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Low molecular weight quaternised chitosan (I): synthesis and characterisation

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For a better understanding of the behaviour of macromolecules *in vitro* and *in vivo*, their structural and chemical properties that may be influential as experimental variables need to be characterised. *N*-Trimethyl chitosan chloride and *N*-triethyl chitosan chloride have been synthesised from chitosan to increase the solubility range of these polymers. However, little is known about the effect of the degree of quaternisation, molecular weight, viscosity and different substitution groups on the polymer's ability to enhance the transport of large hydrophilic compounds, such as peptide and protein drugs, across intestinal and nasal epithelia and on their toxicity profile. This study describes the synthesis of various quaternised chitosan polymers from low and medium molecular weight chitosan. These polymers were characterised to determine if any relationships between their degree of quaternisation, molecular weight and viscosity could be found which will determine their behaviour as absorption enhancers in future studies.

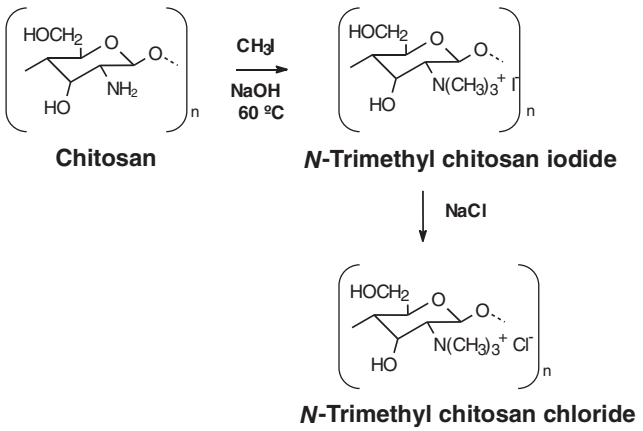
1. Introduction

Chitosan has become well known as a multifunctional biopolymer with a wide range of applications both in the medical and pharmaceutical arena. Chitosan is non-toxic and inexpensive to produce [1]. New research has been predominantly focused on novel applications and uses originating from research indicating that chitosan is an excellent candidate for drug release formulations for most physiological drug delivery pathways. Chitosan oligomers have been suggested as non-viral DNA delivery systems, since they readily form complexes with DNA and protect the DNA against degradation and is also biodegradable [2, 3]. Chitosan oligomers of low molecular weight possess favourable characteristics such as low viscosity and may be suitable for drug inclusion matrices that necessitate an increased rate of drug release compared to the high molecular weight chitosan polymers.

It has been shown that chitosan enhances the absorption of peptide and protein drugs across nasal [4] and intestinal [5] epithelia in acidic environments. Chitosan has an apparent pK_a value of 5.60 and is only soluble in acidic solutions with pH values lower than 6 [6]. This interferes with the biomedical application of chitosan, especially at the physiological pH value (pH = 7.4) where chitosan is insoluble and ineffective as an absorption enhancer. *N*-Trimethyl chitosan chloride is a partially quaternised derivative of chitosan with superior water solubility and absorption enhancing properties for peptide and protein drugs, especially in neutral and basic environments and has been synthesised in the past from high molecular weight chitosan for this purpose [7, 8].

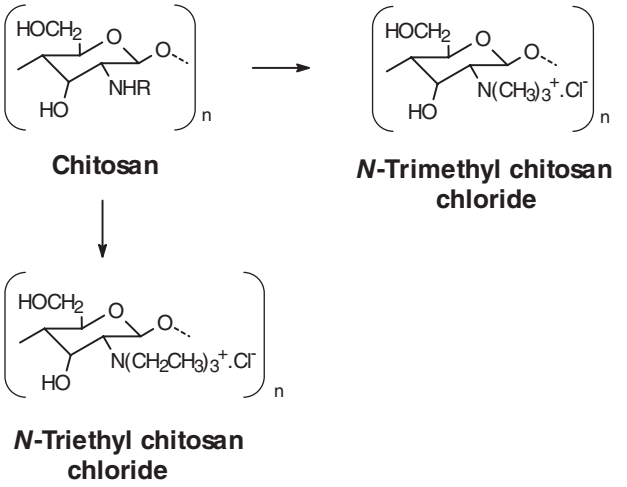
The charge density, as determined by the degree of quaternisation, and the molecular weight (MW) are important factors that influence the absorption enhancing effects and molecular conformation of this polymer. During synthesis, substitution with methyl groups to the amino groups on the C-2 position of chitosan forms quaternary amino groups with fixed positive charges in the TMC structure as shown in Scheme 1. The degree of quaternisation gives an indication of the reaction time during which the molecule was exposed to an alkaline environment and increased temperatures. There is also a significant degradation of the polymer chain with a decrease in the molecular weight corresponding with the respective reaction time for the polymer [9]. Starting with a different parent molecule (medium molecular weight chitosan and low molecular

