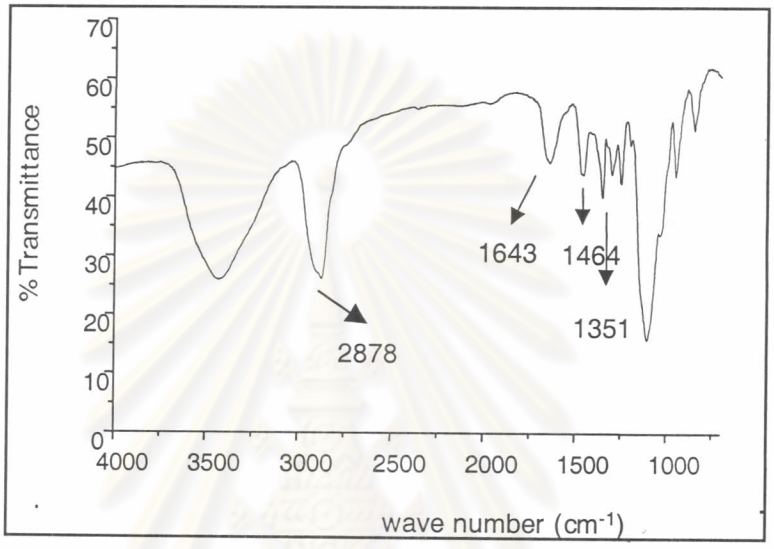


Figure 3.7 FT-IR spectrum of chitosan by grafting with MPEG-ald

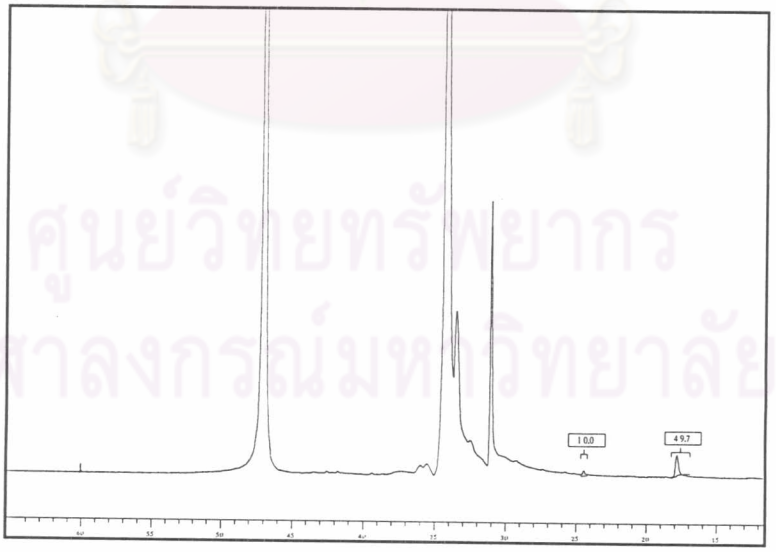


Figure 3.8 ^1H NMR spectrum of chitosan-g-MPEG (solvent: 1% CD_3COOD in D_2O, 25 °C)

From Figure 3.8, the bond between chitosan and MPEG-ald, -NH-C$\underline{H}_2$-CH_2-O- groups were identified by ^{1}H NMR peak at 2.44 ppm. From the data in Table 3.4, %degree of substitution (%DS) of chitosan-g-MPEG is 3.64 %.

Table 3.4 Information obtained from ^{1}H NMR spectrum of chitosan-g-MPEG.

	δ ppm	**Integration**
-C$\underline{H}_2$NH- of GlcN	2.44	10/2
-C$\underline{H}_3$ of GlcNAc	1.84	49.7/3

3.3.2 Grafting of chitosan films

3.3.2.1 Reaction with butyraldehyde (heterogeneous state)

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. Absorption peaks at *ca.* 1650 and 1590 cm^{-1} were assigned to the carbonyl stretching of secondary amides (amide I band), N-H bending vibrations of 2-aminoglucosamine primary amines.

