Malaysian Journal of Analytical Sciences (MJAS)
Published by Malaysian Analytical Sciences Society

SITE-SELECTIVE CARBOXYMETHYLATION OF CHITOSAN UNDER HETEROGENEOUS CONDITIONS

(Penentuan Tapak bagi Proses Pengkarboksimetil pada Kitosan dalam Keadaan Heterogen)

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Received: 11 March 2021; Accepted: 22 May 2021; Published: 27 June 2021

Abstract

The substitution sites on chitosan are affected by the presence of a base. Herein, the effects of pH on the site-selective carboxymethylation of chitosan were investigated. Carboxymethyl chitosan was synthesized by reacting chitosan with monochloroacetic acid at different pH under heterogeneous conditions. Fourier transform infrared-attenuated total reflectance (FTIR-ATR) spectroscopy confirmed that carboxymethylation was successful, with the appearance of peaks around 1326-1320 cm^{-1} (C–N groups) and 1257-1253 cm^{-1} (C–O–C groups) and allowed differentiation between the carboxymethyl substitution sites on chitosan. Additionally, the peaks at approximately 3.28 and 4.12 ppm in the ^{1}H nuclear magnetic resonance (NMR) spectra confirmed that substitution occurred at amine and hydroxyl groups, respectively. Overall, the carboxymethylation of chitosan under heterogeneous conditions at pH 8.5-11 gave O-substitution, at pH 12-13 gave N,O-substitution, and at pH 14 gave N-substitution. This pH dependence of the site-selective substitution of chitosan is important for polymer electrolyte application.

Keywords: carboxymethyl chitosan, pH effect, substitution site, heterogeneous conditions

Abstrak

Tapak penggantian kitosan dipengaruhi oleh kehadiran bes. Dalam kajian ini, kesan pH terhadap tapak penggantian pengkarboksimetil kitosan telah dikaji. Karboksimetil kitosan disintesis melalui tindak balas kitosan dengan asid monokloroasetik pada pH berbeza dalam keadaan heterogen. Spektroskopi inframerah transformasi Fourier (ATR-FTIR) mengesahkan bahawa karboksimetilasi telah berjaya dilakukan dengan kemunculan puncak sekitar 1326–1320 cm^{-1} (kumpulan C–N) dan 1257–1253 cm^{-1} (kumpulan C–O–C), yang menunjukkan perbezaan tapak penggantian karboksimetil pada kitosan. Selain itu, puncak sekitar 3.28 dan 4.12 ppm dalam spektrum resonans magnetik nukleus (^{1}H NMR) mengesahkan bahawa penggantian berlaku pada kumpulan amina dan hidroksil. Secara keseluruhan, karboksimetilasi kitosan dalam keadaan heterogen pada pH 8.5-11 memberikan penggantian pada tapak O, pH 12-13 memberikan penggantian pada tapak N dan O, dan pH 14 memberikan penggantian pada tapak N. Kebergantungan tapak pemilihan untuk penggantian kitosan dengan pH ini adalah penting untuk aplikasi elektrolit polimer.

Kata kunci: karboksimetil kitosan, kesan pH, tapak penggantian, keadaan heterogen

Introduction

Chitosan has been widely studied for various applications in the biomedical field owing to its biocompatible, biodegradable, nontoxic, and antimicrobial properties [1]. However, the limited solubility of chitosan in water and other organic solvents has restricted its potential in drug delivery and tissue engineering [2]. As a water-soluble derivative of chitosan, carboxymethyl chitosan (CM-chitosan) has better biological and physicochemical properties [3]. Furthermore, CM-chitosan is easy to synthesize and can be used for many applications in cosmetic, biomedical, environmental, food industry, and polymer electrolyte systems [4].

Several researchers have claimed that substitution occurs preferentially at the hydroxyl group at C6 in the chitosan structure rather than at the hydroxyl group at C3 and the amine group [4]. This selectivity is influenced by the steric hindrance at the hydroxyl group at C3 and the amine group. However, the choice of the appropriate reaction conditions and reagents allows the preparation of CM-chitosan derivatives with site-selective substitution at either the primary hydroxyl or amine group of chitosan to produce O-carboxymethyl chitosan (O-CM-chitosan), N-carboxymethyl chitosan (N-CM-chitosan), N,O-carboxymethyl chitosan (N,O-CM-chitosan), or N,N-carboxymethyl chitosan (N,N-CM-chitosan), as shown in Figure 1 [3-5].

The selection of a particular substitution site is important, as it influences the performance of chitosan derivatives for specific applications. According to a previous study, O-CM-chitosan has a higher moisture retention–absorption ability than N-CM-chitosan [6]. High moisture retention-absorption properties are crucial for applications in cosmetics, such as moisturizing skincare [7]. In contrast, low moisture retention–absorption properties are important for applications such as battery membranes [8].

