Depolymerization of HMW into a predicted LMW chitosan and determination of the degree of deacetylation to guarantee its quality for research use.

Amjad M. Qandil[*a,b], **Tamara J. Marji**[c], **Bashar M. Al-Taani**[d], **Aseel H. Khaled**[e], **Adnan A. Badwan**[*e]

[a] Pharmaceutical Sciences Department, College of Pharmacy, King Saud bin Abdulaziz University for Health Sciences, Riyadh 11426, Saudi Arabia
[b] Department of Medicinal Chemistry and Pharmacognosy, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid 22110, Jordan
[c] Al Rayyan Campus, University of Calgary in Qatar, Doha, Qatar
[d] Department of Pharmaceutical Technology, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid 22110, Jordan
[e] Jordanian Pharmaceutical Manufacturing Company, Naor 1170, Jordan

Received: April 11, 2018; *Accepted:* April 15, 2018

Original Article

ABSTRACT

Low molecular weight chitosan (LMWC) is a semi synthetic biopolymer with potential uses as a pharmaceutical excipient. However, made-to-specification, consistent and reliable grades of pure LMWC are scarce. Credible and competent preparation and characterization methods are therefore of utmost importance to specialized laboratories. Although acid hydrolysis of chitosan has been reported, existing protocols produce oligomer mixtures of high polydispersity and may not predict the resultant molecular weights. This study aimed to optimize a mild acid-catalyzed depolymerization of high molecular weight chitosan (HMWC) of 100 kDa and 93% degree of deacetylation, as well as, develop a standardized protocol that would enable predetermination of the resultant molecular weight and re-acetylation within the ranges of 1.4-28.1 kDa and 55.1-81.1%, respectively. Molecular weight measurements were extracted and verified based on dynamic viscosity and dynamic light scattering. The degree of deacetylation was measured by H^1 NMR. These protocols were found to be efficient, time- and cost-effective, reproducible, and could produce LMWC with the desired specifications, quality and yield for research purposes.

KEYWORDS: Low molecular weight chitosan, LMWC, depolymerization, acid-catalyzed hydrolysis, acetylation, degree of deacetylation, DDA, excipients

INTRODUCTION

High molecular weight chitosan (HMWC) is usually derived from chitin through base-catalyzed hydrolysis (1-4). Both chitin and chitosan consist of a repertoire of glucosamine and N-acetylglucosamine units linked by β-(1→4) glycoside bonds, as shown in Figure 1 (5, 6). In chitosan, glucosamine constitutes more than 50% of the building units, hence, the degree of deacetylation (DDA) of chitosan ranges between 50-100%.

HMWC is a hygroscopic polymer, soluble in dilute acid solutions e.g., dilute hydrochloric and acetic acids (7) and forms highly viscous solutions (8). HMWC has been investigated in immediate and controlled release drug formulations (9,10). Until now, however, no commercial products have reached the market. Low Molecular Weight Chitosan (LMWC) can be useful for research and in the pharmaceutical

---

*Corresponding address: Pharmaceutical Sciences Department, College of Pharmacy, King Saud bin Abdulaziz University for Health Sciences, Riyadh 11426, Saudi Arabia; Tel.: +966-11-4295007, E-Mail: qandila@ksau-hs.edu.sa

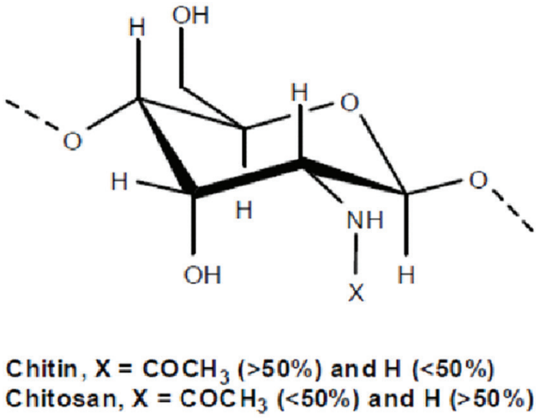

Figure 1 Chitin X = COCH3 (>50%) and H (<50%), Chitosan X=COCH3 (<50%) and H(>50%)

industry as the molecular weight (MW) and degree of deacetylation (DDA) can significantly affect chitosan's physicochemical properties, such as solubility and viscosity (11,12). LMWC with a high DDA is soluble in a much wider ranges of pH and forms much less viscous solutions compared to HMWC (2). It has been shown that 100% deacetylated LMWC can form complexes with ibuprofen (13) and diclofenac (14). Furthermore, it has been demonstrated that ibuprofen can be linked to LMWC through ionic interactions (13). Diclofenac-chitosan complexes showed promising results when incorporated into controlled release formulations (14). It has also been reported that when LMWC is solubilized in the interior of reverse micelles, nano-sized particles are generated made by dispersing aqueous surfactant-cosurfactant mixtures in oleic acid, (15). Furthermore, xanthan gum and LMWC have been used as a binary mixture to form a universal matrix designed to be used in controlled release products. In such matrices, the incorporated drug release can mimic zero order (10). Recently, LMWC was utilized in the formulation of protein drugs, e.g., insulin (16,17), which increases its scope and function as an excipient in protein drug delivery systems. Furthermore, derivatizing LMWC may disrupt inter- and/or intra- molecular bonds affecting its crystallinity and alter its solubility in various solvents. For example, hydrophobic substitution, such as with fatty acyl amides, generates water-insoluble derivatives by hydrophobic self-assembly (18,19).

Such a wide spectrum of features may necessitate specific preparation of chitosan polymers to produce materials with specific physicochemical properties, e.g., molecular weight, degree of deacetylation and polydispersity. Although the literature is replete with methods to depolymerize chitosan, there are few empirical studies that allow for prediction of depolymerized chitosan characteristics.

Depolymerization of HMWC yields LMWC with MW < 50kDa, with various DDA. The depolymerization can be performed by a variety of methods including acid hydrolysis (3, 7, 20, 21), enzymatic degradation (21, 22, 23) or thermal degradation (24-26). Industrially, acid degradation using hydrochloric acid is preferred (7) as this method is usually simple, easy (20) and produces reasonably predictable mixtures of LMWC (19). Nevertheless, using concentrated hydrochloric acid produces low yields of LMWC and high yields of monomers (3, 7, 21). This study applied an optimized acid-catalyzed depolymerization of HMWC of 100 kDa and 93% DDA into sufficient quantities of LMWC, and developed standardized protocols that enable in-lab predetermination of the resultant molecular weight and re-acetylation degree in the ranges of 1.4-28.1 kDa and 55.1-81.1%, respectively.