Scheme 1



Chitosan **N-Trimethyl chitosan iodide** **N-Trimethyl chitosan chloride**

Scheme 2



weight chitosan oligosaccharide) addition of methyl and ethyl groups to the polymer chain backbone will result in specific polymers with unique and controllable characteristics. The chemical structures of the different polymers synthesised in this study are shown in Scheme 2.

Due to the differences in polymer structure and chemical characteristics it is important to completely characterise derivatives of chitosan to understand the behaviour of these polymers in future absorption enhancement and toxicity experiments. In this study several medium molecular weight polymers [*N*-trimethyl (TMC-M) and *N*-triethyl (TEC-M)] and quaternised chitosan oligomers [*N*-trimethyl (TMO) and *N*-triethyl (TEO)] were synthesised with low (L) and high (H) degrees of quaternisation by reductive methylation of the parent polymer chain with variation in the number and duration of the reaction steps [6, 8]. These synthesised polymers were characterised to set parameters for future absorption enhancing experiments, DNA delivery experiments, for the development of novel dosage forms and for ciliary beat frequency measurements to evaluate their cellular toxicity profile.

2. Investigations, results and discussion

2.1. Degree of quaternisation

The degrees of quaternisation (DQ) as calculated from the ^{1}H NMR spectra of the synthesised polymers are shown in Table 1. The DQ of the medium molecular weight TMC polymers (TMC-M) were found to be in the proximity of methylated high molecular weight TMC polymers synthesised previously by a similar reaction protocol [10]. It was noted that even when a shorter reaction time was used for the methylation of the oligosaccharides it resulted in a higher DQ than expected (Table 2). However, the DQ of these oligomers differed significantly and would be sufficient for investigation of polymer charge related characteristics. The DQ of the ethylated oligosaccharides and the ethylated medium molecular weight polymers were found to be lower compared to the methylated oligosaccharides, even when exposed to longer reaction times and higher temperatures. This may be due to the lower reactivity of ethyl iodide when compared to methyl iodide as well as sterical hindrances between monomers during the reaction. The differences between the DQ for the ethylated polymers were also sufficient for further relevant investigations.

Table 1: Degree of quaternisation (DQ), weight average molecular weight (MW), polydispersity (Mw/Mn), intrinsic ([η]) and relative viscosity (η_{rel}) of the synthesised polymers

Polymer	DQ (%)	MW (g/mol)	Mw/Mn	[η]	η_{Rel}
Primex Chitoclear	–	77670	2.05	8.082	–
TMC-ML	23.98	74180	1.85	2.255	–
TMC-MH	62.73	62250	1.98	0.669	–
TEC-ML	10.21	91120	1.90	3.537	–
TEC-MH	26.71	82810	2.03	1.670	–
Primex oligomer	–	13490	1.52	–	1.046
TMO-L	31.2	9815	1.29	–	1.036
TMO-H	54.6	7597	1.95	–	1.016
TEO-L	15.1	11020	1.48	–	1.038
TEO-H	27.9	9032	2.04	–	1.031

Table 2: Number of reaction steps, time and temperature used to synthesise the quaternised polymers

Polymer	Temperature (°C)	Number of reaction steps	Time (min)
TMO-L	60	1	30
TMO-H	60	3	3×30
TMC-ML	60	1	45
TMC-MH	60	4	$3 \times 45 + 30$
TEO-L	80	1	30
TEO-H	80	3	3×30
TEC-ML	80	1	45
TEC-MH	80	4	$3 \times 45 + 30$

2.2. Molecular weight determination

The weight average molecular weights (Mw) as obtained by SEC/MALLS characterisation is shown in Table 1. Degradation of the medium molecular weight TMC polymers during the synthesis was noted, similar to those reported previously for high molecular weight TMC [9]. There was also significant degradation during synthesis of the methylated and ethylated oligosaccharides. As the degree of quaternisation of the polymer increased, the Mw decreased as a function of the reaction time used for the synthesis. Together with the decrease in the molecular weight of the polymers, an increase in the polydispersity for the polymer samples was found, indicating that degradation of the original polymer resulted in a greater distribution of shorter chain lengths (Table 2). Polymerisation of the TEC-M polymers was noted, resulting in a higher Mw for these products. A plot of the Mw degradation of the quater-