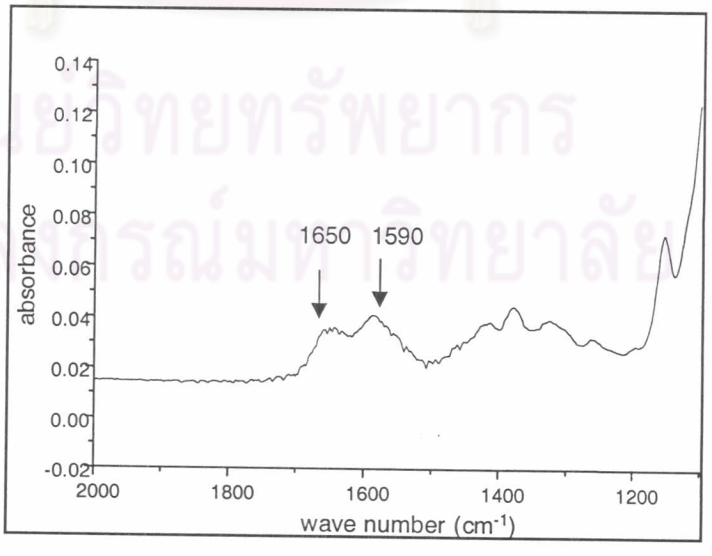


Figure 3.9 ATR-IR spectrum of non-modified chitosan films

Modified chitosan showed new peaks at 1463 cm^{-1} for the C-H deformation ($CH_{2\,(s)}$, $CH_{3\,(as)}$) and at 1379 cm^{-1} for the C-H deformation ($CH_{3\,(s)}$).

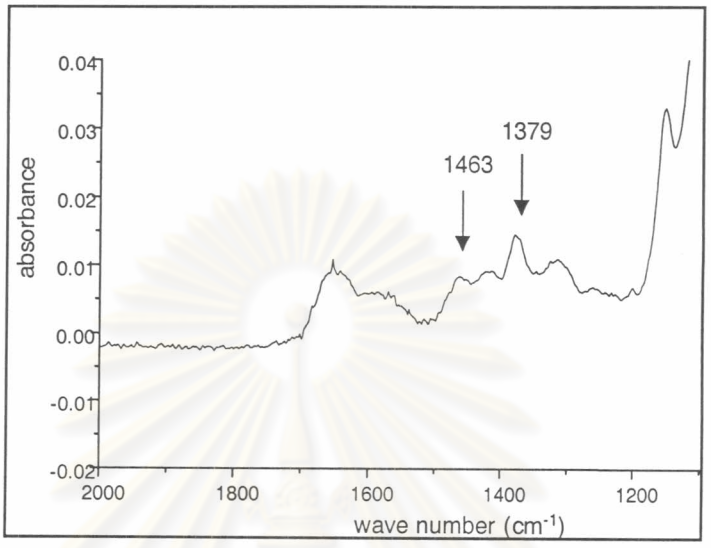


Figure 3.10 ATR-IR spectrum of modified chitosan films by grafting with butyraldehyde

The grafting step was carried out by using excess amount of butyraldehyde and $NaCNBH_3$, i.e. chitosan:butyraldehyde:$NaCNBH_3$ = 1:10:10. Using NMR technique, the grafting amount was determined (Figure 3.11, Table 3.5). It must be noted here that NMR technique is a bulk analysis method. Therefore the % conversion calculated from the NMR could be a lot lower than it would be if the analysis was done only on the sample surface.

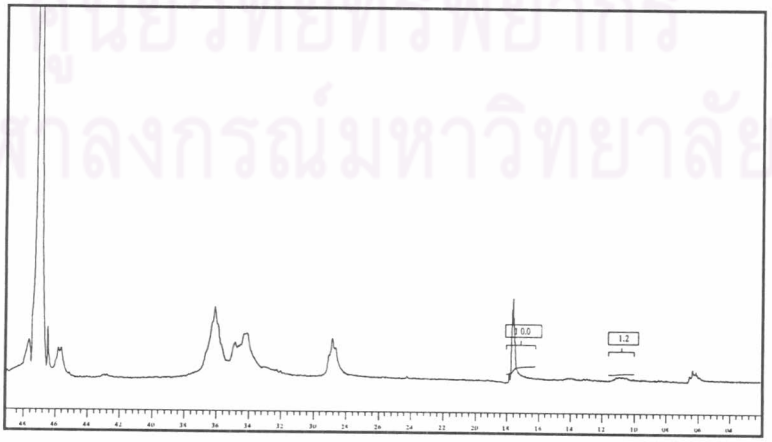


Figure 3.11 ^1H NMR spectrum of modified chitosan films by grafting with butyraldehyde (solvent: 1% CD_3COOD in D_2O, 25 °C)

Table 3.5 Information obtained from ^{1}H NMR spectrum of modified chitosan films by grafting with butyraldehyde

	δ ppm	**Integration**
CH_3-$C\underline{H}_2$-CH_2 of butyraldehyde	1.07	1.2/2
-$C\underline{H}_3$ of GlcNAc	1.76	10/3

From the data in Table 3.5, at least 2.17 % of butyraldehyde was grafted on the chitosan films.

