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International Journal of Pharmaceutics 281 (2004) 45–54
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

The depolymerization of chitosan: effects on physicochemical and biological properties

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Received 12 January 2004; received in revised form 17 May 2004; accepted 20 May 2004

Available online 10 July 2004

Abstract

Chitosan has been extensively used as an absorption enhancer for macromolecules and as gene delivery vehicle. Both properties are molecular weight (MW) dependent. Here, we investigate factors affecting the oxidative depolymerization of chitosan and physicochemical properties of the resulting polymer fractions including their cytotoxicity. The molecular weight of the depolymerized chitosan was influenced by the initial concentration and the source of chitosan. At constant initial concentrations, the molecular weight decreased linearly with the chitosan/$NaNO_2$ ratio and was a function of logarithm of the reaction time. Chitosan with larger molecular weight was more sensitive to depolymerization. No structural change was observed during the depolymerization process by infrared and proton nuclear magnetic resonance spectroscopy. In addition, thermal properties of chitosan fragments were studied by thermal gravimetric analysis and it was found that the decomposition temperature was molecular weight dependent. Furthermore, the solubility of different molecular weight chitosan was assayed as a function of pH and it increased with decreasing molecular weight. The cytotoxicity of chitosan was concentration dependent but almost molecular weight independent according to MTT assay using L929 cell line recommended by USP26. In summary, low molecular weight fractions of chitosan may potentially useful for the design of drug delivery systems due to the improved solubility properties.

Keywords: Chitosan; Depolymerization; Intrinsic viscosity; Solubility; Cytotoxicity

1. Introduction

Chitosan (poly[β-(1-4)-2-amino-2-deoxy-D-glucopyranose]) is a non-toxic and biocompatible cationic polysaccharide produced by partial deacetylation of chitin isolated from naturally occurring crustacean shells. Due to its specific properties, chitosan has found a number of applications in drug delivery including that of as an absorption enhancer of hydrophilic macromolecular drugs (Artursson et al., 1994; Illum et al., 1994; Luessen et al., 1996; Singla and Chawla, 2001) and as gene delivery system (Erbacher, 1998; Richardson Simon et al., 1999).

The term chitosan embraces a series of polymers, which vary in molecular weight (MW) and degree of deacetylation (DD). Although a number of investigations have been performed to elucidate the relationship between MW and cytotoxicity, the results were controversial. A series of toxicity studies indicated that chitosan was toxic and the toxicity

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doi:10.1016/j.ijpharm.2004.05.019

was dependent upon their molecular weight, degree of deacetylation and salt form (Sgouras and Duncan, 1990; Heller et al., 1996; Carrecuno-Gomez and Duncan, 1997). By contrast, other investigations suggested that the toxicity of chitosan was negligible (Aspden et al., 1995, 1996, 1997a, 1997b). Additionally, the transfection efficiency of chitosan with molecular weight >100 kDa was reduced compared to 15 and 52 kDa and chitosans of 10–50 kDa seem to be promising as gene transfer reagents (Sato et al., 2001). Lee et al. (2001) indicated that low molecular weight chitosan (molecular weight of 22 kDa) showed higher transfection efficiency than poly-L-lysine.

However, most commercially available chitosans possess quite large MWs. Due to promising properties of low molecular weight chitosans in the pharmaceutical field, it is essential to establish a reproducible and straightforward method for generating low MW chitosans. Generally, low molecular weight chitosans can be prepared from high molecular weight chitosan by depolymerization using enzymatic degradation (Chang et al., 1998), oxidative degradation (Li et al., 1999), acidic cleavage and ultrasonic degradation (Chen and Chen, 2000). The rate of molecular weight degradation was irregular during the time course of ultrasound treatment. Liu studied the depolymerization of chitosan using $NaNO_2$, H_2O_2, and HCl. They found that $NaNO_2$ showed the best performance (Liu et al., 1997). These results were recently confirmed, but no detailed experimental information was provided (Janes and Alonso, 2003).