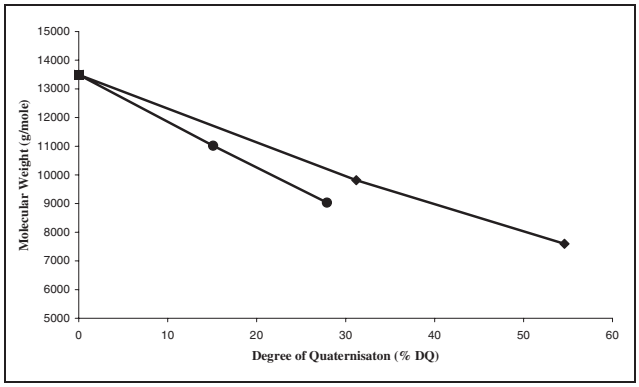


Fig. 1: Molecular weight degradation for the synthesised oligomers. Chitosan (■), TMO (◆), TEO (●)

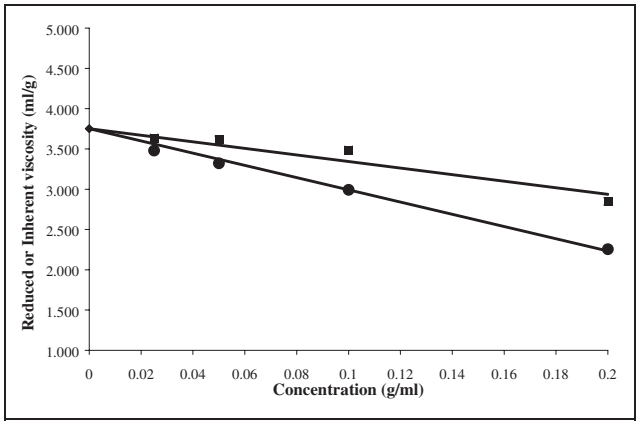


Fig. 2: Intrinsic viscosity [η] as determined from the plot of reduced (η_{Red}) (■) and inherent viscosity (η_{inh}) (•) of TMC-MH against concentration

nised oligomers is given in Fig. 1 where the Mw is plotted against the DQ as an indication of the reaction time.

2.3. Viscosity determination

The trends found with SEC/MALLS characterisation were also shown by the viscosity experiments (Table 1). The viscosities of the synthesised polymers decreased compared to the parent polymer and can be related to the DQ of the polymers. Significant molecular weight degradation was found for the medium molecular weight TMC polymers as the viscosity of the polymers is a function of the Mw as described in the following equation (eq. 1)

$$[\eta] = KM^{\alpha} \qquad (1)$$

where K and α are conformation constants for a given polymer-solvent system and M is the molecular weight of the polymer. An example of the plot of η_{red} and η_{inh} against the concentration of the polymer solution used to determine the intrinsic viscosity (y-intersection) is given in Fig. 2. Similar plots were obtained for the other chitosan derivatives.
As the viscosity of the low molecular weight polymers were found to be very low only the relative viscosities of these polymers are given as compared to the solvent in eq. (4). A decrease in the Mw of the oligomers was also observed.

2.4. IR characterisation

IR spectral results of all samples were compared to determine possible differences with regard to structural changes during synthesis. Fig. 3 shows the IR spectra of TMC-M (L and H) and the IR spectra of the original Primex Chitoclear®. Similar differences were found when the other polymers were compared against the IR spectrum of the parent polymer. According to Silverstein and Bassler [11] the bands between 1220–1020 cm^{-1} in the figures are usually assigned to C-N stretching vibrations. The increase in this band may be attributed to the increase in C-N bonds during the methylation and ethylation of the chitosan polymers.
It can be concluded that significant differences were found between the novel polymers synthesised after variation in the existing reaction protocol for high molecular weight TMC [10]. The differences in the degree of quaternisation, Mw and viscosities of these polymers may have an important effect on the behaviour of these polymers *in vitro* and *in vivo*. Increased knowledge of the behaviour of these polymer properties will lead to a better understanding of the effect of these polymers on their absorption enhancing capabilities, toxicity and properties in solid dosage from design.