O-CM-chitosan can be synthesized through direct alkylation by reacting chitosan, sodium hydroxide (NaOH), and monochloroacetic acid ($ClCH_2COOH$) at different temperatures and reactant ratios under heterogeneous conditions [9, 10]. Meanwhile, N-CM-chitosan can be obtained through a reductive N-alkylation reaction [11]. The N-alkylation of chitosan can be carried out in the presence of various bases. The pK_a of the reaction condition should be higher than 6.3 [12]. Previously, N-CM-chitosan and N,N-CM-chitosan have been prepared at pH 8 and pH 8.5, respectively. These reactions were carried out under homogeneous conditions and the pH was adjusted to 8 or 8.5 after the addition of $ClCH_2COOH$ [13, 14]. Other than these N-alkylation reactions, a prior study reported the unsuccessful preparation of N-octylchitosan under heterogeneous conditions, likely due to the high steric hindrance caused by using chitosan with a degree of deacetylation (DD) lower than 90% [15]. To the best of our knowledge, there have been no reports on the successful synthesis of N-CM-chitosan under heterogeneous conditions.

Therefore, in the present study, the effect of pH under heterogeneous conditions for the synthesis of N-CM-chitosan have been investigated. We also discussed the effect of pH on the degree of substitution (DS), as research has shown that the DS is influenced by the reaction conditions, with low DS values obtained for heterogeneous reactions and high DS values for homogeneous conditions [16]. We used Fourier transform infrared-attenuated total reflectance (FTIR-ATR) spectroscopy, ^1H nuclear magnetic resonance spectroscopy (NMR), and the DS to characterize and analyze the synthesized CM-chitosan derivatives. Computational modeling was also performed to understand the charges in all the structures.

Materials and Methods

Materials

Chitosan (DD = 89%, as calculated by ^1H NMR spectroscopy) was commercially obtained from ChitoChem. NaOH and $ClCH_2COOH$ were purchased from Sigma-Aldrich. Isopropanol (IPA), ethanol (EtOH), and acetic acid (CH_3COOH) were purchased from Systerm. All materials were used without further purification.

Preparation of CM-chitosan derivatives

The carboxymethylation of chitosan was carried out based on previously reported work with minor modifications [17]. Briefly, 5 g of chitosan was suspended in 25 mL of IPA in a flask. Then, a mixture of 5 g of NaOH in 25 mL of water was added to the flask. The resulting mixture was stirred with a magnetic stirrer at 50 °C for 1 hour on a hot plate. The pH of the mixture (pH 8.5-14) was adjusted by adding NaOH dropwise using a micropipette. Subsequently, 7.5 g of $ClCH_2COOH$ dissolved in 25 mL of IPA was added to the reaction mixture. After stirring for 4 hours at 50 °C, the reaction was terminated by adding 70% EtOH (100 mL). The precipitate was filtered and rinsed with 70%, 80%, and 90% EtOH to obtain the final product. The final product, which was obtained as the Na-salt of CM-chitosan, was stored in a desiccator for 3-5 days. The obtained products were denoted as CM-chitosan-8.5 (pH 8.5), CM-chitosan-9 (pH 9), CM-chitosan-10 (pH 10), CM-chitosan-11 (pH 11), CM-chitosan-12 (pH 12), CM-chitosan-13 (pH 13), and CM-chitosan-14 (pH 14). The masses of the obtained products were 7.8375 g (CM-chitosan-8.5), 7.0290 g (CM-chitosan-9), 6.2343 g (CM-chitosan-10), 6.4860 g (CM-chitosan-11), 6.1240 g (CM-chitosan-12), 6.2510 g (CM-chitosan-13), and 6.562 g (CM-chitosan-14).

FTIR-ATR spectroscopy

FTIR-ATR spectra were recorded using a PerkinElmer Spectrum 2000 spectrometer in the range of 4000–650 cm^{-1} with a scanning resolution of 2 cm^{-1}. The FTIR-ATR analysis was conducted to observe the changes in the functional groups before and after chitosan modification.

^{1}H NMR spectroscopy

NMR analysis was performed to confirm the N- and O-substitution of the CM-chitosan derivatives. ^{1}H NMR spectra were recorded using a Bruker Avance 111 spectrometer (400 MHz). Chitosan and the CM-chitosan derivatives were dissolved in a mixture of 1% CD_3COOD in D_2O [18].

DS of CM-chitosan derivatives

To determine the DS of each CM-chitosan derivatives, the Na form was changed to the H-form [19, 20]. First, 1.5 g of Na-form CM-chitosan was suspended in 80% EtOH (100 mL) and then 37% hydrochloric acid (10 mL) was added. After stirring the mixture was with a magnetic stirrer for 30 min, the precipitate was filtered and rinsed with 70–90% EtOH. The products were stored in a desiccator for 2–3 days.

The DS represents the average number of substitutions per anhydroglucose unit (AGU). The DS values of the CM-chitosan derivatives were determined by the standard ASTM D1439 method, as previously reported [21]. First, 0.5 g of dry H-form CM-chitosan was dispersed in 100 mL of distilled water in a flask. Then, 25 mL of NaOH solution (0.3 M) was added to the suspension and the resulting mixture was stirred for 15 min at 40 °C. Three drops of phenolphthalein were then added to the mixture as an indicator. Finally, the mixture was titrated with 0.3 M HCl until the indicator changed from pink to colorless. A blank test also was carried out using the same procedure without adding a CM-chitosan derivative.