3.3.2.2 Reaction with MTEG-ald (heterogeneous state)

Modified chitosan showed new peaks at the 1456 cm^{-1} for C-H deformation ($CH_{2\,(s)}$, $CH_{3\,(as)}$) and at 1354 cm^{-1} for the C-H deformation ($CH_{3\,(as)}$).

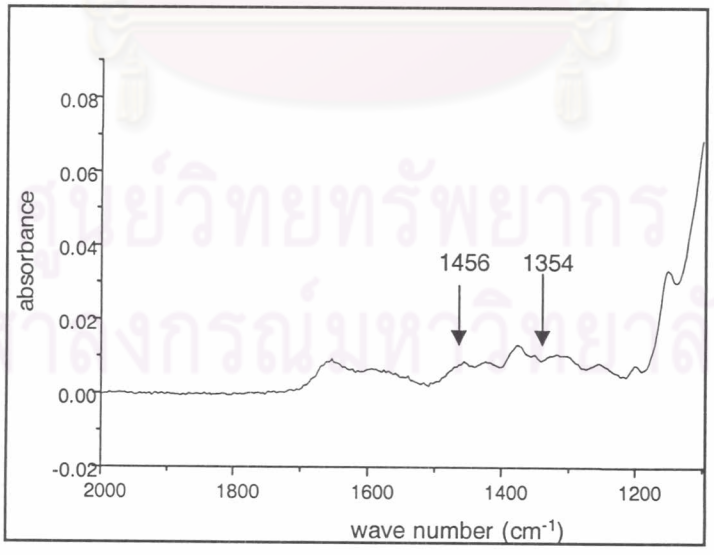


Figure 3.12 ATR-IR spectrum of modified chitosan films by grafting with MTEG-ald

The grafting step was carried out by using excess amount of MTEG-ald and $NaCNBH_3$, i.e. chitosan:MTEG-ald:$NaCNBH_3$ = 1:10:10. Using NMR technique, the grafting amount could be approximated. (Figure 3.13, Table 3.6).

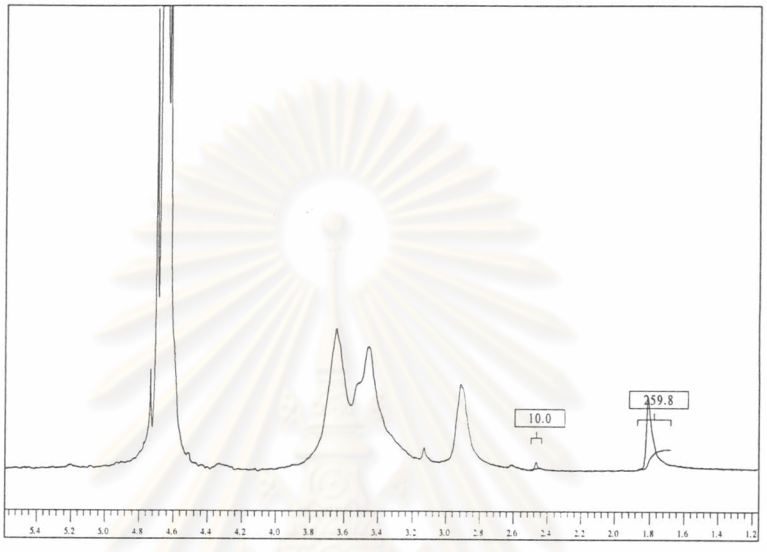


Figure 3.13 ^1H NMR spectrum of modified chitosan films by grafting with MTEG-ald (solvent: 1% CD_3COOD in D_2O, 25 °C)

Table 3.6 Information obtained from ^1H NMR spectrum of modified chitosan films by grafting with MTEG-ald

	δ ppm	**Integration**
-C<u>H</u>$_2$NH- of GlcN	2.47	10/2
-C<u>H</u>$_3$ of GlcNAc	1.81	259.8/3

From the data in Table 3.6, at least 0.70 % of MTEG was grafted on the chitosan films

3.3.2.3 Reaction with MPEG-ald (heterogeneous state)

Modified chitosan showed new peaks at 1452 cm^{-1} for the C-H deformation ($CH_{2\,(s)}$, $CH_{3\,(as)}$) and at 1352 cm^{-1} for the C-H deformation ($CH_{3\,(s)}$).