The purpose of this paper, therefore, is to study the factors affecting the depolymerization process of chitosan using sodium nitrite and to clarify the relationship between chitosan MW and cytotoxicity. Using intrinsic viscosity measurements, these factors were investigated systematically and the structure identification of chitosan during depolymerization was performed with IR and ^{1}H NMR methods. In addition, the solubility of different chitosans was characterized as a function of pH value. The cytotoxicity of chitosans was characterized by MTT assay using L929 fibroblast cell line.

2. Materials and methods

2.1. Materials

Three different commercially available chitosans F-LMW (150 kDa), F-MMW (400 kDa), F-HMW (600 kDa), with a nominal degree of deacetylation of 84.5%, 84.7%, and 85.0%, respectively, were purchased from Fluka (Neu-Ulm, Germany). Acetic acid (HAc), sodium acetate (NaAc), sodium hydroxide (NaOH) and sodium nitrite ($NaNO_2$) were of analytical grade. Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Eggenstein, Germany). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma (Deisenhofen, Germany), dimethylsulfoxide (DMSO) was from Merck (Darmstadt, Germany).

The chitosans selected for this study had similar degrees of deacetylation, but differed markedly in their molecular weights. The degrees of deacetylation, viscosity-average molecular weights, and moisture contents of the commercial chitosans used in this study were determined experimentally and compared with the values reported by the supplier, as listed in Table 1.

Table 1
Characteristics of chitosans used in this study

Chitosan	Degree of deacetylation (%)		Molecular weight (kDa)		Moisture content (%)	
	Labeled[a]	Determined[b]	Labeled[a]	Determined[c]	Labeled[a] (%)	Determined[d]
F-LMW	84.5	83.0	150	225	≤10	10.0
F-MMW	84.7	85.1	400	540	≤10	10.4
F-HMW	85.0	84.9	600	610	≤10	10.3

[a] Values supplied by the supplier.
[b] Determined by ^{1}H NMR.
[c] Calculated from the intrinsic viscosity using the classical Mark-Houwink equation $[\eta] = K(M_v)^a$, where the constants $K = 1.38 \times 10^{-5}$ and $a = 0.85$.
[d] Calculated from TGA measurement.

2.2. Depolymerization of chitosan

The low molecular weight chitosans were prepared by oxidative degradation with $NaNO_2$ at room temperature. Briefly, 1% (w/w) chitosan was dissolved in 1% acetic acid solution under magnetic stirring. When chitosan was completely dissolved, the appropriate amount of 0.1 M $NaNO_2$, as indicated below, was added dropwise and the reaction was performed at room temperature for 3h unless indicated otherwise. The reaction mixture was subsequently neutralized with 1N NaOH to pH 8.0 to precipitate chitosan. The precipitated chitosan was recovered by centrifugation, washed several times with deionized water, and dried by lyophilisation.

2.3. Characterization of chitosan

Fourier transformed infrared spectroscopy (FTIR) was conducted on a FT-IR 510P spectrometer (Nicolet) in the range between 4000 and 400 cm^{-1}, with a resolution of 2 cm^{-1}. All powder samples were compressed into KBr disks for the FTIR measurement.

Thermogravimetric analysis (TGA) was performed on a thermogravimetric analyser TGA 7 with a thermal analysis controller TAC 7/DX from Perkin-Elmer using an approximately 5 mg polymer sample. The scanning rate was 20 °C/min, and the thermograms were recorded within the temperature range 30–550 °C. Analysis was performed under a nitrogen gas atmosphere in platinum crucibles.

2.4. Determination of intrinsic viscosity

Intrinsic viscosity of chitosans in 2% HAc/0.2 M NaAc were measured using an automated Ubbelohde capillary viscometer (Model Schott AVS-360, Germany) in a constant-temperature water bath at 25 ± 0.01 °C in triplicate. The capillary diameter used was 0.63 mm. Solution concentrations were adjusted based on the viscosity of the samples and the flow through time was kept in the range of 100–150 s. Six different concentrations were tested for each sample. The intrinsic viscosity was determined by the common intercept of both Huggins ($\eta_{sp}/C \sim C$) and Kraemer ($\eta_{inh} \sim C$) plots on the ordinate at $C = 0$. As a representative example, the intrinsic viscosity calculation method of HMW chitosan was shown in Fig. 1.