The percentage of CM groups (%CM) and the DS were calculated using equations (1) and (2), respectively.

$$\%CM = \frac{[(Vo-Vn)M \times 0.059 \times 100]}{m} \quad (1)$$

$$DS = \frac{162 \times \%CM}{[5900-(58 \times \%CM)]} \quad (2)$$

where V_o is the volume of HCl used for the blank test (mL), V_n is the volume of HCl used for sample titration (mL), M is the molar concentration of HCl (M), m is the amount of sample (g), 162 is the molar mass of the AGU, and 59 is the molar mass of a CM group (–CH_2COOH).

The differences between means were analyzed for statistical significance using Student's t-test. A value of $p < 0.05$ was considered significant [22].

Computational modeling studies

Density functional theory (DFT) calculations implemented in Gaussian 09 were used to investigate the Mulliken charge and stability of all the CM-chitosan structures. The GaussView 5.0 program was used for

molecular visualization. The gas-phase molecular structure of chitosan and all the CM-chitosan derivatives in the ground state were optimized by DFT in its restricted form without any symmetry constraint. To produce accurate ground state geometries, chitosan and the CM-chitosan derivatives were modeled using two monomers. The chitosan monomer consists of glucosamine and *N*-acetylglucosamine. For O-CM-chitosan, the hydroxyl groups on the chitosan chain were substituted with CM groups (–CH_2COOH). For N-CM-chitosan, the CM groups were substituted at the amine groups. For N,O-CM-chitosan, both the hydroxyl and amine groups were substituted with CM groups. All the structures were minimized using the B3LYP/6-31++G (d,p) basis set.

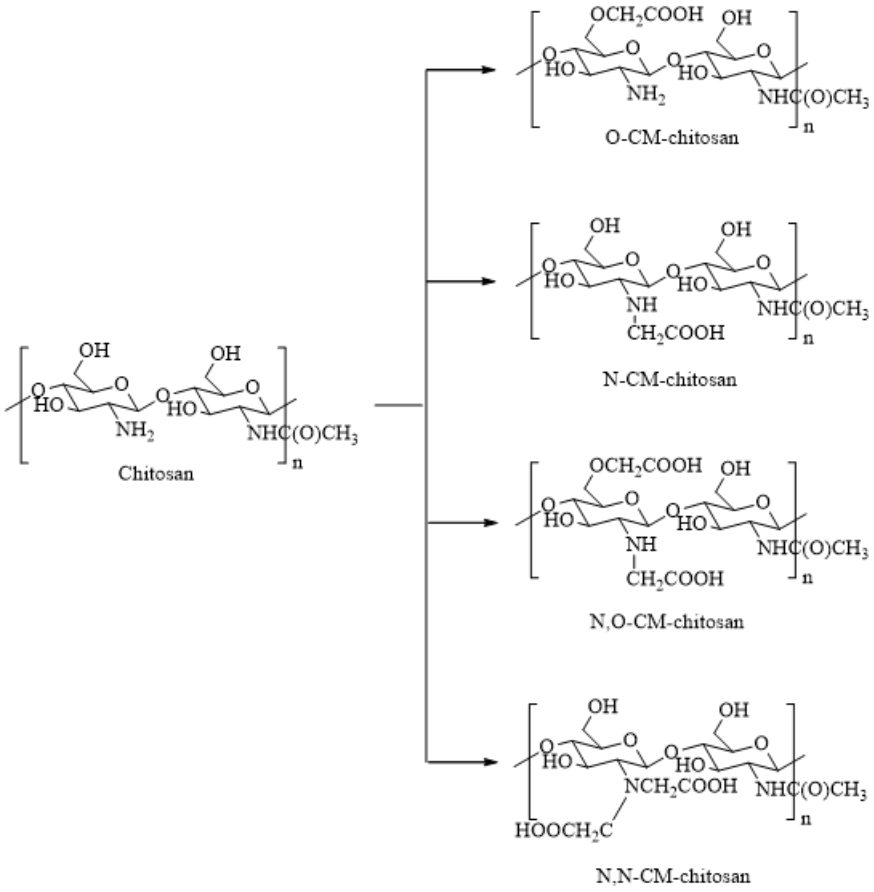


Figure 1. Structures of chitosan and CM-chitosan derivatives

Results and Discussion

Characterization by FTIR-ATR spectroscopy

CM-chitosan can be synthesized through a direct alkylation process. Figure 2 shows the FTIR-ATR spectra of chitosan and the various CM-chitosan derivatives. In the FTIR-ATR spectrum of chitosan, the peaks at 3283, 2868, 1644, 1585, and 1060 cm^{-1} represent O–H stretching, C–H stretching, the N–C(O)–CH_3 carbonyl, N–H bending, and C–O stretching, respectively. The positions of these peaks are similar to those reported in a previous study [23]. In contrast, in the FTIR-ATR spectra of all the CM-chitosan derivatives, a new peak appears around 2922–2954 cm^{-1}, corresponding to the methylene of the CM group,

which confirmed the carboxymethylation of chitosan. Additional new peaks at 1648-1634 cm^{-1} and 1588-1585 cm^{-1} correspond to the N-acetyl and carboxylate of the CM group, respectively.