MATERIALS AND METHODS

Materials and Methods

High molecular weight chitosan (HMWC, 100 kDa, 93% DDA) was purchased from Hongjo Chemical Company Ltd® (China) and used as is without further purification. N-acetylglucosamine (>98.0% for biochemistry) was purchased from Merck (Darmstadt, Germany). Acetic anhydride was purchased from Acros Organics® (Geels, Belgium). Concentrated hydrochloric acid and absolute ethanol were all of analytical grade were obtained through local vendors. All chemicals were used as received without further purification. Potassium bromide for IR and deuterium oxide (D_2O) or deuterium chloride (DCl) for NMR were purchased from Acros Organics® (Geels, Belgium). Water was double distilled (S < 2 µS/cm). Centrifugation was performed using a Sorvall

SuperSpeed RC2-B centrifuge (Ivan Sorvall-Inc.®, Norwal, CT, USA). The prepared LMWC polymers were dried using a Heto PowerDry PL9000 freeze dryer (Thermo Fisher Scientific Inc®, Waltham, MA, USA). MestReNova 8.1 was used to process the H^1 NMR spectra (Mestrelab Research®, Spain).

ChemDraw Ultra 10 was used for chemical drawing (Cambridgesoft®, USA). Viscosity was measured using Sinewave Vibro SV-10/SV-100 Vibro viscometer (KSV Instruments®, Helsinki, Finland). Viscosity Standards were purchased from Poulten Selfe and Lee® (UK). DLS was performed using a Zetasizer nano-ZS (Malvern, Worcestershire, UK).

Preparation of low molecular chitosan (LMWC) by acid-catalyzed hydrolysis of high molecular weight chitosan (HMWC)

HMWC of 100±14.7 KDa (10.0g) was dissolved in 0.1 M hydrochloric acid (830mL) at room temperature forming a solution with pH 1.4. Concentrated 37% hydrochloric acid (170 mL) was added to give a final concentration of 2 M for hydrochloric acid and 10 g/L for HMWC. The reaction mixture was stirred with a magnetic stirrer (750 RPM) at 100°C for 1, 2, 3, 4, 6, 12, and 24 hours. After each specified time interval, absolute ethanol (1.5 L) was added to the reaction while hot, and the mixture was left overnight to form a precipitate. The precipitate was filtered, then, washed with ethanol to remove excess HCl (indicated by a non-changing pH value) and centrifuged at 4000 g for 30 minutes after each wash. The residue was then freeze-dried at a condenser temperature of 10°C, drum temperature of -85°C and a pressure of 90 hPa, to obtain the desired LMWC polymers in the form of hydrochloride salts. The resultant moisture content of the LMWC was not measured. To perform the reactions in 1 M hydrochloric acid; 0.1 M HCl (925 mL) was used to dissolve HMWC (10.0 g) and 37% HCl (75 mL) was subsequently added. For reactions in 3 M hydrochloric acid; 0.1M HCl (750 mL) was used to dissolve the HMWC (10.0 g) and 37% HCl (250 mL) was subsequently added.

Preparation of Acetylated LM WC (Actl-LMWC)

Fully deacetylated LMWC hydrochloride (1.0 g) was dissolved in de-ionized water (100 mL) while stirring. The pH was adjusted to 6.25 using 6 M NaOH. Depending on the intended acetylation percent, different molar ratios of LMWC to acetic anhydride (Ac_2O) were used, with Ac_2O being the limiting reagent. The amount of Ac_2O required for each reaction was calculated using Equation 1.

$$\text{Weight of } Ac_2O = \frac{MR_{\textit{Acetic anhydride}}}{rMW_{\textit{Glucosamine HCl}}} \times MW_{\textit{Acetic anhydride}} \qquad \text{Eq. 1}$$

Where, $rMW_{\text{Glucosamine HCl}}$ is the relative molecular weight of glucosamine HCl equivalent to the molecular weight glucosamine HCl minus the molecular weight of a water molecule (197.62 g/mol) and $MW_{\text{Acetic anhydride}}$ is the molecular weight of Ac_2O (102.09 g/mol): For molar ratio (1: 0.15) (LMWC:Ac_2O), $MR_{\text{Acetic anhydride}}$ = 0.15; the solution was stirred for 10 minutes after the addition of 77.4 mg Ac_2O. For the molar ratio (1:0.6) (LMWC:Ac_2O): 309.7 mg was added in two equal portions, 5 minutes apart, and the pH was adjusted to 6.25 using 6 M NaOH before adding the second portion of acetic anhydride. The reaction mixture was transferred into a dialysis tube with specified pore size depending on molecular weight of LMWC used. The tube was then placed in a beaker containing de-ionized water (4 L) and was gently agitated for 24 hours in order to remove any by-products. Finally, the solution left in the tube, which contains the desired product, was poured in Petri dishes and allowed to dry in the oven at a temperature of 40 ± 3°C.

Determination of the average molecular weight (MW) of LMWC using viscosity measurements

Samples from hydrolyzed products (2.5 g or 5 g) were dissolved in 0.1 M HCl or distilled water (50 mL), respectively. Serial dilutions of the stock solution were made in distilled water to give working concentrations of 5, 4, 3, 2, 1 w/v (%) or 10, 7, 5, 3, 1 w/v(%). Viscosity was measured at 25±0.5°C. All measurements performed in triplicate and the average was used for calculations. A two-point calibration was performed using standards of 3.5 and 7.4 mPa.s at 25±0.5°C.

The average molecular weights of the different LMWC polymers were determined using the Mark-Houwink equation shown in Equation 2.

$$[\eta] = \mathrm{kMa} \qquad \text{Eq. 2}$$

Where: [η] is the intrinsic viscosity; M is the viscosity average molecular weight; and k and a are coefficients whose values were taken to be 0.00058 and 0.69, respectively, based on a previous study (27).

Determination of the weight average molecular weight (MWW) of LMWC by dynamic light scattering

Determination of the average molecular weight using dynamic light scattering is very sensitive to dirt or dust in the samples, hence, all solvents were filtered, and the prepared solutions were allowed to stand for up to 24 hours, depending on the sample, to ensure complete dissolution. For each sample, the following concentrations were prepared: 1, 0.8, 0.6, 0.4, 0.2 and 0.1 mg/mL. Light scattering intensity of the solvent was measured initially. Rayleigh scattering was assumed for molecular weight measurements. The software reduced the Rayleigh equation to a linear form from which a Debye plot was generated to show the variation in the average intensity versus concentration. The intercept corresponding to zero concentration was determined and then used to calculate the average molecular weight of polymers. Instrument settings were 25°C at an scattering angle of 173° with vertical polarization at 633 nm.

