RESEARCH ARTICLE

Preparation and Characterization of Surface Active Chitosan Derivative

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ABSTRACT

In this research, synthesizing of two derivatives of chitosan, namely lauroyl chitosan with two different concentrations (5 and 15%) of substitution, was performed. The reaction of low chitosan molecular weight was carried out with different fatty acid chlorides. Characterization of The chemical structure of the derivatives was conducted utilizing ^{1}HNMR and FTIR. Following that, an investigation of the physical properties of the prepared compounds utilizing thermal analysis (DSC/TGA) techniques. The interfacial analysis of aqueous solutions showed that Lauroyl chitosan derivatives exhibit weak amphiphilic behavior and hard to self-aggregate and to form micelle due to the length of Lauroyl (12 carbons). Cytotoxicity examination was performed via 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay to verify particle applicability as a drug carrier, and all Chitosan derivatives were found non-toxic on human epithelial colorectal adenocarcinoma cell lines.

Keywords: Chitosan, Derivatives, Fatty acids, Lauroyl chitosan, Micelle, Substitution.

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INTRODUCTION

Chitosan (CS) consider as a linear polysaccharide of glucosamine (GlcN) and N-acetyl glucosamine (GlcNAc) units that are bound through β-(1–4) linkages. CS can be prepared conventionally through partial chitin alkaline deacetylation.[1] It is also found and occurs abundantly in nature. Additionally, CS has excellent biocompatibility besides its several other advantages subsequent to its sole polymer cationic character, making it highly suitable in pharmaceutical applications.[2,3] The macromolecular features of chitosan can be designed by modifying the average molecular weight and acetylation degree.[4] However, the high molecular weight of CS orients it to have poor solubility property in aqueous solutions at neutral pH concentration. Therefore, the polymers family gains restrictions in the biomedical applications regarding this issue. On the other hand, the water-soluble property is preferable for Chitosans at low molecular weight (LMW) over a wide range of pH.[5] Concerning organic solvents; chitosan has low solubility in dimethyl sulfoxide and p-toluene sulfonic acid. This poor soluble property is limiting the treating of chitosan slowing its chemical modification.[5] So, to avoid this issue of solubility in both water and organic solvents, modification concerning chemical reactions must be performed in different methods based on the application[6]

Chitosan in Drug Delivery

The exceptional properties of chitosan place it as a unique material for developing the diversity of biomedical applications. Recently, CS utilized many different types of drug carriers in various administration drug routes such as transdermal, nasal, and oral.[7] Different shapes and geometries can be found for chitosan, such as nanoparticles, microspheres, and membranes. However, many advantages are gained in accordance to the shape and formation; such as; capacity to control the release of active agents, ease of manufacturing Chitosan-based particles in the absence of hazardous organic solvents, linear polyamine that contains a particular of free amine groups are readily existing for crosslinking. The cationic nature of chitosan allows for ionic crosslinking with multivalent anions.[8,9]

Elsabee *et al*., 2009[6] have described the activity of Chitosan surface along with its aggregation properties in water to be around 71.45 m/Nm at 25°C. This value is very near to pure water's surface tension, which is 71.99 m/Nm at 25°C. However, this value of surface tension indicated that chitosan molecules are left out from the air/solution interface, in which surface activity is absent.[10]

Modified Chitosan

Normally, fluorescence spectroscopy, surface tension methodology, and viscosity are typical measurement methods of derivatives of Hydrophobically chitosan and the critical micelle concentration of chitosan.[11,12] Therefore, several researchers have paid attention to these chemical modifications[13] to improve, on the other hand, increasing the surface aggregation by increasing the interaction of chitosan

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with hydrophobic groups, leading to an increase in the self-aggregation of these hydrophobized water-soluble chitosan derivatives.[11,14-16]

A type of modification is to introduce alkyl side chains into the polymer backbone chemically that modifies chitosan, creating what is commonly called a Hydrophobically modified chitosan. Because the coexistence of both hydrophilic and hydrophobic parts could be accompanying polymers display amphiphilic character in an aqueous solution.[5,7]