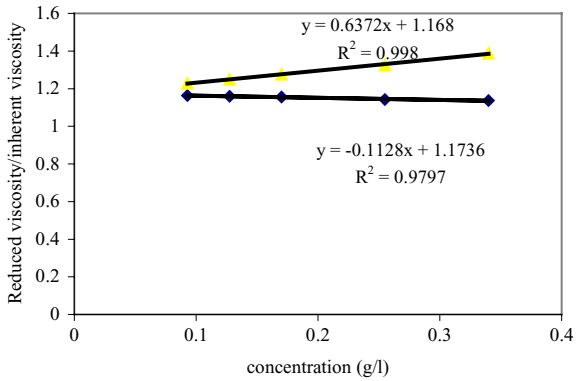

Fig. 1. A representative plot for intrinsic viscosity calculation (HMW chitosan). Both η_{sp}/C and η_{inh} are plotted on the same graph and the common intercept of the plots on the ordinate at $C = 0$ is the intrinsic viscosity.

While reduced viscosity of polymers is defined as:

$$\frac{\eta_{sp}}{C} = \frac{t - t_0}{t_0 C}$$

and inherent viscosity is defined as:

$$\eta_{inh} = \frac{\ln(t/t_0)}{C}$$

where t_0 is the flow time for solvent and t is the flow time for tested solution.

Both η_{sp}/C and η_{inh} are plotted on the same graph in Fig. 1. The common intercept of the plots on the ordinate at $C = 0$ gives:

$$[\eta] = \left(\frac{\eta_{sp}}{C}\right)_{C=0} = (\eta_{inh})_{C=0} = 1.168\,\mathrm{L/g}$$

The viscosity-average molecular weights of chitosans were calculated using the classical Mark-Houwink equation:

$$[\eta] = K(M_v)^a$$

where $[\eta]$ is the intrinsic viscosity of the depolymerized chitosan, K and a are constants for given solute–solvent system and temperature. For chitosan, they are influenced by the degree of deacetylation, pH, and ionic strength of the solvent (Kasaai et al., 2000). As to the chitosans with a DD value of 85%, the constants $K = 1.38 \times 10^{-5}$ and $a = 0.85$ were reported (Gamzazade et al., 1985). The viscosity-average

molecular weight of the HMW chitosan was therefore calculated as follows:

$$\overline{M_v} = \left(\frac{1.168}{1.38 \times 10^{-5}}\right)^{1/0.85} \approx 610\,\text{kDa}$$

2.5. Determination of degree of deacetylation

In order to assess any structure modification occurred during the depolymerization process, the structure of original chitosans and the depolymerized chitosans were determined by ^{1}H NMR spectroscopy and infrared spectrum. ^{1}H NMR spectra were recorded on a FT-NMR spectrometer (AMX500, 500 MHz, Bruker) at 80 °C using D_2O containing 5% CD_3COOD as the solvent. The NMR experiment was performed at higher temperatures in order to shift the signal of HOD to a higher field, which allowed quantifying the H-1 signals of glucosamine residues. The samples were dissolved at a concentration of 10 mg/ml. The DD was determined from the integral of the CH_3 signal at 1.97 ppm compared with that of H-1 signals of glucosamine and *N*-acetylglucosamine (Varum et al., 1991).

2.6. Solubility testing

Solubility of various chitosans was measured at different pH values. Briefly, chitosans were dissolved in 0.25% HAC solution (2 mg/ml), the pH of the solution was adjusted by the addition of 1N NaOH solution and the transmittance of the solution at 600 nm as a function of pH value was recorded on a UV–vis spectrophotometer (UV-160, Shimadzu) (Park et al., 2003). Cloud point pH values, which are defined as the pH when the transmittance was no less than 98%, were determined at the same time (Anderson and Mallapragada, 2002) in triplicate.