However, intense peaks around 1533-1529 cm^{-1}, 1386-1379 cm^{-1}, and 1257-1253 cm^{-1} are only observed for CM-chitosan-8.5 to CM-chitosan-13. These peaks can be assigned to the amide II group, symmetrical $–COO^-$ stretching, and ether groups, respectively. In contrast, the intense peaks observed around 1326-1320 cm^{-1} for CM-chitosan-12 and CM-chitosan-13 correspond to C–N stretching. In addition, CM-chitosan-14 exhibits peaks around 1414, 1376, and 1327 cm^{-1}, representing symmetrical $–COO^-$ stretching, the amide III group, and C–N stretching, respectively. These peaks have also been reported in a previous study [24]. As well as confirming that carboxymethylation occurred, these observations provided an initial indication that the CM groups had been substituted at hydroxyl groups for CM-chitosan-8.5 to CM-chitosan-13 and at amine groups for CM-chitosan-12 to CM-chitosan-14. These findings were further confirmed by ^{1}H NMR analysis and determination of the DS.

Characterization by ^{1}H NMR spectroscopy

Figure 3 shows the ^{1}H NMR spectra of chitosan and the CM-chitosan derivatives obtained under different pH conditions. The proton assignments for chitosan are as follows. ^{1}H NMR (400 MHz, 1% CD_3COOD/D_2O), δ (ppm): 2.17 (H7), 3.11 (H2), 3.66–3.84 (H3, H4, H5, H6), 4.81 (H1). The DD was calculated based on the intensity of the ^{1}H NMR peaks using Equation (3) [25].

$$DD(\%) = \{1 - \left(\frac{1}{3} I_{CH3} / \frac{1}{6} I_{H2-H6}\right)\} \times 100 \quad (3)$$

Following the substitution of CM groups, new peaks, assigned as H8a and H8b, should be observed. Furthermore, the H1 peak should appear at approximately 4.80 ppm as reported by previous study [26–28] but this peak was not apparent owing to overlap with the solvent signals.

The proton assignments for the CM-chitosan derivatives are as follows: CM-chitosan-8.5. ^{1}H NMR (400 MHz, 1% CD_3COOD/D_2O), δ (ppm): 2.18 (H7), 3.13 (H2), 3.67–3.84 (H3, H4, H5, H6), 4.11 (H8a), 4.81 (H1). CM-chitosan-9. ^{1}H NMR (400 MHz, 1% CD_3COOD/D_2O), δ (ppm): 2.17 (H7), 3.13 (H2), 3.67–3.85 (H3, H4, H5, H6), 4.12 (H8a), 4.81 (H1). CM-chitosan-10. ^{1}H NMR (400 MHz, 1% CD_3COOD/D_2O), δ (ppm): 2.17 (H7), 3.12 (H2), 3.67–3.87 (H3, H4, H5, H6), 4.12 (H8a), 4.81 (H1). CM-chitosan-11. ^{1}H NMR (400 MHz, 1% CD_3COOD/D_2O), δ (ppm): 2.17 (H7), 3.12 (H2), 3.67–3.85 (H3, H4, H5, H6), 4.13 (H8a), 4.81 (H1). CM-chitosan-12. ^{1}H NMR (400 MHz, 1% CD_3COOD/D_2O), δ (ppm): 2.16 (H7), 3.11 (H2), 3.28 (H8b), 3.66–3.84 (H3, H4, H5, H6), 4.12 (H8a), 4.81 (H1). CM-chitosan-13. ^{1}H NMR (400 MHz, 1% CD_3COOD/D_2O), δ (ppm): 2.17 (H7), 3.12 (H2), 3.27 (H8b), 3.64–3.84 (H3, H4, H5, H6), 4.11 (H8a), 4.81 (H1). CM-chitosan-14. ^{1}H NMR (400 MHz, 1% CD_3COOD/D_2O), δ (ppm): 2.17 (H7), 3.11 (H2), 3.66–3.84 (H3, H4, H5, H6), 3.23 (H8b), 4.81 (H1).

Based on the ^{1}H NMR spectra in Figure 3, peaks at 4.11–4.12 ppm are observed for CM-chitosan-8.5, CM-chitosan-9, CM-chitosan-10, CM-chitosan-11, CM-chitosan-12, and CM-chitosan-13. Furthermore, peaks at 3.23–3.28 ppm are observed for CM-chitosan-12, CM-chitosan-13, and CM-chitosan-14. The peaks in the region of 4.05–4.55 ppm correspond to protons of CM groups ($–OCH_2–COOD$) substituted at the hydroxyl groups at the C3 and C6 positions, as described in the previous study [27, 28]. In contrast, the peaks in the region of 3.25 ppm correspond to the protons of $N–CH_2–COOH$ groups, which are confirmed by the previous study that reported the substitution of CM groups at the amine groups at around 3.2 ppm [26, 29]. These results indicate that O-CM-chitosan can be attained at pH 8.5-11, whereas pH 12-13 gives N,O-CM-chitosan, and N-CM-chitosan can only be obtained at pH 14.

DS of CM-chitosan derivatives

The DS indicates the number of substituent groups attached per monomeric unit. Because chitosan has two hydroxyl groups and one amine group, the maximum DS value is 3 [20,21]. As depicted in Table 1, the DS values are close to 1 for all the CM-chitosan derivatives. Based on the NMR spectra, at pH 8.5–11, substitution occurred

at the hydroxyl groups. However, as the pH increases in this range, the DS decreases. Thus, the deprotonated hydroxyl group is expected to play an important role in producing O-CM-chitosan, as shown in Scheme 1.