Chitosan chemical modification with hydrophobic fatty acids, namely linoleic and oleic acid, was successfully performed recently. The reaction of fatty acids with chitosan was catalyzed with 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide-mediated reaction (EDC), which reacts with carboxyl groups of fatty acids to form an active ester intermediate.[17,18] Consequently, to form an amide bond, a reaction of primary amine groups of chitosan with the intermediates can be performed.[19]

Additionally, the advantage of using EDC as a catalyst is as easy removal from the reaction medium, and more importantly, the reaction can be executed in mild conditions. The product of modification: amphiphilic derivatives were capable to self-assemble into micelles.[20]

Based on the above review, the study aims to evaluate the hypothesis that fatty acid derivatives of chitosan can be synthesized and will exhibit surface-active properties that may be useful in micellar drug delivery.

EXPERIMENTAL PROCEDURE

N-Acylation of Chitosan

CS solution (5 g in 300 mL of 1% v/v acetic acid) was prepared by stirring it for the overnight duration to be certain of complete solubility. Then, a slow addition in the vigorous stirring of 0.5 M NaOH to adjust pH to the neutral value. (0.233g) of lauroyl chloride was mixed with the neutralized CS solution, and the reaction allowed to continue stirring for 48 h at room temperature. Correspondingly, different weights of highly reactive lauroyl chloride were utilized to have various substitution degrees.

On the other hand, the reaction medium was normalized by adding 0.1 M NaOH and CS derivative; then the liquid precipitated in acetone. Centrifugation at 3000 rpm was assigned to collect the precipitation, followed by washing thrice in hot methanol to remove unreacted acid chloride. The final step was drying the product in oven overnight and stored at room temperature.[21] Table 1 shows all the raw materials utilized in this experiment.

The degree of N-acylation, which can be distinguished as the number of fatty acid groups per 100, an hydroglucose units of chitosan, was 5 and 15%.

Insoluble impurities were removed from each derivative employing purification procedure, the process conducted by yield dissolving, afterword the solution placed in a dialysis bag where deionized water was used for 48 hours at a range of changing the water every 8 hours. Finally, freeze-dried the solution.[11]

Cytotoxicity Assay

Cell culture medium (CCM) was ready by mixing 500 mL of Dulbecco's modified eagle's medium (DMEM) with 50 mL of fetal bovine serum (FBC), 5 mL of 100 x of L-glutamine solution, and 5 mL of 100x of antibiotics (penicillin + streptomycin).

MTT Assay

The MTT assay was seeded using $CaCo_2$ cells on 96 well plates; the density of seeding is 2*10^4 cells per well in100 µL of DMEM culture medium. All cells were grown in a controlled atmosphere of 95% air and 5% CO_2 at 37°C for 24 hours until obtaining a continuous monolayer. Subsequently, 100 µL added the test solution at the predetermined concentrations range of each derivative to the cultured cells. The experiment was performed three times for each concentration, and the cells and particles incubated for 48h. Then, 20 µL of MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution added to each wall, and the plates incubated for 4 hours.

The medium was distant, and 200 µL of DMSO and the formed crystals were solubilized in each well. The absorbance of the well was measured using spectrophotometry at a range of 570 nm absorbance background was corrected utilizing a 630 nm wavelength of Elisa reader and Gen5 software. All cells that treated in the medium were utilized as control, the viability calculated as follow[21]:

Viability % = (mean absorbance of sample) / (mean absorbance of control) *100

Chemical and Physical Characterization of *N*-acylated Chitosan Derivatives

The ^{1}H NMR spectra and FTIR were employed to characterize the existence of specific chemical groups in Chitosan and Chitosan derivatives. Thermal analysis DSC/TGA for 5 mg ± 0.1