2.7. Cytotoxicity testing (MTT assay)

A mouse connective tissue fibroblast cell line, L929 was selected to evaluate cytotoxicity as a direct contact test, as recommended by USP 26. The experiment was carried out according to the method described previously (Fischer et al., 1999). Briefly, L929 was cultured in DMEM supplemented with 10% fetal calf serum and 2 mM glutamine without antibiotics. The cells were cultivated in an incubator at 37 °C, 95% RH and 10% CO_2. Chitosan was first dissolved in 0.5% HAC solution, and then diluted with equal volume of double DMEM medium. The pH of this stock solution was adjusted to 6.5 for all the chitosans tested. Thereafter, DMEM (pH 6.5) was used to prepare serial dilutions of the polymer. L929 cells were seeded into 96-well microtiter plates at a density of 8000 cells/well. Twenty-four hours later, culture medium was replaced with 100 μl serial dilutions of chitosan (0.6–10 mg/ml) and cells were incubated for 24 h. Subsequently, 20 μl MTT (5 mg/ml) was added to each well. After 4 h, unreacted dye was aspirated and the formazon crystals were dissolved in 200 μl/well DMSO. Absorption was measured at 570 nm with a background correction at 690 nm using a Titertek Plus MS 212 ELISA reader (ICN, Eschwege, Germany). The relative cell viability compared to control cells containing cell culture medium (pH 6.5) without polymer was calculated by $[A]_{test}/[A]_{control}$ ($n = 7$).

2.8. Calculation and statistics

Results are depicted as mean ± S.D. from at least three measurements. Significance between the mean values was calculated using ANOVA one-way analysis (Origin 7.0 SRO, Northampton, MA, USA). Probability values $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Depolymerization of chitosan

The concentration of sodium nitrite could be expected to play a significant role in the depolymerization process. In order to facilitate the control of the molecular weight of chitosan fragment, keeping chitosan concentration at 1%, the depolymerization was carried out in a 1% acetic acid solution by varying chitosan/$NaNO_2$ molar ratio, using three types of chitosan as original material. The relationship between chitosan/$NaNO_2$ molar ratio and the MW of chitosan fragment is shown in Fig. 2.

Regression analysis of chitosan/$NaNO_2$ molar ratio as a function of the molecular weight of chitosan fragment yielded an almost linear correlation with correlation coefficients of 0.98, 0.99 and 0.97 for chitosan

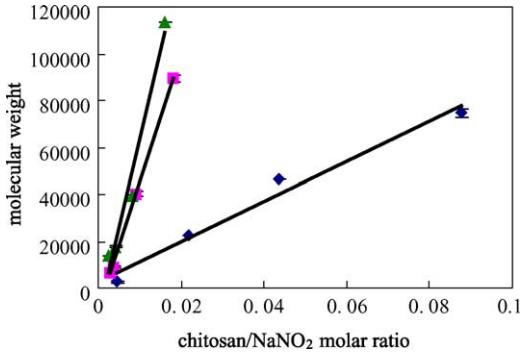

Fig. 2. . The relationship between chitosan/$NaNO_2$ molar ratio and chitosan molecular weight: (◆) Chitosan 150 kDa; (■) chitosan 400 kDa; and (▲) chitosan 600 kDa. The measurements were carried out in triplicate and the results are expressed as mean ± S.D. Different molecular weight chitosans (1%) were degraded by sodium nitrite in 1% acetic acid solution for 3 h at room temperature.

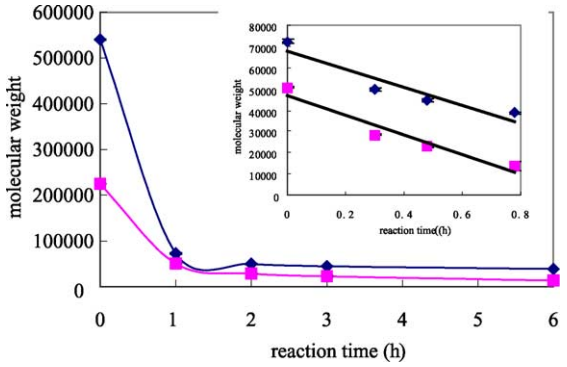

Fig. 3. Effect of reaction time on the molecular weight of depolymerized chitosan: (■) Low MW chitosan; and (◆) medium MW chitosan. The measurements were carried out in triplicate and the results are expressed as mean ± S.D. Chitosan (1%) was degraded in 1% acetic acid solution with chitosan/$NaNO_2$ molar ratio 0.009.