Degree of Deacetylation (DDA) of LMWC and Acetyl-LMWC using UV Spectroscopy

The degree of deacetylation (DDA) for LMWC and acetyl-LMWC was determined using UV spectroscopy (28, 29). A sample product (0.25 g) was dissolved in distilled water (50.0 mL). 1.0 mL of the resultant solution was diluted to 100 mL with distilled water. Absorbance was measured at λ = 200-205 nm as the first derivative of the absorbance curve. The pH of the solution was measured.

To prepare a calibration curve, solutions of N-acetylglucosamine in distilled water (1.0, 5.0, 15.0 and 35.0 µg/mL) were prepared and their absorbance measured at λ = 200-205 nm. A standard curve was constructed by plotting the first derivative of absorbance as a function of the concentration of N-acetylglucosamine, and the slope of the curve was calculated by least squares linear regression. The standard curve was then used to determine the equivalent amount of N-acetylglucosamine for the samples to be examined. Equation 3 was used to calculate the degree of deacetylation (DDA):

$$\mathrm{DDA}\,\frac{(100 \times \mathrm{M1}(\mathrm{C1}\text{-}\mathrm{C2}))}{((\mathrm{m1} \times \mathrm{C1}) - (\mathrm{M1}\text{-}\mathrm{M3}) \times \mathrm{C2}} \qquad \text{Eq. 3}$$

Where; C1 is the concentration (µg/mL) of LMWC/Actl-LMWC in the test solution, C2 is the concentration of N-acetylglucosamine (µg/mL) in the test solution, as determined from the standard curve prepared using the reference solution; M1 is the relative molecular mass of N-acetylglucosamine unit equal to the molecular weight of N- acetylglucosamine minus the molecular weight of a water molecule (203.2 g/mol); and M3 is the relative molecular mass of LMWC/Actl-LMWC as calculated from the pH of the solution, using a $\mathrm{pK_a}$ of glucosamine = 6.8 using Equations 4, 5 and 6.

$$\mathrm{M3} = \mathrm{f} \times \mathrm{M2} + (1 - \mathrm{f})\,\mathrm{X}\,(\mathrm{M2} + 36.3) \qquad \text{Eq. 4}$$

$$\mathrm{f} = \frac{\mathrm{p}}{1 + \mathrm{p}} \qquad \text{Eq. 5}$$

$$\mathrm{p} = 10^{(\mathrm{pH}\text{-}\mathrm{pKa})} \qquad \text{Eq. 6}$$

Where; M2 is the relative molecular weight of glucosamine equal to the molecular weight of glucosamine minus that of a water molecule (197.62 g/mol), f is the fraction of ionized species in the aqueous phase, and p is the partition-coefficient that refers to the concentration ratio of un-ionized species.

Structure and DDA determination of LMWC and Actl-LMWC using H^1 NMR Spectroscopy

H^1 NMR spectra were obtained using a Burker Avance Ultrasheild 400 MHz spectrometer and processed using MestReNova 8.1 software (Mestrelab Research, Spain). Each sample (30 mg) was dissolved in D_2O (1 mL) and was placed in 5 mm, 7" NMR tubes and at least 64 scans were acquired at ambient temperature. For the calculation of DDA by H^1 NMR, Lavertu's *et. al.* validated procedure was used (30) and Equations 7 and 8 were applied.

$$\mathrm{DDA}(\%) = \frac{(\mathrm{H1D}}{(\mathrm{H1D} + (1/3)\,\mathrm{H\text{-}Ac}))} \mathrm{x}100 \qquad \text{Eq. 7}$$

$$\mathrm{DDA}\left(\%\right) = \frac{(1\text{-}((1/3)\,\mathrm{H\text{-}Ac}}{(1/6)\,\mathrm{H\text{-}2\text{-}6}))} \mathrm{x}100 \qquad \text{Eq. 8}$$

Where; H-1D is the integral of the peak corresponding to the proton of H-1 of the deacetylated monomer and H-Ac is the integral of the peak of the three protons of the acetyl group, while H-2-6 is the integral of the peak corresponding to H-2D, H-3D, H-4D, H-5D, H-6D, of the deacetylated monomer and H-6 of the acetylated monomer.

RESULTS AND DISCUSSION

A report for the acid catalyzed hydrolysis of HMWC investigated the effects of acid concentration, temperature, and reaction time on the depolymerization of chitosan with MW > 440 kDa (31). However, the authors did not characterize the product structure, and very small scale reactions (10 mg) were performed, which limited synthetic scalability, and adequate yields for experimentation. Another report by Tsao et. al. did not mention the amount of HMWC used for the reaction, although it did specify the weight % of HMWC used (32). The latter study investigated the kinetics of hydrolysis using acetic acid of various concentrations. Although both reports provided very important information for the reaction and its kinetics, this current work offers an empirical reproducible systematic procedure for the production of relatively large amounts of made-to-specification LMWC with yields adequate for use in further studies.

Preparation of low molecular weight chitosan (LMWC) by acid-catalyzed hydrolysis of high molecular weight chitosan (HMWC)

Aqueous acid digestion of HMWC was performed at 100°C for specified time intervals. The resultant LMWC was precipitated using ethanol followed by centrifugation. The precipitate was filtered, then freeze-dried. The yields of the dried product, shown in Table 1, were calculated taking into account the loss of the acetyl group during depolymerization. These yields were greater at lower concentrations of HCl and/or at shorter reaction times. When 3 M HCl was used, the reaction was unpredictable when allowed to progress for a duration of greater than three hours (see below).

Table 1 The viscosity average molecular weights (MW), percent yields and degree of deacetylation (DDA) of depolymerized chitosan using different concentrations of hydrochloric acid for different times (up to 24 hours for 1 and 2 M HCl reactions, and up to 3 hours for the 3 M HCl reaction) measured by first derivative UV detection.