Although the NMR spectra showed that substitution occurred at both the amine and hydroxyl groups at pH 12 and 13, DS values of 2 were not observed under these conditions. The DS values of 0.80 and 0.66 at pH 12 and 13, respectively as reported in Table 1, indicates that incomplete substitution occurred at both functional groups at these pH values. However, it is possible that the degree of substitution at each site differed depending on the pH. Finally, at pH 14, the NMR spectrum confirmed that substitution only occurred at the amine group with a DS of 0.75. At such a high pH, the amine group should act as a stronger nucleophile than the hydroxyl group [30], and a plausible mechanism for N-CM-chitosan formation is shown in Scheme 2.

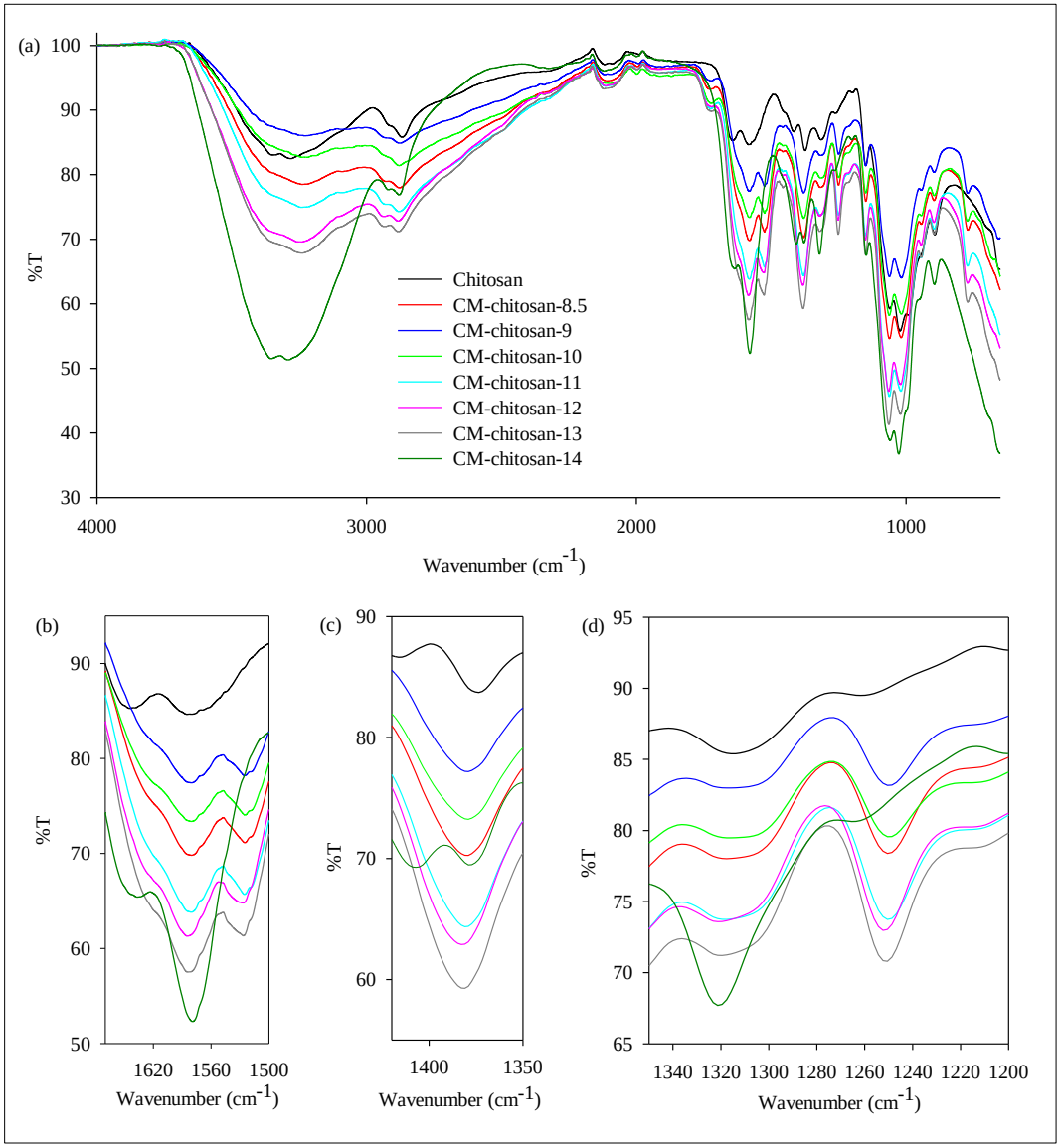


Figure 2. (a) Full FTIR-ATR spectra of chitosan and CM-chitosan derivatives. Enlarged spectra in the regions of (b) 1680–1500 cm^{-1}, (c) 1420–1350 cm^{-1}, and (d) 1360–1200 cm^{-1}