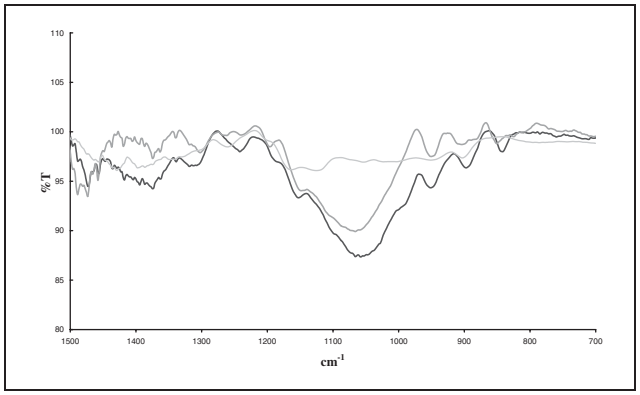


Fig. 3: IR spectra of TMC-ML (————) and TMC-MH (————) superimposed on the spectrum of the original Primex Chitoclear® (————).

3. Experimental

3.1. Materials

Primex Chitoclear® medium molecular weight (93.2% deacetylated chitosan) and Primex chitosan oligosaccharide (79.1% deacetylated chitosan oligosaccharide) was a gift from Primex Ingredients ASA (Avaldsnes, Norway). The chitosan polymers were used as received. Iodomethane, sodium hydroxide pellets, sodium chloride, acetic acid, ammonium acetate, absolute ethanol, diethylether and *N*-methyl-2-pyrrolidone (Riedel-de Haën, South Africa) sodium iodide and iodoethane (Fluka, Switzerland) and potassium bromide (Merck, Germany) were used as received. All chemicals used in the synthesis process were of analytical grade.

3.2. Synthesis of the quaternised polymers

Quaternised chitosan polymers were synthesised by reductive methylation and ethylation of chitosan that was accomplished by a chemical reaction between chitosan and iodomethane/iodoethane in the presence of sodium hydroxide based on methods previously described [6, 10, 12]. The reaction step was repeated several times with the product obtained from each step to increase the degree of substitution. A compilation of the number and duration of reaction steps and the temperature used to synthesise the different polymers is shown in Table 2.

3.3. Degree of quaternisation

3.3.1. TMC-M and TMO

^{1}H NMR spectra of the TMC polymers were obtained with a 600 MHz Bruker DMX-600 spectrometer (Karlsruhe, Germany) by dissolving samples of the polymers in D_2O at 80 °C. The degree of quaternisation was calculated with data obtained from the ^{1}H NMR spectra according to a previously described method [6, 10] with eq. (2):

$$\%DQ = \left[\frac{[(CH_3)_3]}{[H]} \times \frac{1}{9}\right] \times 100 \qquad (2)$$

where: % DQ is the degree of quaternisation as a percentage, $[(CH_3)_3]$ is the integral of the trimethyl amino group at 3.1 ppm and [H] is the integral of the ^{1}H peaks between 4.7 and 5.7 ppm.

3.3.2. TEC-M and TEO

The degree of quaternisation for the ethylated polymers was calculated with data obtained from the ^{1}H NMR spectra according to a previously described method [12] with eq. (3):

$$\%DQ = \left[\frac{[(CH_2CH_3)_3)]}{[H]} \times \frac{1}{15}\right] \times 100 \qquad (3)$$

where: % DQ is the degree of quaternisation as a percentage, $[(CH_2CH_3)_3]$ is the integral of the triethyl amino group at 1.3 ppm and [H] is the integral of the ^{1}H peaks between 4.7 and 5.7 ppm.

3.4. Molecular weight determination

The weight average molecular weights (Mw) of the synthesised polymers were measured with a size exclusion chromatograph (SEC) (Hewlett Packard 1100, USA) connected to a multi-angle laser light scattering detector (MALLS) that consisted of a laser photometer (Dawn DSP, Wyatt Technology Corporation, USA) coupled to a refracting index detector (ERC 7515A, Japan).