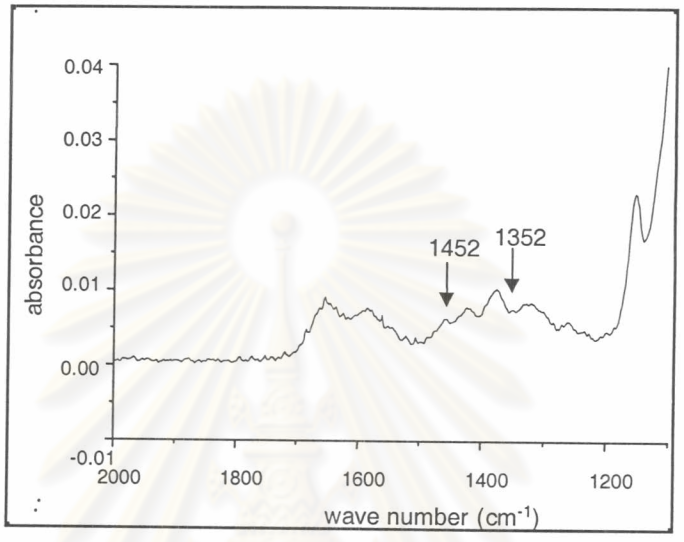


Figure 3.14 ATR-IR spectrum of modified chitosan films by grafting with MPEG-ald

The grafting step was carried out by using excess amount of MPEG-ald and $NaCNBH_3$, i.e. chitosan: MPEG-ald: $NaCNBH_3$ = 1:10:10. Using NMR technique, the grafting amount could be approximated (Figure 3.15, Table 3.7).

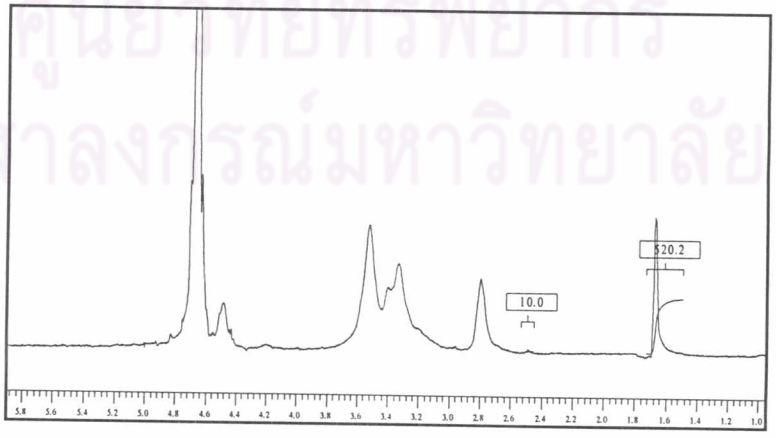


Figure 3.15 ^1H NMR spectrum of modified chitosan films by grafting with MPEG-ald (solvent: 1% CD_3COOD in D_2O, 25 °C)

Table 3.7 Information obtained from ^{1}H NMR spectrum of modified chitosan films by grafting with MPEG-ald

	δ ppm	**Integration**
-C<u>H</u>$_2$NH- of GlcN	2.48	10/2
-C<u>H</u>$_3$ of GlcNAc	1.66	520.2/3

From the data in Table 3.7, at least 0.35 % of MPEG was grafted on the chitosan films.

3.4 Air-water contact angle

Air–water contact angle technique was used to determine the hydrophilicity of the film surface. In general, the contact angle of a hydrophobic surface is bigger than that of the hydrophilic surface. Since chitosan films were immersed in MeOH or DMF during the reaction, it is therefore necessary to investigate the effect of solvent on the contact angle. From Table 3,8, blank chitosan films were soaked in either MeOH or DMF for the same length of time as when doing the reaction. The films were then dried in vacuum for 2 days prior to contact angle measurement. It was found that the contact angle became lower than the non-soaked films. This suggests that a trace amount of solvent remains in the films. Therefore, for correct comparison, the data of all modified films should be compared to the values obtained from the soaked films

From Table 3.8, the hydrophilicity increased as expected after the chitosan films reacted with the hydrophilic MTEG-ald and MPEG-ald. On the other hand, when reacted with butyradehyde the films became slightly more hydrophobic, due to its hydrocarbon moiety. Changes of hydrophobicity of the modified chitosan films have been reported earlier for the chitosan reacted with stearoyl chloride ($C_{17}H_{35}C$ (O)Cl) and succinic anhydride.[15]

Table 3.8 Air-contact angle of chitosan films and modified chitosan films (8 repetitions)