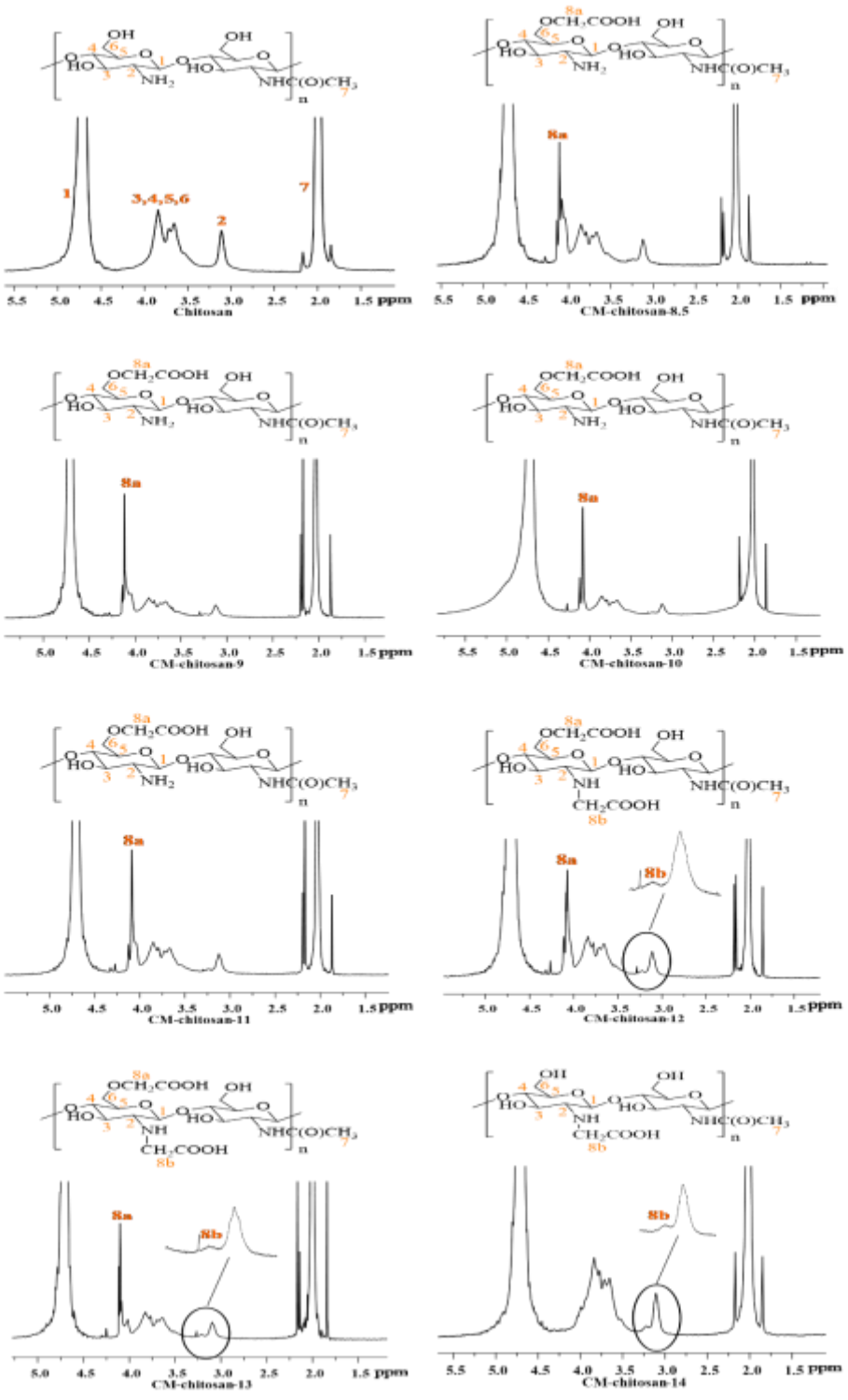
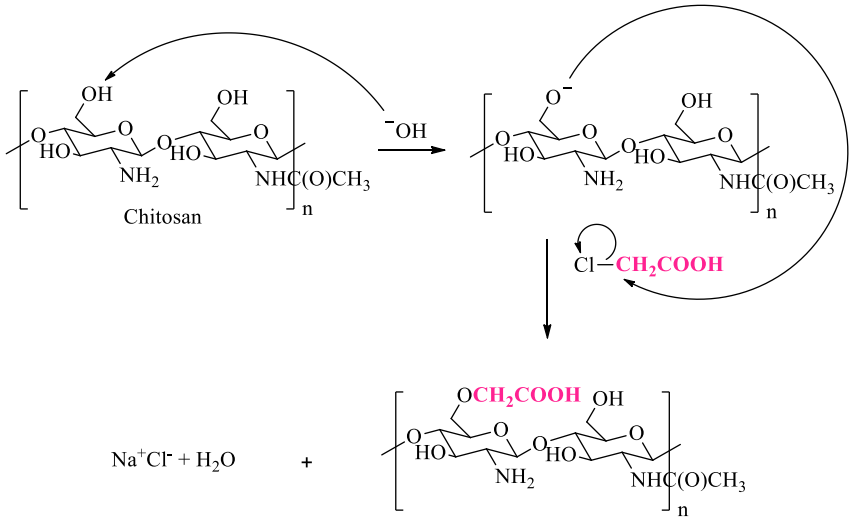