CONCENTRATION OF HCl (M)	REACTION TIME (h)	MW (kDa)	YIELD (%)	DDA (%)
1	1	44.3±2.3	92.1	101.4
	2	30.7±0.8	95.5	103.4
	4	23.5±3.2	84.5	104.4
	6	19.9±0.2	89.1	104.4
	12	16.1±0.2	88.4	105.4
	24	14.2±0.3	85.2	103.4
2	1	28.1±0.2	80.8	104.4
	2	15.6±0.2	84.5	105.4
	3	10.1±0.8	83.2	104.4
	4	6.8±0.1	82.0	104.4
	6	5.1±0.0	87.7	103.4
	12	2.7±0.0	68.8	103.4
	24	2.7±0.3	42.0	99.6
3	1	4.7±0.9	95.4	100.4
	2	5.9±0.5	90.6	100.4
	3	4.1±0.0	81.8	98.4

An interpretation of a typical NMR spectrum of a fully deacetylated LMWC is summarized in Figure 2(a). The effect of the concentration of the depolymerizing acid is evident in the NMR spectra shown in Figure 3, the more concentrated the acid, the higher the resolution of NMR spectrum of LMWC. The spectra shown in Figures 3a and 3b have the highest and the least resolution respectively. This augmentation in the resolution can be attributed to the reduction of the average molecular weight of produced LMWC as the concentration of hydrochloric acid was increased. An explanation could be the fact that as the molecular weight of LMWC decreased, there was less averaging of proton signals, which in turn resulted in sharper peaks in the NMR spectrum.

A similar effect was noticed for the reaction time as seen in Figures 4 and 5. For the same concentration of the depolymerizing acid, as the time of the reaction increased the resolution of the spectrum increased. This was also attributed to the progressive reduction in MW. The effect of MW on NMR can be seen when comparing the spectrum of HMWC in Figure 4a to that of LMWC (b-g) produced using 2 M HCl for variable reaction time intervals. These spectra also provide indirect evidence for the narrow dispersion of molecular weights of the LMWC produced from each reaction and also imply that negligible, if any, amounts of monomers were isolated with the purified products. The first claim can be explained by the fact that the general features in the NMR spectra; peak shape and chemical shift, are common between all the produced LMWC regardless of the molecular weight, and the only major change is the enhanced resolution as the molecular weight decreases. The second claim can be attributed to the absence of any significant peaks that correspond to the glucosamine monomer, indicating high yields reported of true LMWC for most reactions. It can also be observed that none of the spectra show an acetyl peak at around $\delta = 2$ ppm, which may be interpreted to imply that acid hydrolysis of the parent HMWC (100kDa, 93% DDA) produced fully deacetylated LMWC polymers (2, 33).

Determination of the viscosity average molecular weight (MW) of LMWC

Different methods were used for the determination of chitosan molecular weight such as light scattering techniques (2-4, 34), Ostwald's viscometer (35) and Ubbelohde capillary viscometer (3, 36, 37), which utilizes the Mark-Houwink equation (Equation 2). In this equation, the intrinsic viscosity is the intercept of the regression line of the inherent viscosity or the reduced viscosity vs. the concentration of the sample, w/v (%). In general, the closer the values of the two intercepts, the more accurate the viscosity measurements and hence the more accurate the calculation of average molecular weights. Viscosity of the sample solution, η, is divided by viscosity of solvent, η_o, to obtain the relative viscosity, η_{rel}. The inherent viscosity, η_{inh}, equals to $\ln(\eta_{rel})/C$, where C is the concentration of the sample as w/v (%), whereas, the reduced viscosity, η_{red}, equals to $(\eta_{rel}-1)/C$. Two intrinsic viscosity values will be obtained for each of LMWC produced by the hydrolytic reactions (i.e. one from inherent viscosity and another from reduced viscosity). The viscosity average of these two values was used to calculate the average molecular weight of product. Table 1 summarizes viscosity average molecular weights and yields of LMWC obtained from each reaction, as well as the DDA percent calculated from the first derivative UV method.

Figure 6(a) provides a graphical representation of the decrease in average molecular weight of LMWC as a function of reaction time for the reactions in 1 M and 2 M hydrochloric acid. For the reactions performed in 1 M HCl, the curve fits a predictive power equation where molecular weight = 40.604 × (Reaction Time)-0.359 with $R^2 > 0.97$ while for the reactions performed in 2M HCl, the equation was, molecular weight = 28.165 × (Reaction Time)-0.951 with $R^2 > 0.99$ shown in Figure 6(a). The foregoing was confirmed by the linear relationship obtained when log MW was plotted against log time for the 1 and 2 M HCL experiments (Figures 6b and 6c). To validate the equation for LMWC depolymerization in 2 M HCl, the average MW of some independently prepared samples were determined in the same manner as explained and were then compared to their average MW calculated from the equation obtained. Figure 6(d) shows acceptable agreement between the calculated values of molecular weight and the experimental data with slope = 1.0376. Hence, these equations can be used to predetermine

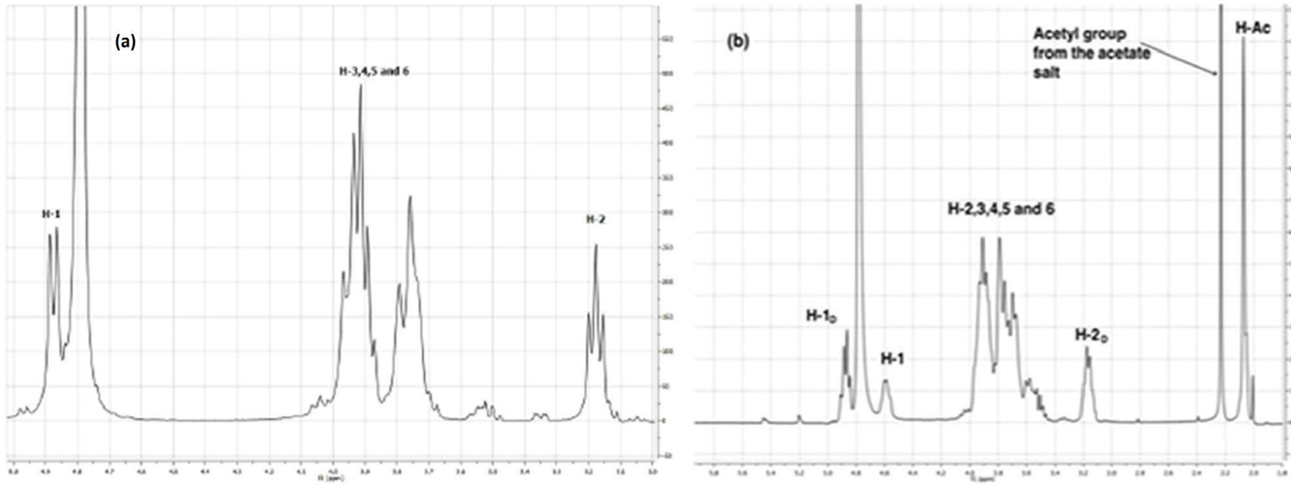

Figure 2 (a) NMR Spectrum for fully deacetylated LMWC hydrochloride salt (b) H^1 NMR spectrum for partially acetylated LMWC acetate salt.