LMW, MMW, HMW, respectively. Furthermore, from the slope of the profiles, one can conclude that HMW chitosan is more sensitive to $NaNO_2$ degradation compared to LMW chitosan. This may be explained by larger molecular dimensions of HMW chitosan in solution, which increases the contact area with $NaNO_2$.

3.2. Effect of reaction time on the molecular weight of chitosan

In order to investigate the effect of reaction time, keeping chitosan/$NaNO_2$ molar ratio at 0.009, chitosan (1%) was degraded by $NaNO_2$ in 1% acetic acid solution and the reaction was stopped at 1, 2, 3 and 6 h, respectively, and the corresponding samples were processed in the same way as that described before. LMW and MMW chitosan were used as starting materials. The intrinsic viscosities of the samples were determined and the molecular weights were calculated according to the MHS equation. The results are illustrated in Fig. 3.

It was noted that the depolymerization occurred mainly in the first hour, and then slowed down significantly. This behavior was expected since the concentration of sodium nitrite decreased with the reaction time. Moreover, it could be deduced from the slope that MMW chitosan was more sensitive to $NaNO_2$ degradation compared with that of LMW chitosan. In addition, the molecular weight of depolymerized chitosans was plotted versus the logarithm of the reaction time, and a linear relationship was observed with correlation coefficients 0.9991 and 0.9985, respectively, for LMW and MMW chitosan, indicating that the degradation process belongs to first-order kinetics.

3.3. Effect of chitosan initial concentration

In order to investigate whether chitosan/$NaNO_2$ molar ratio and reaction time were the only factors influencing the molecular weight of chitosan fragment, taking MMW chitosan as an example, the chitosan/$NaNO_2$ molar ratio was kept at 0.009 and reaction time 3 h, two different chitosan initial concentrations, 0.5% and 1%, respectively, were studied. The results are shown in Fig. 4, with regression coefficients of 0.99 and 0.97, respectively, for 1% and 0.5% initial concentration of chitosan.

It seems that the depolymerization of chitosan was not only influenced by the ratio of chitosan/$NaNO_2$, but also by the initial concentration of chitosan. When the concentration was low, chitosan was more sensitive to depolymerization despite of the same chitosan/$NaNO_2$ ratio. This phenomenon is related to the structure of chitosans in solution. Normally chitosans take the shape of an extended random coil in solution. When the concentration is high, due to the larger viscosity of the solution and strong intermolecular interactions, accessible chain segments can only stretch in a limited area, decreasing the contact prob-

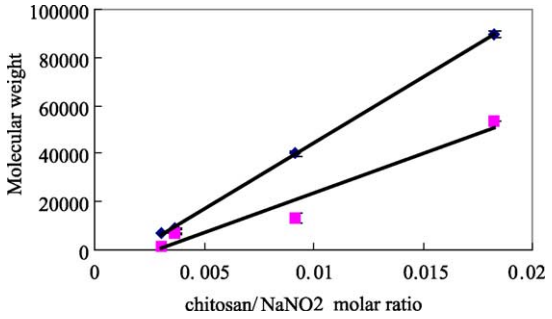

Fig. 4. Effect of MMW chitosan initial concentration on the molecular weight of chitosan fragments: (■) 0.5%; and (◆) 1.0%. The measurements were carried out in triplicate and the results are expressed as mean ± S.D. Medium molecular weight chitosans were degraded in 1% acetic acid solution with different chitosan/$NaNO_2$ ratios for 3 h at room temperature.

ability with sodium nitrite, thus resulting in a lower degradation rate. In this case, low chitosan concentration would be preferred to yield very small molecular weight chitosan in a short time.