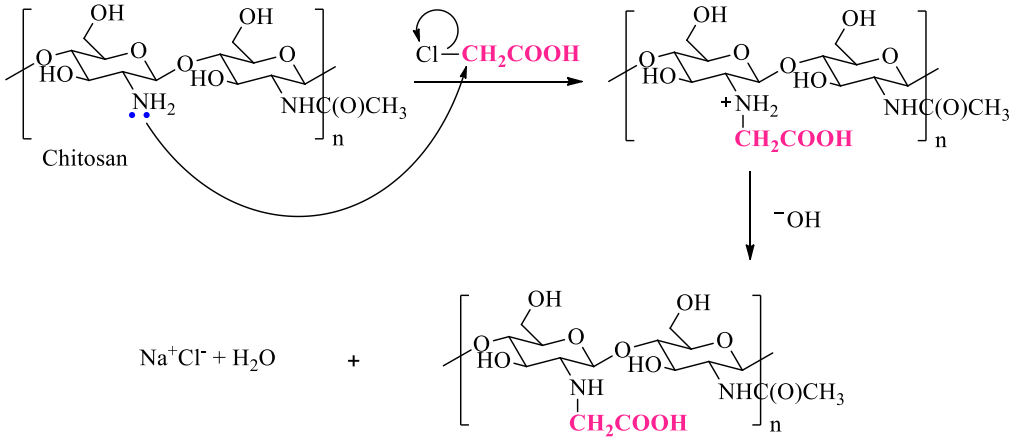
Figure 3. ^{1}H NMR spectra of chitosan and CM-chitosan derivatives

Table 1. DS values for CM-chitosan derivative synthesized at various pH values

Sample	DS
CM-chitosan-8.5	0.80 ± 0.10
CM-chitosan-9	0.81 ± 0.12
CM-chitosan-10	0.78 ± 0.12
CM-chitosan-11	0.70 ± 0.10
CM-chitosan-12	0.80 ± 0.10
CM-chitosan-13	0.66 ± 1.10
CM-chitosan-14	0.75 ± 0.26



Scheme 1. Plausible mechanism for the synthesis of O-CM-chitosan from chitosan



Scheme 2. Plausible mechanism for the synthesis of N-CM-chitosan from chitosan

The student's *t*-test was used to observe the significance between pH selection and substitution site. pH values of 8.5-11 and pH 12-13 were selected as substitutions occurred at hydroxyl and amine groups, respectively. Based on the results, there were no significant differences in the DS values ($p > 0.05$), which showed that CM substitution can be carried out at the hydroxyl groups at pH 8.5-11 and the amine group at pH 12-13.

Computational modeling studies

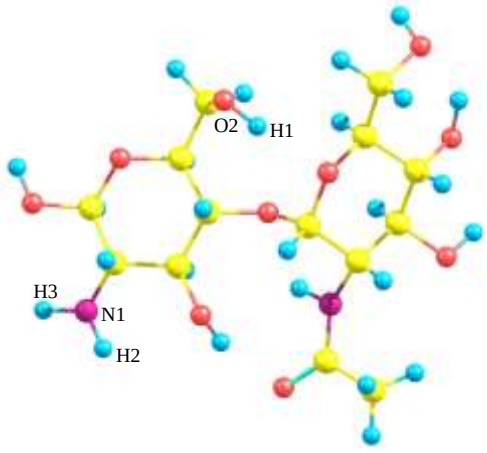
The optimized structures of chitosan, O-CM-chitosan, N,O-CM-chitosan, and N-CM-chitosan are depicted in Figure 4. The structural optimization was performed at the B3LYP/6-31++G (d,p) level.

The Mulliken charges at O2, H1, N1, H2, and H3 of chitosan are shown in Table 2. The hydrogen atom at O2, which is H1, has a higher positive value than both the hydrogen atoms at N1, which are H2 and H3, because the oxygen atom is more electronegative than the nitrogen atom. As a result, the oxygen has a greater ability than the nitrogen atom to pull electrons from a hydrogen atom towards it.

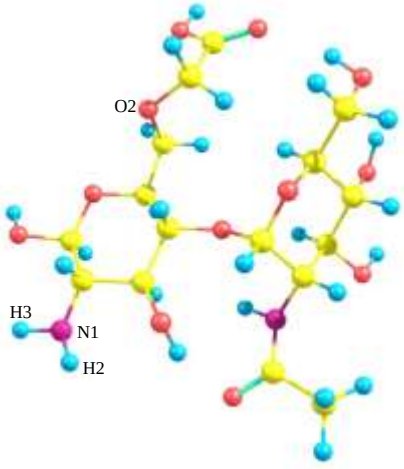
Furthermore, the pK_a of oxygen is higher than that of nitrogen. The pK_a is a measure of how tightly a proton is held by an acid. Therefore, the electron density of the hydrogen bonded to oxygen decreased and the resulting increase in electropositivity made nucleophilic substitution of H1 easier than that of H2 or H3.

In addition, Mulliken charge values became more positive after CM group substitution. A similar outcome had been reported in a previous study. The study showed an increase of Mulliken charges for the structure with electron withdrawing substituents [31]. The Mulliken charge of O2 in O-CM-chitosan increased from −0.6334 to −0.2080, and a similar increase occurred for N1 in N-CM-chitosan (from −0.7153 to −0.2295). As shown in Table 2, the values for N,O-CM-chitosan were more positive, likely because the carboxylate groups, which are electron-withdrawing groups, were able to pull electrons from both the oxygen and nitrogen atoms [31]. However, the Mulliken charge not only affected the substituted atom, as the other atoms in the molecule also became more positive. Similar effects were observed for all the CM-chitosan derivatives. Previous study reported that this was due to the weakening of inter- and intramolecular hydrogen bonds after the introduction of CM groups [32].