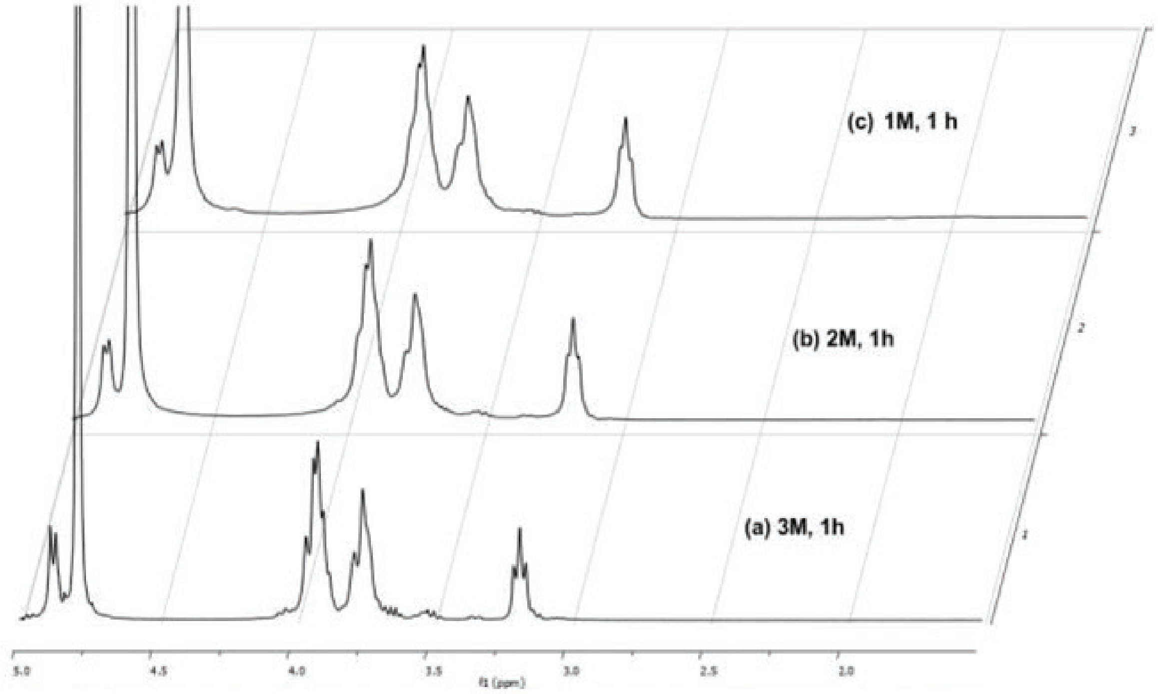

Figure 3 H^1 NMR spectra of LMWC produced after 1 hour using (a) 3M HCl (b) 2M HCl and 1M HCl

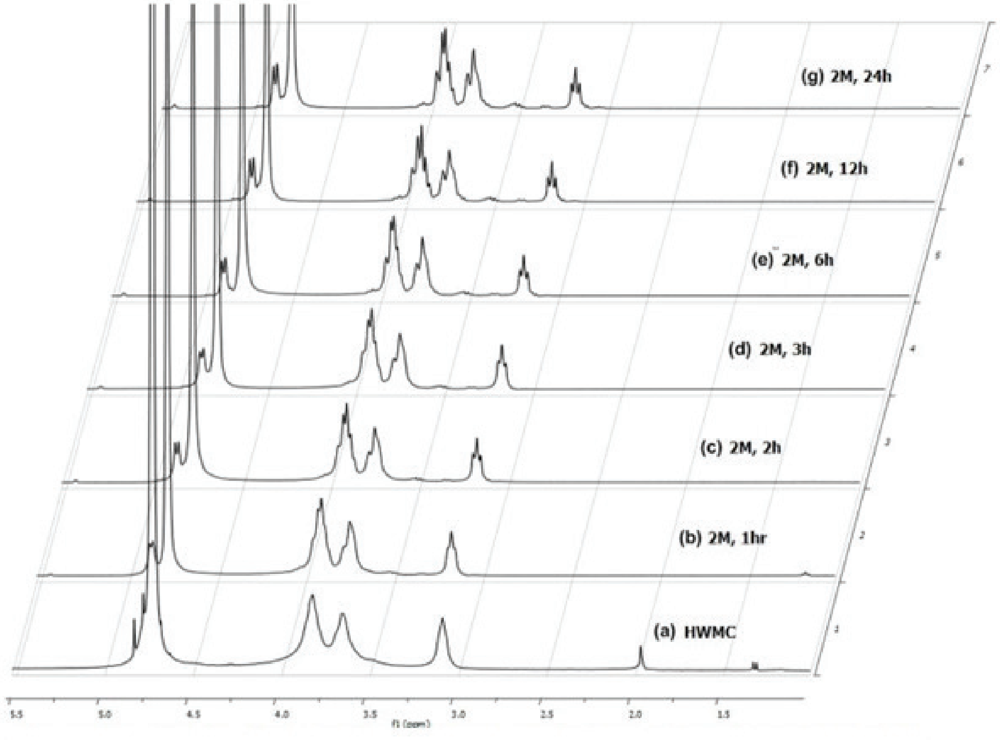

Figure 4 NMR spectra of (a) HMWC and LMWC produced after (b) 1 hour, (c) 2 hours, (d) 3 hours, (e) 6 hours, (f) 12 hours and (g) 24 hours in 2M HCl