3.4. Investigation on the reproducibility of the degradation process

While the reproducibility of a method is of extremely importance, chitosan samples of LMW and MMW were degraded in triplicate to test the deviation of this oxidative degradation method. The chitosan/$NaNO_2$ molar ratio was adjusted based on the purposed molecular weight of chitosan. Briefly, 1% chitsoan was degraded in 1% acetic acid solution for 3 h with chitosan/$NaNO_2$ molar ratio 0.004 and 0.009, respectively, for LMW and MMW chitosans to get different molecular weight products. The resulting molecular weights were 2800 ± 200 and 49000 ± 3600, respectively. Coefficients of variation for both samples were approximately 7% and no significant difference was indicated by *t*-test in the same array. Consequently, it could be concluded that this method has a good reproducibility and can be used to prepare chitosans with desired molecular weight.

3.5. Structure identification during the depolymerization process

In order to investigate any structural changes during the depolymerization process, IR spectra of the

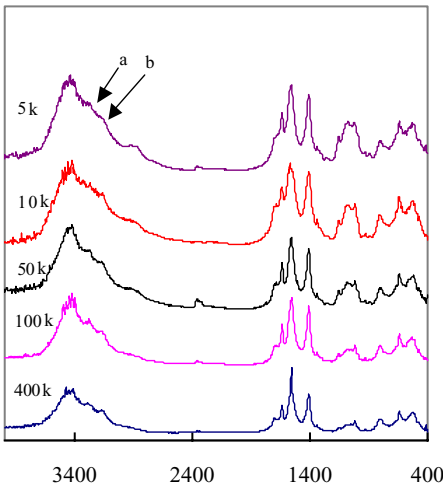

Fig. 5. IR spectra of chitosan fragment with different molecular weights. In case of great changes in the degree of deacetylation, there should be some change in the absorption bands a (3253 cm^{-1}) and b (3143 cm^{-1}) which increase with decreasing DD.

depolymerized chitosans were recorded, as shown in Fig. 5. The IR spectrum clearly shows that the process has no significant influence on the structure. In case of great changes in the DD value, there should be some change in the absorption bands a (3253 cm^{-1}) and b (3143 cm^{-1}) which increase with decreasing DD. The decrease of peak a indicates the reduction of intermolecular C (2_1) NH···O=C (7_3) hydrogen bonds, and the decrease of peak b indicates the reduction of intermolecular C (6_1) OH···HOC (6_2) hydrogen bonds (Cho et al., 2000). To quantify the absorption intensity as an indirect indicator for the change of DD, the absorption ratios at 3253 and 3143 cm^{-1} compared with that of at 1551 cm^{-1} were calculated, respectively, and the results are shown in Table 2. Significance between different molecular weight samples was calculated using ANOVA one-way analysis

Table 2
Characteristics of different molecular weight chitosans prepared from MMW chitosan

M_v	5000	10000	500000	100000	400000
A_{3143}/A_{1551}[a]	0.6713	0.7049	0.7195	0.5100	0.5295
A_{3259}/A_{1551}[a]	0.7946	0.8085	0.8456	0.6462	0.6928
DD (%)[b]	85.42	86.27	89.93	85.39	85.11

[a] Absorption ratios calculated by infrared spectroscopy.
[b] Degree of deacetylation calculated by ^1H NMR.

(Origin 7.0 SRO, USA) and no statistically significant difference ($P > 0.05$) was indicated despite that the value was to some extent higher for chitosan 10 and 50 kDa.

While IR is a coarse method for DD determination, the DD values were further measured by ^{1}H NMR and the results are listed in Table 2. It was consistent with the result from IR spectra and the DD values of different molecular weight samples had only a negligible difference.

3.6. Thermoanalytical characterization of different molecular weight chitosans

The thermal properties of chitosan were further characterized by TGA. Molecular weight dependent degradation behaviour was observed, as illustrated in Fig. 6. Chitosan with MW of 250–500 kDa showed a maximum degradation temperature at about 280 °C, low MW chitosan degraded at lower temperature, at 220, 180 °C for MW 25–100 and 2.5–5 kDa, respectively. A similar MW dependent degradation behaviour was observed for polyethylenimine and polyethylene glycol (Petersen et al., 2002).