The polymers were dried in a vacuum oven at 40 °C for 24 h and prepared in solutions of 5 mg/ml in deionised water from which 0.8 ml samples were filtered through 0.2 μm membrane filters and collected in chromatographic sample vials. The mobile phase consisted of a 0.2 M ammonium acetate solution and the pH was adjusted to 4.50 with acetic acid. The experimental setup consisted of a HP 1100 vacuum degasser, isocratic pump and auto sampler connected to a TSK-guard PWH (Toso Haas, Japan) inline column. The size exclusion columns included a TSK G6000 PW (Toso Haas, Japan, inside diameter = 7.5 mm, length = 30 cm, particle size > 17 μm, pore size > 1000 Å) column connected in series with a TSK G5000 PW (Toso Haas, Japan, inside diameter = 7.5 mm, length = 30 cm, particle size = 17 μm, pore size = 1000 Å) column. Samples of the polymer solutions (100 μl) were injected at a flow rate of 0.8 ml/min and were analysed with the laser photometer (He/Ne laser, $\lambda = 633$ nm) and the refracting index detector. The data from the detector was interpreted with a computer using Astra® for Windows (Wyatt Technology Corporation, USA) and the weight average molecular weight and polydispersity of the samples were calculated.

3.5. Viscosity determination

Intrinsic viscosities of the synthesised polymers were measured according to the method described in the British Pharmacopoeia (2000) for dextran with a size D tube for the medium molecular weight polymers and a size CF 50 glass U-tube viscometer for the low molecular weight polymers at 25.0 °C. Solutions of the polymers were prepared in 0.2% v/v acetic acid in concentrations of 0.2, 0.1, 0.05 and 0.025% w/v. The temperature of the solutions was kept at 25.0 °C in a waterbath throughout the experiment. The flow-through time of each solution and the solvent were measured in the U-tube. The viscosity ratio for each solution is expressed by the ratio of the viscosity of the solution (η) to the viscosity of the pure solvent (η_o) at the same temperature [13]. This ratio is referred to as the relative viscosity (η_{rel}) as shown in eq. (4).

$$\eta_{rel} = \eta/\eta_o \quad (4)$$

The reduced (η_{red}) and inherent (η_{inh}) viscosity of the solution are calculated by the following equations:

$$\eta_{red} = (\eta_{rel} - 1)/c \quad (5)$$

$$\eta_{inh} = (\ln \eta_{rel})/c \quad (6)$$

The extrapolated value (where $c = 0$) of the straight lines when η_{red} and η_{inh} is plotted as a function of c (concentration) is the intrinsic viscosity ($[\eta]$) of the polymer, which gives an indication of the molecular weight according to the eq. (1).

3.6. IR characterisation

IR spectra were recorded on a Nicolet Nexus 470 FT IR ESP spectrometer (Madison, USA) over a range of 4000–400 cm^{-1} using the KBr reference technique. Samples weighing approximately 2 mg were collected after drying for 1 h at 40 °C and mixed with 200 mg of KBr before analysis.

References

1 Paul, W.; Sharma, C. P.: S. T. P. Pharma Sci. **10**, 5 (2000)
2 Florea, B. I.; Thanou, M.; Geldof, M.; Meaney, C.; Junginger, H. E.; Borchard, G.: Proceedings of the 27th International Symposium on Controlled Release of Bioactive Materials. 27 : 7315 (2000)
3 Huang, L.: Proceedings of the 27th International Symposium on Controlled Release of Bioactive Materials. 27 : 337 (2000)
4 Illum, L.; Farraj, N. F.; Davis, S. S.: Pharm. Res. **8**, 1186 (1994)
5 Artursson, P.; Lindmark, T.; Davis, S. S.; Illum, L.: Pharm. Res. **9**, 1358 (1994)
6 Sieval, A. B.; Thanou, M.; Kotzé, A. F.; Verhoef, J. C.; Brussee, J.; Junginger, H. E.: Carbohydr. Polym. **36**, 157 (1998)
7 Domard, A.; Rinaudo, M.; Terassin, C.: Int. J. Biol. Macromolecul. **8**, 105 (1986)
8 Kotze, A. F.; Luessen, H. L.; De Boer, A. G.; Verhoef, J. C.; Junginger, H. E.: Eur. J. Pharm. Sci. **7**, 145 (1999)
9 Snyman, D.; Hamman, J. H.; Kotzé, J. S.; Rollings, J. E.; Kotzé, A. F.: Carbohydr. Polym. **50**, 145 (2002)
10 Hamman, J. H.; Kotzé, A. F.: Drug Devel. Ind. Pharm. **27**, 373 (2001)
11 Silverstein, R. M.; Bassler, G. C.: Spectrometric identification of organic compounds, p. 124, Marcell Dekker, New York, N. Y.
12 Nud'ga, L. A.; Plisko, E. A.; Danilov, S. M.: Zhurnal Obschei Khimii **43**, 2733 (1973)
13 Martin, A.: Physical Pharmacy, 4th ed., p. 622, Lippincott Williams and Wilkins, Baltimore 1993