Table 1: The chemicals and lab tools used in this work

Material	*Source*	*Patch number*
LMW chitosan Deacetylation (75-85%) MW=35Kda.	Sigma-Aldrich,USA	MKBH1108V
Absolute Methanol	VWR international,USA	10L150510
Absolute Ethanol	VWR international,USA	12G260506
Acetone	Daejung, Korea	A0033MG5
Dichloromethane DCM	AZ chem, USA	FG10081601
Dimethylsulfoxide DMSO	Tedia company, USA	907363
Sodium hydroxide NaOH	Gain Land Chemical Company, UK	1285
Glacial Acetic acid	Scharlau, Germany	29728/1468
Lauroyl chloride	Sigma-Aldrich, USA	101414735
Potassium bromide KBr (FT-IR grade)	Sigma-Aldrich, USA	SZBC3070V
Dialysis membrane MW cut off 1000 Da.	Spectra/Por, USA	132105

Table 2: Percentage of viable cells after cytotoxicity studies for Lauroyl chitosan with 5% and 15% degree of substitution (LCS 5 and 15%) with a mean value ± standard deviation = 105.98 and 96.06

Sample µg/ml	*Viability 5%*	*Viability 15%*
2.5	98.386	88.58585
2	99.42845	86.29514
1.5	90.2261	88.62164
1	107.3727	117.6492
0.5	107.804	84.82766
0.2	107.9119	88.55006
0.1	107.9119	98.5361
0.05	80.91592	92.91671
0.01	112.9084	87.35101
0.005	128.3296	109.5243
0.0025	124.6271	113.891

Table 3: 1H-NMR chemical shift assignments of chitosan and the derivatives

ppm	*Proton assignments*
3.0 to 3.2	CH (H2 of chitosan)
3.4 to 3.8	CH (H3,H4,H5,H6,H6‘)of the ring of chitosan
4.5 to 5	CH (H1 of chitosan)
2.0 to 2.2	CH3 (N-acetyl proton of GlcNAc)
0.7 to 0.9	CH3 of acyl groups
1.2 to 1.9	CH2 of the long chain of acyl groups

sample weigh, constant heating rates of 10°C/min until reach a target temperature of 350°C was performed on all samples.[22]

Surface tension was conducted on CS derivatives and Modified chitosan derivatives.[12,23]

RESULTS AND DISCUSSIONS

The experimental results on the determination of Viability % for Chitosan derivatives are given below in Table 2. It can be observed that all samples are non-toxic, even with very high concentrations.

Characterization of Chitosan and *N*-acylated Chitosan Derivatives

The original Chitosan and N-acylated chitosan derivatives with two different degrees of substitutions (5 and 15%) were chemically characterized using ^{1}H-NMR as shown in Table 3 and Figure 1.

From 1H NMR spectra of all Chitosan derivatives that were prepared, new peaks at 0.7, 1.2, and 1.9 ppm recognized respectively to CH3 and CH2 of the acyl residue appeared with intensities fluctuating directly with the degree of substitution.

Structure change of chitosan after acylation was confirmed by FT-IR spectra and the results are shown in Figure 2.

The manufacturer indicated that the native chitosan used as a starting material had a deacetylation level of 75–85% due to the acetylation group's existence. Chitosan has a peak at (1430 cm^{-1}) attributed to the N–H bending vibration mode of non-acylated amino glucose primary amines (-NH2), and a peek at (1643 cm^{-1}) due to the presence of the acetylated groups.