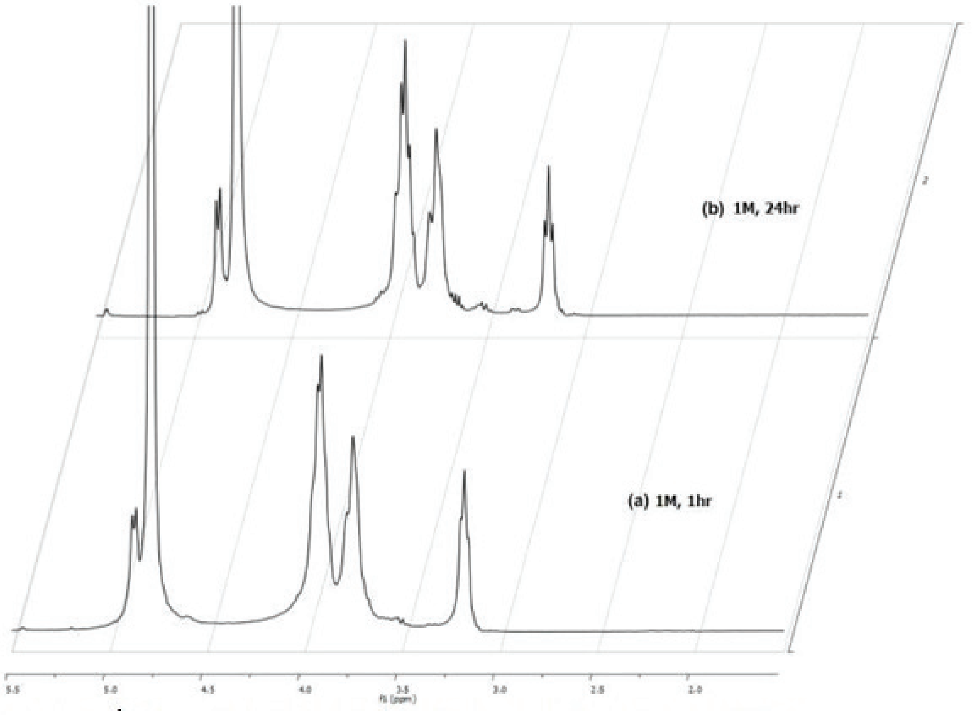

Figure 5 H^1 NMR spectra of LMWC produced after (a) 1 hour and (b) 24 hours using 1M HCl

the reaction time that is needed to obtain LMWC polymer using 1 M and 2 M hydrochloric acid. The LMWC produced by depolymerization in 3 M HCl, however, showed mathematically predictable viscosity data for reaction times 1, 2 and 3 hours only. Longer reaction times gave highly scattered viscosity values, which could indicate that the products were of a polydisperse nature.

Determination of the weight average molecular weight (MWW)

To evaluate the polydispersity of the prepared LMWC, their weight average molecular weights were measured using dynamic light scattering (DLS) (38). In addition, static light scattering (SLS) has been used to study the behavior of chitosan polymers with variable DDA during its neutralization in aqueous solutions (39). The weight average molecular weight of two LMWC samples, prepared in 2 M HCl, were examined. These two samples had viscosity-based average MW of 28.1 kDa (1 hour hydrolysis) and 1.4 kDa (24 hours hydrolysis) and their DLS-based MW were 28.5 kDa and 1.25 kDa respectively. These values indicate a narrow range of polydispersity of the prepared polymers. Since the isolation of a particular MW of chitosan is tedious, polydispersity of LMWC can be a useful indicator to guarantee reproducibility.

Determination of the degree of deacetylation (DDA) using UV Spectroscopy

The calibration curve was constructed using known concentrations of the N-D- glucosamine standard plotted against the first derivative of absorbance measured at ƛ = 202 nm. The absorbance of each sample solution was measured at ƛ = 200-205 nm. The pH of all samples was determined at room temperature, and the equations mentioned earlier were used to determine the DDA.

Preparation of acetylated LMWC (Actl-LMWC) and determination of its Degree of Deacetylation using H^1 NMR

The preparation of acetylated LMWC (Actl-LMWC) was performed by a simple acyl substitution reaction in which acetic anhydride was reacted with LMWC at relatively neutral conditions. The pH was adjusted to de-protonate the amino groups in the glucosamine subunits in LMWC. The reaction was performed at ambient temperature and the product was purified by simple dialysis. Yields ranged between 85-90%. The H^1 NMR spectrum of a typical acetylated LMWC polymer (1.3 kD) in HOD is shown in Figure 2. As can be seen, the peak corresponding to the three protons of acetyl group (from acetamide, H-Ac) appears as a singlet at 2.07 ppm and that proton H-2 appears as a multiplet at 3.18 ppm, whereas, the peak corresponding to the protons of the deacetylated monomer H-1 appears as a multiplet at 4.88 ppm.

H^1 NMR spectroscopy was adopted as the method of choice for reliable quantitation of the degree of deacetylation of chitosan polymers (37) for this part of the experimentation and can be used interchangeably with the first derivative UV method. Lavertu et. al. have described a validated H^1 NMR method for the measurement of DDA of chitosan by liquid phase H^1 NMR in which the spectra are recorded in D_2O/DCl at 70°C (33). The obtained DDA% for each of LMWC used in the acetylation reaction is detailed in Table 2. It is evident that as the molar ratio of acetic anhydride increases, DDA% decreases due to higher extent of acetylation. In addition, for a certain LMWC:Ac_2O ratio, the degree of deacetylation is the same regardless of the molecular weight of LMWC used. This is because the molar ratio used in the reaction is for acetic acid to glucosamine units, which is constant for any given amount of LMWC regardless of the molecular weight.

CONCLUSIONS

Although acid-catalyzed depolymerization has been described previously, the method reported here uses lower concentrations of hydrochloric acid at a lesser temperature thus yielding a simpler and reproducible protocol for the preparation of LMWC. The empiricalprocedure proved to be cost and time-effective, and applicable to processing relatively large amounts of HMWC (10.0 g), with the advantage of allowing