3.7. Solubility of different molecular weight chitosans

The water solubility of chitosan was assayed as a function of pH. A good correlation between pH and transmittance at 600 nm was established for all the chitosans investigated. pH, which is defined as the pH_{50} value when the transmittance reached 50%, was calculated from the equation and was employed to express the solubility difference of different chitosans. pH_{50} and the cloud point pH of different molecular weight chitosans are shown in Fig. 7.

We observed that pH_{50} and cloud point pH increased with decreasing molecular weight. This was expected because, the presence of rigid crystalline domains, formed by intra- and/or intermolecular hydrogen bonding, was considered to be responsible for the poor solubility of chitosan in high pH solutions (Nishimura et al., 1991). The hydrogen bonding will be disturbed during the depolymerization process, resulting in the improved solubility. It should be noted that these tests were only carried out at one concentration and were simply used to verify that the solubility of chitosan could be improved by decreasing molecular weight.

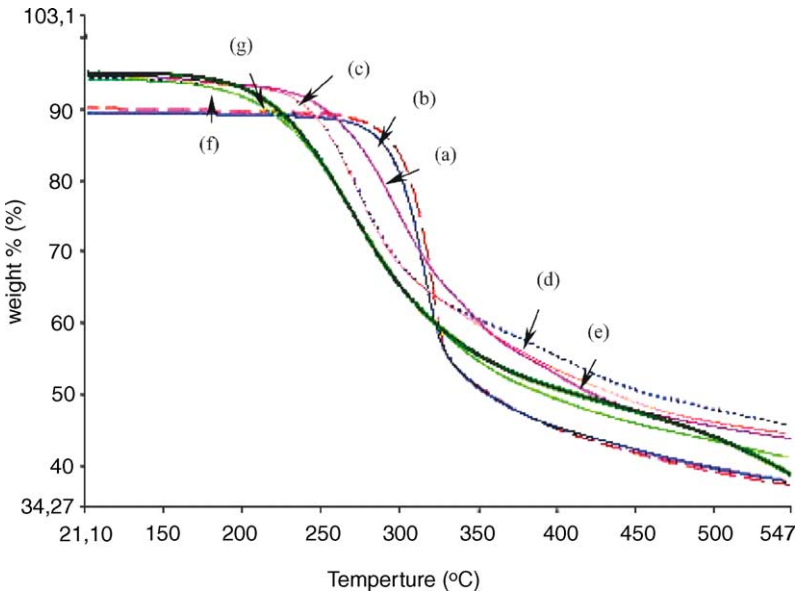

Fig. 6. Degradation behavior of different molecular weight chitosans measured by thermal gravimetric analysis: (a) MW 400 kDa; (b) MW 250 kDa; (c) MW 100 kDa; (d) MW 50 kDa; (e) MW 25 kDa; (f) MW 5 kDa; and (g) MW 2.5 kDa. Molecular weight dependent degradation behaviour was observed.

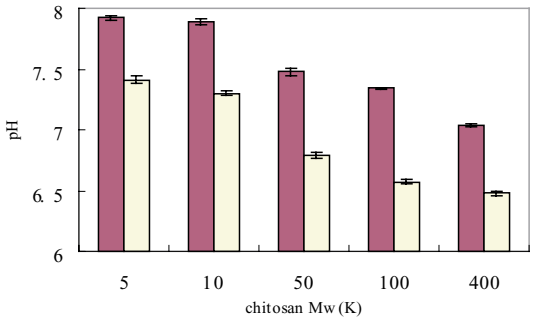

Fig. 7. Solubility properties of different molecular weight chitosans: (■) pH_{50}; and (□) critical pH. pH_{50} is defined as the pH value when the transmittance of the solution at 600 nm reached 50%. Cloud pH is defined as the pH when the transmittance at 600 nm was no less than 98%.