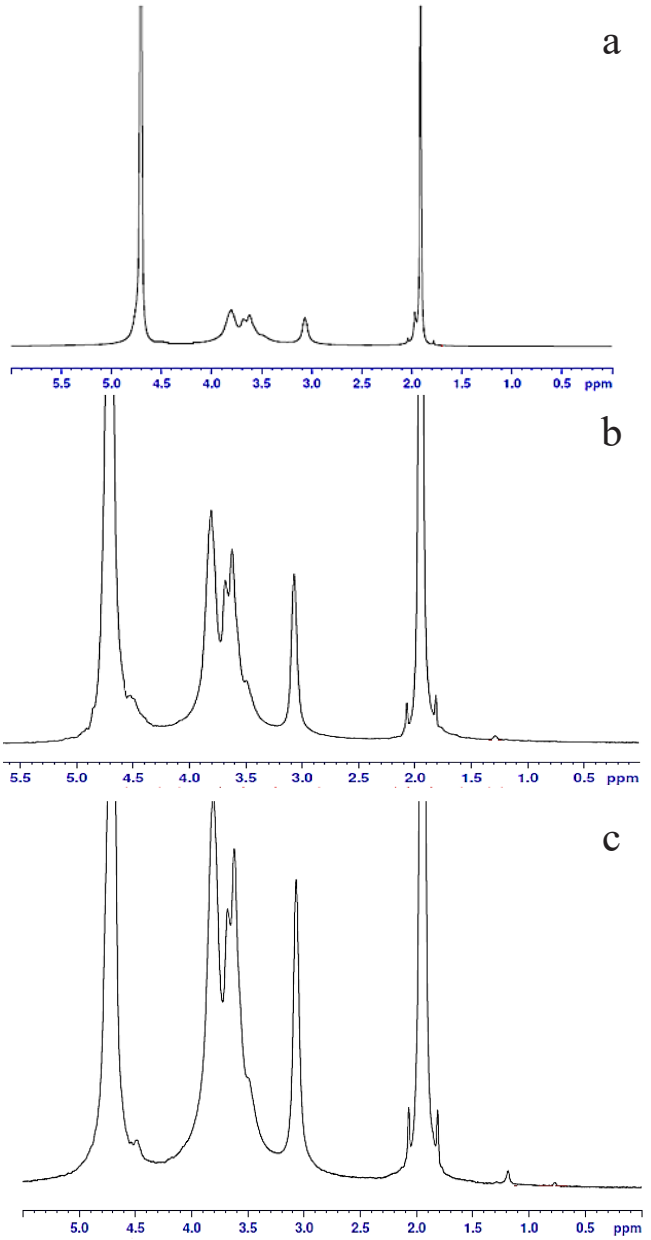

Figure 1: 1H-NMR spectrum a) Chitosan, b) LCS 5% derivative, c) LCS 15% derivative

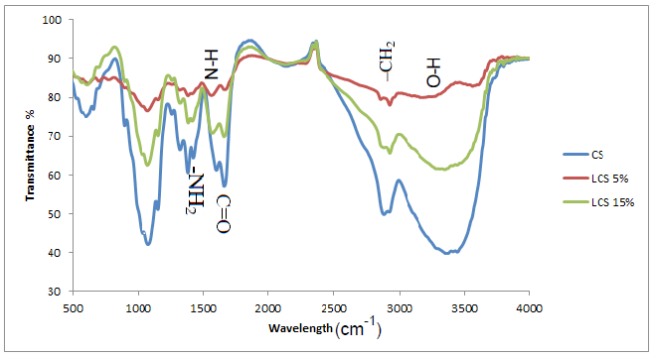

Figure 2: FTIR spectrum of CS and LCS derivative with 5 and 15% DS

In addition, the broadband between (3200–3500cm^{-1}) is related to the bonding of free OH vibration stretching

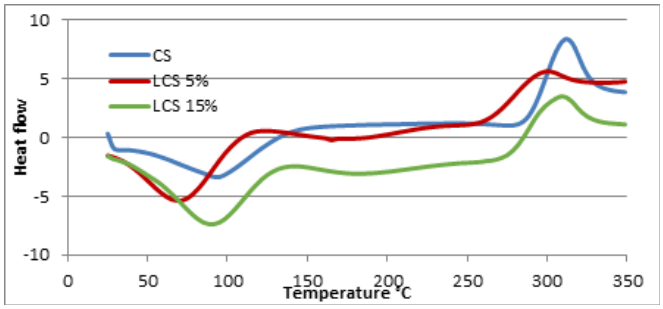

Figure 3: DSC thermograms of (a) Chitosan, (b) LCS 5% and (c) LCS 15% with a temperature range 25-350 °C and a heating rate of 10°C/min.