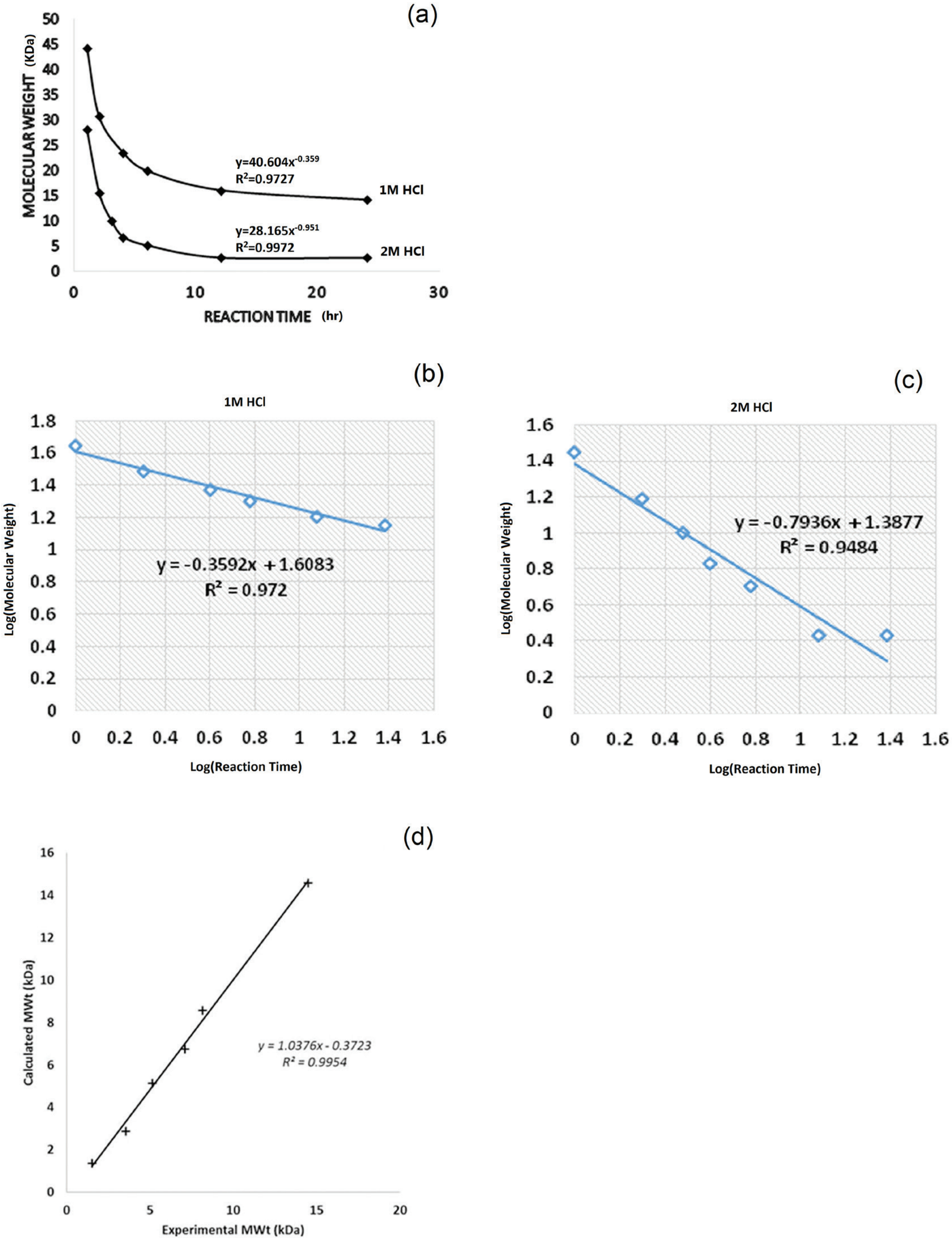

Figure 6 The average molecular weight of LMWC versus reaction time using 1M HCl (x) and 2M HCl (glyph)

Table 2 Degree of deacetylation (DDA) post re-acetylation of LMWC* with Acetic anhydride (Ac_2O) calculated using the peaks corresponding to H-1D** and H-Ac. Ratio (1:0.60) consisted of a 2-step addition of Ac_2O.

MOLECULAR WEIGHT (kDa)	MOLAR RATIO ($LMWC:Ac_2O$)	INTEGRAL OF THE PEAK CORRESPONDING TO H-Ac	DDA (%)
30	1:0.15	0.70	81.08
	1:0.60	2.30	56.60
18	1:0.15	0.74	80.21
	1:0.60	2.35	56.07
13	1:0.15	0.84	78.13
	1:0.60	2.41	55.45
6	1:0.15	0.75	80.00
	1:0.60	2.50	54.55
1.3	1:0.15	0.77	79.58
	1:0.60	2.45	55.05

*DDA of LMWC used for the reactions was 100%

**The integral of the peak corresponding to H-1D was normalized to 1

the predetermination of the resulting MW of LMWC as a function of reaction time, as well as the predetermination of DDA.

ACKNOWLEDGMENTS

This work was supported by a grant from the Deanship of Research at Jordan University of Science and Technology and contributions from The Jordanian Pharmaceutical Manufacturing Company (JPM). No studies were performed on human or animal subjects.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 Kumar, A.B.V.; Tharanathan, R.N. A comparative study on depolymerization of chitosan by proteolytic enzymes. Carbohydrate Polymers, 58:275-283, 2004.

1 Rinaudo, M. Chitin and chitosan: Properties and applications. Progress in Polymer Science, 31:603-632, 2006.

2 Cabrera, J.C.; Van Cutsem, P. Preparation of chitooligosaccharides with degree of polymerization higher than 6 by acid or enzymatic degradation of chitosan. Biochemical Engineering Journal, 25:165-172, 2005.

3 Kumar, R.; Majeti, N.V. A review of chitin and chitosan applications. Reactive and Functional Polymers, 46:1-27, 2000.

4 Guinesi, L.S.; Cavalheiro, E.T.G. The use of dsc curves to determine the acetylation degree of chitin/chitosan samples. Thermochimica Acta, 444:128-133, 2006.

5 Knill, C.; Kennedy, J.; Mistry, J.; Miraftab, M.; Smart, G.; Groocock, M.; Williams, H. Acid hydrolysis of commercial chitosans. Journal of Chemical Technology & Biotechnology, 80:1291-1129, 2005.

6 Varum, K.M.; Ottoy, M.H.; Smidsrod, O. Acid hydrolysis of chitosan. Carbohydrate Polymers, 46:89-98, 2001.

7 Dodane, V.; Vilivalam, V.D. Pharmaceutical applications of chitosan. PSTT 1988, 1.

8 Ikeda, I.; Sugano, M.; Yashida, K.; Sasaki, E.; Iwamoto, Y.; Hatono, K. Dietary fibre in health and disease. Kritchevsky, D.; Bonefield, C., Eds. Eagan Press: 1995; p 95.

9 Eftaiha, A.a.F.; Qinna, N.; Rashid, I.S.; Al Remawi, M.M.; Al Shami, M.R.; Arafat, T.A.; Badwan, A.A. Bioadhesive controlled metronidazole release matrix based on chitosan and xanthan gum. Marine Drugs 2010, 8, 1716-1730.

10 Jeon, Y.-J.; Shahidi, F.; Kim, S.-K. Preparation of chitin and chitosan oligomers and their applications in physiological functional foods Food Reviews International, 16:159–176, 2000.

11 Li, J.; Du, Y.; Yang, J.; Feng, T.; Li, A.; Chen, P. Preparation and characterisation of low molecular weight chitosan and chito-oligomers by a commercial enzyme. Polymer Degradation and Stability, 87:441-448, 2005.

12 Qandil, A.; Obaidat, A.; Ali, M.; Al-Taani, B.; Tashtoush, B.; Al-Jbour, N.; Al Remawi, M.; Al-Sou'od, K.; Badwan, A. Investigation of the interactions in complexes of low molecular weight chitosan with ibuprofen. Journal of Solution Chemistry, 38:695-712, 2009.