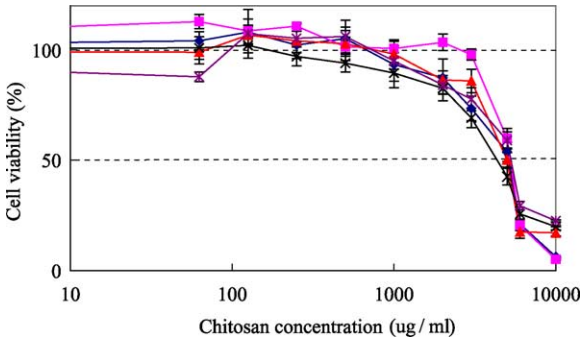

Fig. 8. Cell viability upon incubation with increasing concentrations of chitosans for 24 h, as demonstrated by MTT assay with L929 cells. Values shown are mean ± S.D. ($n = 7$): CH400kDa (◆), CH100kDa (■), CH50kDa (▲), CH10kDa (×), CH5kDa (∗).

3.8. Biocompatibility studies

While chitosan is considered as a degradable polymer due to its susceptibility to various enzymes (Zhang and Neau, 2001), we performed in this work a cytotoxicity study to clarify the relationship between molecular weight and toxicity.

Generally, the determination of cell viability is an ordinary assay to evaluate the in vitro cytotoxicity of biomaterials. Common methods for determining cell viability depend upon membrane integrity (e.g. trypan blue exclusion), or incorporation of nucleotides during cell proliferation (e.g. BrdU or 3H-thymidine). However, these methods are limited by the impracticality of processing large number of samples, or by the requirement for handling hazardous materials. The MTT assay, in contrast, provides a rapid and versatile method for assessing cell viability (Mosmann, 1983). This test is a quantitative colorimetric method to determine cell proliferation and it utilizes the yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] which is metabolized by mitochondrial succinic dehydrogenase activity of proliferating cells to yield a purple formazan reaction product which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilization of the cells results in the liberation of the product that can readily be detected using a simple colorimetric assay. The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity, which, in turn, may be interpreted as a measure of viability and/or cell number. Comparison of results between control and test cultures provides an indication of the cytotoxic effect of test compounds.

The results are described in Fig. 8. The cytotoxicity of chitosan was concentration dependent, as expected, but all the chitosans tested were relatively non-toxic at concentration up to 1 mg/ml. Chitosan 400 kDa showed an IC_{50} of 4200 mg/ml, whereas approximately 5000 mg/ml were found for chitosan 100–5 kDa. Taking the deviation of this method into account, it is reasonable to conclude that the cytotoxicity of chitosan was molecular weight independent although it was not in agreement with the result of Sgouras and Duncan (1990), who found a molecular weight dependence of the cytotoxicity. This difference can be attributed to different cell lines used in those experiments. The L929 fibroblast cell line used in our experiments is recommended by USP and several other pharmacopoeias as a standard method for cytotoxicity testing.

4. Conclusions

The depolymerization of chitosan could be carried out by oxidative degradation using sodium nitrite; a large series of chitosans with desired molecular weights could be obtained by changing chitosan/$NaNO_2$ molar ratio, chitosan initial concentration and reaction time. The molecular weight of the depolymerized chitosan was linear with

chitosan/$NaNO_2$ ratio, and decreased as a function of the logarithm of reaction time. Chitosan with larger molecular weight was more sensitive to depolymerization. IR spectrum and ^1H NMR spectroscopy demonstrated that there was no structure change during depolymerization process. The reproducibility of this method was fairly good. The decomposition temperature of chitosan was molecular weight dependent and the solubility of chitosan increased with decreasing molecular weight. The cytotoxicity of chitosan was almost molecular weight independent according to MTT assay using L929 cell line. Chitosan was nontoxic when the concentration was as high as 1 mg/ml.

In summary, using the oxidative depolymerization of chitosan, low molecular weight fragments can be easily and reproducibly obtained.

Acknowledgements

Shirui Mao cordially thanks DAAD (Deutsche Akademische Austauschdienst) for the financial support.

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