Film samples	Solvent	Contact angle (degree)
Chitosan	none	78.4±3.2
	MeOH	73.6±2.4
	DMF	77.4±1.3
Chitosan: butyraldehyde: $NaCNBH_3$	MeOH	78.1±1.4
Chitosan: MTEG-ald: $NaCNBH_3$		
1:10:10	MeOH	58.4±2.9
1:30:30	MeOH	64.1± 2.6
1:10:10	DMF	62.0±1.5
Chitosan: MPEG-ald: $NaCNBH_3$		
1:10:10	MeOH	59.3± 2.5
1:30:30	MeOH	60.7± 1.4
1:10:10	DMF	53.8±1.3

3.5 Protein adsorption study

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

The color intensity of protein solution after adding BCA working solution depends on the concentration of protein solution (Figure 3.16). The calibration curve showing the correlation between the absorbance at 562 nm and protein concentration is shown in Figure 3.17.

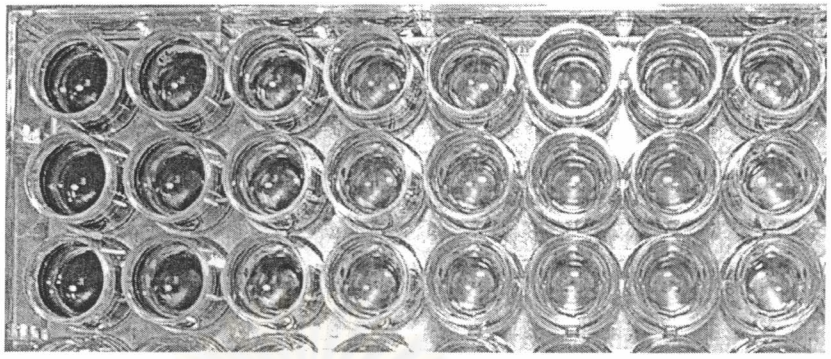


Figure 3.16 Color intensity of protein solution, 16 h after adding BCA working solution, at different amounts of proteins

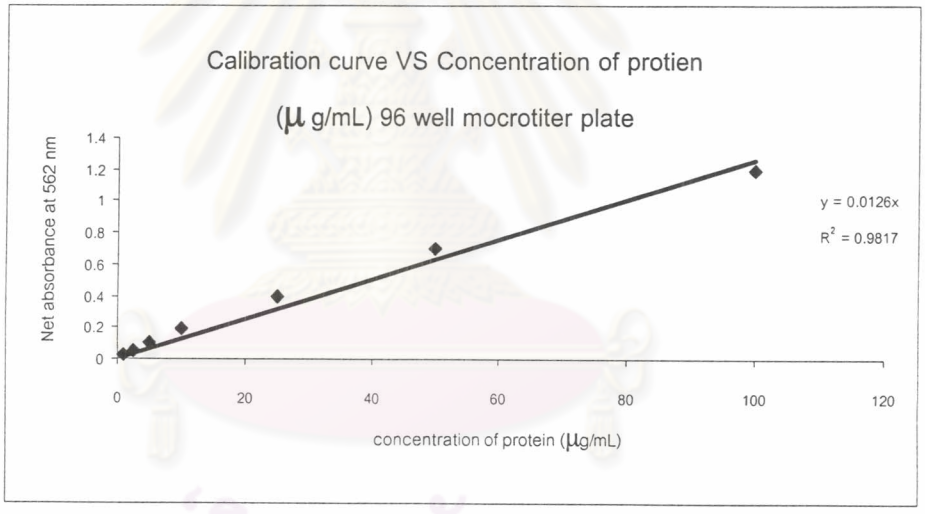


Figure 3.17 Standard curve between absorbance at 562 nm and protein concentration

Table 3.9 shows the amount of proteins adsorbed on the surface of chitosan films. In the case of *n*-butyl chitosan, the amounts of protein adsorbed were not much different from the unmodified films. On the other hand, both types of proteins were adsorbed less on the EG-grafted surface. This result agrees with the finding made by Aiba[20] and Mansoor[21] that the PEG-grafted chitosan chain reduced protein and platelet adhesion. It was postulated that the highly mobile EG chain repelled the

approaching protein molecules. It was also found from this study that the EG-grafted films could not differentiate the structure difference of albumin and lysozyme.

Table 3.9 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	Ave.	Set I	Set II	Ave.
Chitosan	1.60	1.53	1.57	2.81	2.19	2.86
n-butyl chitosan	1.43	1.38	1.41	3.63	2.62	3.13
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