13 Athamneh, N.A.; Tashtoush, B.M.; Qandil, A.M.; Al-Tanni, B.M.; Obaidat, A.A.; Al- Jbour, N.D.; Qinna, N.A.; Al-Sou'od, K.; Al-Remawi, M.M.; Badwan, A.A. A new controlled-release liquid delivery system based on diclofenac potassium and low molecular weight chitosan complex solubilized in polysorbates. Drug Development and Industrial Pharmacy, 39:1217-1229, 2013.

14 Jiang, G.-B.; Quan, D.; Liao, K.; Wang, H. Preparation of polymeric micelles based on chitosan bearing a small amount of highly hydrophobic groups. Carbohydrate Polymers, 66:514-520, 2006.

15 Zakieh I. Al-Kurdi; Babur Z.Chowdhry; Stephen A. Leharne; Mahmoud M. Al Omari; Adnan A. Badwan. Low Molecular Weight Chitosan–Insulin Polyelectrolyte Complex: Characterization and Stability Studies. Mar. Drugs, 13(4):1765-1784, 2015.

16 ElSayed A.; Khaled A.; AlRemawi M.; Qinna N.; Abu Farsakh H.; Badwan A.; Low Molecular Weight Chitosan-Insulin Complexes Solubilized in a Mixture of Self-Assembled Labrosol and Plurol Oleaque and Their Glucose Reduction Activity in Rats. Mar. Drugs, 16(1):32, 2018.

17 Assaf, S.M.; Al-Jbour, N.D.; Eftaiha, A.a.F.; Elsayed, A.M.; Al-Remawi, M.M.; Qinna, N.A.; Chowdhry, B.; Leharne, S.; Badwan, A.A. Factors involved in formulation of oily delivery system for proteins based on peg-8 caprylic/capric glycerides and polyglyceryl-6 dioleate in a mixture of oleic acid with chitosan. Journal of Dispersion Science and Technology, 32:623-633, 2011.

18 Remawi, M.A.; Al-Akayleh, F.; Salem, M.; Shami, M.A.; Badwan, A. Application of excipient made from chitosan - xanthan as a single component for the controlled release of ambroxol tablet. Journal of Excipients and Food Chemicals, 4:48-57, 2013.

19 Kurita, K. Controlled functionalization of the polysaccharide chitin. Progress in Polymer Science, 26:1921-1971, 2001.

20 Lee, M.-Y.; Var, F.; Shin-Ya, Y.; Kajiuchi, T.; Yang, J.-W. Optimum conditions for the precipitation of chitosan oligomers with dp 5-7 in concentrated hydrochloric acid at low temperature. Process Biochemistry, 34:493-500, 1999.

21 Shin-ya, Y.; Yoshizawa, T.; Hong, K.-J.; Lee, M.-Y.; Kajiuchi, T. Log-normal distribution for a description of the molecular weight distribution of n-acetylated chitosans depolymerized with hydrolytic enzyme. Polymer International, 52:838-842, 2003.

22 Kittur, F.S.; B., A.; Kumar, V.; Tharanatha, R.N. Low molecular weight chitosans preparation by depolymerization with aspergillus niger pectinase, and characterization. Carbohydrate Research, 338:1283-1290, 2003.

23 deBritto, D.; Campana-Filho, S.P. Kinetics of the thermal degradation of chitosan. Thermochimica Acta, 465:73-82, 2007.

24 Holme, H.K.; Foros, H.; Pettersen, H.; Smidrød, O. Thermal depolymerization of chitosan chloride. Carbohydrate Polymers, 46:287-294, 2001.

25 Liu, H.; Bao, J.; Du, Y.; Zhou, X.; Kennedy, J.F. Effect of ultrasonic treatment on the biochemphysical properties of chitosan. Carbohydrate Polymers, 64:553-559, 2006.

26 Kasaai, M.R. Calculation of mark-houwink-sakurada (mhs) equation viscometric constants for chitosan in any solvent-temperature system using experimental reported viscometric constants data. Carbohydrate Polymers, 68:477-488, 2007.

27 Muzzarelli, R.A.A. Chitin. 1st ed.; Pergamon Press: New York, 1977; p 309.

28 British Pharmacopoeia Commission. British pharmacopoeia. Stationery Office: 2004.

29 Lavertu, M.; Xia, Z.; Serreqi, A.N.; Berrada, M.; Rodrigues, A.; Wang, D.; Buschmann, M.D.; Gupta, A. A validated 1h nmr method for the determination of the degree of deacetylation of chitosan. Journal of Pharmaceutical and Biomedical Analysis, 32:1149-1158, 2003.

30 Li, B.; Zhang, J.; Bu, F.; Xia, W. Determination of chitosan with a modified acid hydrolysis and hplc method. Carbohydrate Research, 366:50-54, 2013.

31 Tsao C. T.; HaoChang C.; YungLin Y.; FungWu M.; LinHan J.; HuangHsieh K.; Kinetic study of acid depolymerization of chitosan and effects of low molecular weight chitosan on erythrocyte rouleaux formation. Carbohydrate Research. 346(1):94-102, 2011.

32 Nah, J.-W.; Jang, M.-K. Spectroscopic characterization and preparation of low molecular water-soluble chitosan with free-amine group by novel method. Journal of Polymer Science: Part A: Polymer Chemistry, 40:3796-3803, 2002.

33 Pa, J.-H.; Yu, T.L. Light scattering study of chitosan in acetic acid aqueous solutions. Macromolecular Chemistry and Physics, 202:985-991, 2001.

34 Vishu Kumar, A.B.; Tharanathan, R.N. A comparative study on depolymerization of chitosan by proteolytic enzymes. Carbohydrate Polymers, 58:275-283, 2004.

35 Kasaai, M.R. Calculation of mark-houwink-sakurada (mhs) equation viscometric constants for chitosan in any solvent-temperature system using experimental reported viscometric constants data. Carbohydrate Polymers, 68:477-488, 2007.

36 Mao, S.; Shuai, X.; Unger, F.; Simon, M.; Bi, D.; Kissel, T. The depolymerization of chitosan: Effects on physicochemical and biological properties. International Journal of Pharmaceutics, 281:45-54, 2004.

37 Wang, Q.; Huang, X.; Nakamura, A.; Burchard, W.; Hallett, F.R. Molecular characterisation of soybean polysaccharides:

An approach by size exclusion chromatography, dynamic and static light scattering methods. Carbohydrate Research, 340:2637-2644, 2005.

38 Schatz, C.; Pichot, C.; Delair, T.; Viton, C.; Domard, A. Static light scattering studies on chitosan solutions: From macromolecular chains to colloidal dispersions. Langmuir, 19:9896-9903, 2003.