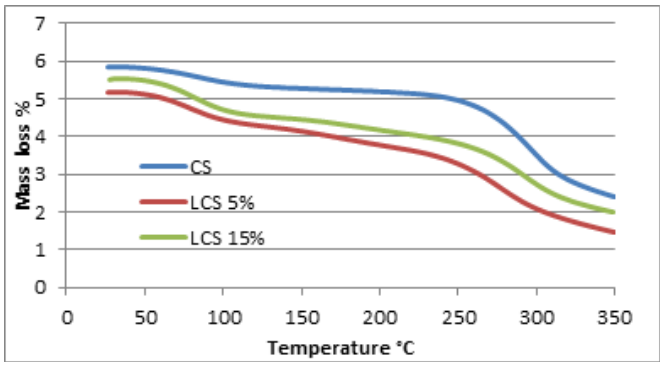

Figure 4: TGA curves for chitosan and two different degrees substituted Lauroyl chitosan with a temperature range of 25-350 °C and a heating rate of 10°C/min.

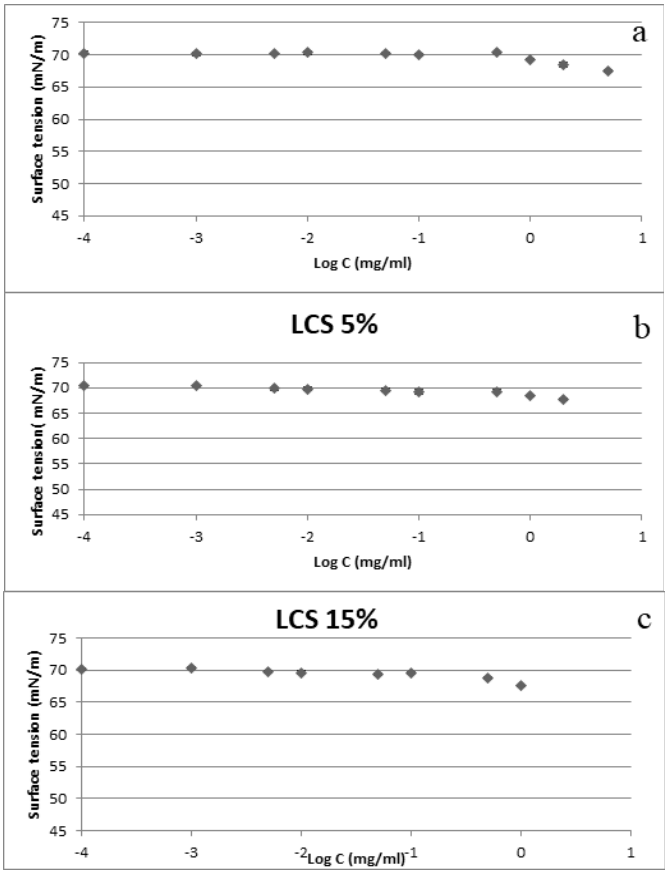

Figure 5: Surface tension–Log concentration plots of a) Chitosan, b) Lauroyl chitosan 5%, c) Lauroyl chitosan 15% no micellar behavior within the measured solubility range.

of Chitosan molecules between inter-and intra-molecular hydrogen. Following acylation, the peak characteristic at (1430 cm^{-1}) is associated with the free NH_2 groups in which it decreased or disappeared after attaching the acyl groups. At the same time, prominent bands at (1655 cm^{-1}) and (1555 cm^{-1}) were detected, which represent the carbonyl (C=O) vibration stretching mode of the secondary amide (amide band I) and amide band II (N-H) bending vibration mode.

The spectra of CS derivatives show new peaks around 2920 and 2850 cm^{-1}, attributed to the asymmetric and symmetric bending vibrations of the methylene (-CH2) group in alkyl chain of the derivatives. These bands are strong in Modified chitosan derivatives in comparison to non-modified CS.[17]

When the acid chloride ratio to chitosan increased, the intensity of vibration of carbonyl (1555 cm^{-1}) and alkyl (2850–2920 cm^{-1}) groups increased clearly.