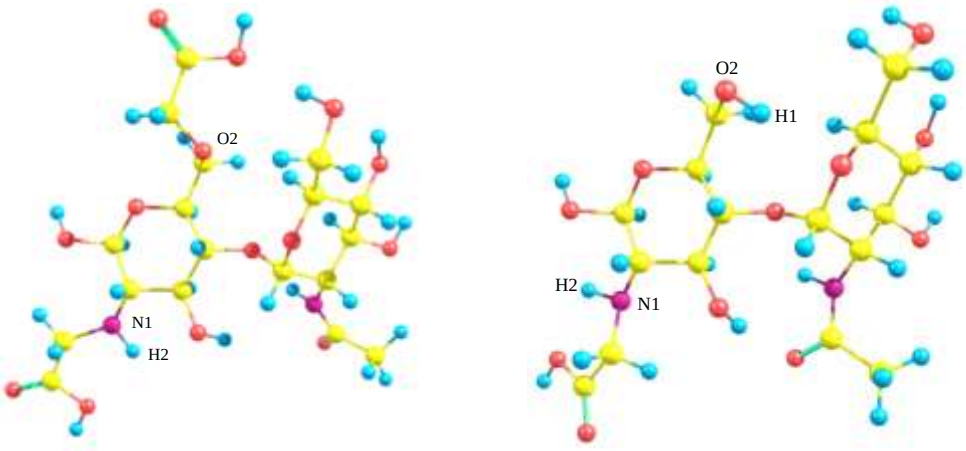
The total energies of the chitosan and CM-chitosan derivative structures are illustrated in Figure 5. N,O-CM-chitosan has the lowest energy (−50796.31 eV) compared with chitosan, N-CM-chitosan, and O-CM-chitosan (−38378.67, −44595.38, and −44595.38 eV, respectively). Thus, among these structures, N,O-CM-chitosan is the most stable, likely because both the nucleophilic sites in N,O-CM-chitosan were substituted with a CM group, which made the structure less reactive and simultaneously increased the stability.



Chitosan



O-CM-chitosan



N,O-CM-chitosan N-CM-chitosan

Figure 4. Optimized structures of chitosan and CM-chitosan derivatives (yellow: carbon; blue: hydrogen; red: oxygen; purple: nitrogen)

Table 2. Mulliken charges of atoms in chitosan and CM-chitosan derivative structures

Atom	Mulliken charges			
	Chitosan	**O-CM-chitosan**	**N,O-CM-chitosan**	**N-CM-chitosan**
O2	−0.6334	−0.2080	−0.1871	−0.4152
H1	0.3977	-	-	0.4555
N1	−0.7153	−0.4480	−0.1356	−0.2295
H2	0.2918	0.3002	0.3880	0.3755
H3	0.2984	0.3376	-	-

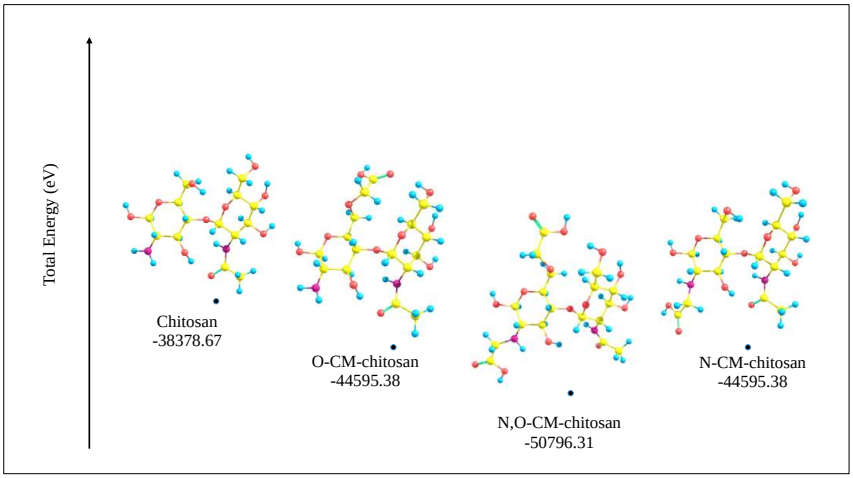


Figure 5. Total energies of chitosan and CM-chitosan derivative structures

Conclusion

In this study, CM-chitosan was successfully prepared under various pH conditions, as confirmed by FTIR-ATR spectroscopy, ^{1}H NMR spectroscopy, and DS calculations. Furthermore, computational modeling showed that the N,O-CM-chitosan structure was more stable than the O-CM-chitosan and N-CM-chitosan structures. The findings from this study make a noteworthy contribution to understanding the site-selectivity of chitosan substitution reactions under heterogeneous conditions. Under heterogeneous conditions, O-CM-chitosan was successfully obtained at pH 8.5–11, N,O-CM-chitosan at pH 12–13, and N-CM-chitosan at pH 14.

Acknowledgement

The authors would like to thank Universiti Kebangsaan Malaysia (UKM) for providing the facilities and research grant GGPM-2017-093.

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