No peaks were observed in the spectra of the *N*-acylated chitosan derivatives in the range of (1710–1760 cm^{-1}, which are revealing of the presence of O -acyl ester. So, no occurrence of substitution on the hydroxyl group of CS. Consequently, the findings obviously showed that *N*-acylated chitosan derivatives were successfully produced, and the reaction *N*-acylation was selective. These observations are in line with the report by[24]

As shown in Figure 3, the thermal stability of parent chitosan is higher than Hydrophobically modified chitosan. Thus, N-acylation works on decreasing the thermal stability of chitosan.

From DSC curves of chitosan and the derivatives, it is generally observed that inserting of fatty-acid groups on the Chitosan polymer backbone reduces the thermal stability. This reduction in thermal stability is related to an increase in the derivatives' chain mobility compared with the parent chitosan, which disrupts the semi-crystalline structure of the parent chitosan. Thus, leading to the interference of the strong intermolecular hydrogen bonding forces prevalent in the parent chitosan.[3]

From the TGA result, it is obviously noticed that all Chitosan derivatives have low thermal stability than chitosan attributed to the weak bonding of the inter-and extra-hydrogen bond. The thermal stability of Chitosan derivatives increased with increased DS because of the introduction of the hydrophobic interaction.[25]

The mass loss in the TGA curves, as can be seen in Figure 4, exhibited two major steps. The first region ranging from 25 to 110°C, is related to the water evaporation that occurred in the inner network of polymeric. This phenomenon is incompatible with a broad endothermic peak of the DSC curves corresponding to 85°C.

The second region starts from 250 to 350°C related to the thermal decomposition of the polymeric chain.[22] Thermal stability of pure chitosan is higher than the modified chitosan derivatives, whereas the decomposition range of the Chitosan derivatives temperature is broader than chitosan. In this contrast, the result is similar to that of [26]

As the DS of Modified chitosan derivatives increased from 5 to 15%, the onset of decomposition of these derivatives showed a shift to a higher temperature.

The surface activities exhibited by Chitosan and Modified chitosan derivatives were estimated through measurements of the variation of surface tension of aqueous solutions against concentration, as shown in Figure 5.

Surface tension measurements of the LMW Chitosan used in this work indicated the absence of surface activity. Conversely, some studies exhibited that amphiphilic chitosan derivatives invariably demonstrate some surface properties of interest in various fields.[11] However, these derivatives have low aqueous solubility, which limits their application in oral drug delivery.

The micelles formation that occurred by self-aggregation of Hydrophobically is determined by their amphiphilicity along with the modified derivatives of chitosan in the aqueous solution due to the DS and acyls chain length. Based on that, weak Chitosans modified amphiphilicity by using Lauroyl because the length of Lauroyl is (12 carbons). Therefore, Lauroyl chitosan was not proper for self-aggregation and to form micelles in an aqueous solution.[23]

CONCLUSION

The results of this work found that a series of *N*-acylated derivatives of chitosan had been developed using fatty acid (C12) chlorides. The degree of substitution was 5 and 15 fatty acids group per 100 anhydroglucose units. From the chemical characterization by FT-IR and ^{1}H-NMR, it was shown that the amide formation was related between amino groups of chitosan and acid chloride carboxyl groups.

According to DSC/TGA data, the thermal stability of Hydrophobically modified chitosans was low while it was increased with increased DS. The surface tension was combined as a function of concentrations. The Lauroyl-modified amphiphilicity of Chitosan derivatives is poor. However, self-aggregation and the formation of the micelles in an aqueous solution.

Within the calculated range of the determined concentrations, the MTT survival assay suggested no toxicity on $Caco_2$ cell lines. These Chitosan derivatives are suggested to be carriers of hydrophobic drugs.

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