

LOW MOLECULAR WEIGHT *O*-CARBOXYMETHYLATED CHITOSANS DERIVED FROM IRRADIATED CHITOSAN AND THEIR ANTIBACTERIAL ACTIVITY*

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Abstract Original chitosan with M_v of 2.7×10^5 was degraded by irradiation with γ -rays and a series of low molecular weight *O*-carboxymethylated chitosans (O-CMCh) were prepared based on the irradiated chitosan. A kinetic model of the irradiation of chitosan was put forward. Results show that the irradiation degradation of chitosan obeys the rule of random degradation and the degree of deacetylation of irradiated chitosan is slightly raised. The antibacterial activity of O-CMCh is significantly influenced by its MW, and a suppositional antibacterial peak appears when M_v is equal to 2×10^5 .

Keywords Chitosan, *O*-Carboxymethylated chitosan, Irradiation degradation, Antibacterial activity, Molecular weight

INTRODUCTION

Chitin is the second most naturally abundant biopolymer and is found in a variety of organisms, including fungal cell walls, the exoskeleton of crustaceans, skeletal tissue of mollusks and the integument of insects.

When treated with alkali, chitin can be deacetylated and turned into chitosan, which is a linear binary heteropolysaccharide composed of (1–4) linked 2-acetamido-2-deoxy- β -*D*-glucopyranose and 2-amino-2-deoxy- β -*D*-glucopyranose residues. Chitosan has a wide variety of application fields such as biomedical, food, and chemical industries because of its good properties^[1–4]. Interestingly, some antibacterial and antifungal activity has been found for chitosan and modified chitosan derivatives^[5–8].

As an antibacterial agent, chitosan shows several advantages over other types of disinfectants, such as higher antibacterial activity, broader spectra of activity, higher killing rate and lower toxicity toward mammalian cells^[9]. However, chitosan shows its antibacterial activity only in acidic medium because of its poor solubility above pH 6.5. Thus, water soluble chitosan derivatives soluble under both acidic and basic physiologic conditions may be good candidates to broaden its application. When chitosan is changed to *O*-carboxymethylated chitosan (O-CMCh) by substituting —OH with O-CM along the chitosan molecular chains, its antibacterial activity becomes much enhanced. Moreover, since O-CMCh can be dissolved in a wide pH range ranging from pH 3 to pH 11, it has a much broader application as an antibacterial agent than chitosan does^[10].

It was reported that chitosan with different molecular weight (MW) usually exhibits different properties^[11–13]. Moreover, some valuable functions of chitosan can appear only when its MW is low enough^[14]. So it is meaningful to prepare a series of low molecular weight O-CMCh and to study their antibacterial activity dependence on MW.

To carry out this study, the present work offered a degradation method for chitosan by using γ -irradiation. Kinetics of chitosan degradation and the effect of irradiation on the degree of deacetylation (DD) were also studied.

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EXPERIMENTAL

Irradiation Degradation of Chitosan

Chitosan (viscosity-average molecular weight (M_v) = 2.7×10^5) flakes of 5 mm diameter was provided by Qingdao Pharmaceutical Institute, China. Chitosan was irradiated in a γ cell (^{60}Co source, 35 Gy/min, Tianjin Technical Physics Institute, Tianjin, China) at 20°C for different interval of times either in air or in vacuum, which led to degradation of the chitosan main chain.

M_v of the irradiated chitosans was calculated from solution viscosity data determined at $(25 \pm 0.01)^\circ\text{C}$ in 0.1 mol/L HAc–0.2 mol/L NaCl solvent. The Mark-Houwink equation $[\eta] = KM_v^\alpha$ was used to calculate M_v . The values of K and α are $1.81 \times 10^{-3} \text{ cm}^3/\text{g}$ and 0.93, respectively^[15]. DD was measured by a potentiometric titration method^[16].

Synthesis of Low MW O-CMCh

Irradiated chitosan (15.0 g), monochloroacetic acid (9.0 g) was added to 150 mL of sodium hydroxide solution (42 wt%). After being stirred for 48 h at 0°C, the pH of the mixture was adjusted to 1.0 using hydrochloric acid. Then, the solid product was filtered and washed with methanol several times. The degree of substitution (DS) determined by pH titration was 0.86^[17].

FTIR spectra of chitosan and O-CMC were obtained with a Nicolet 560 E.S.P. spectrometer.

Antibacterial Assessment

The Shaking Flask Test Method was applied for determining the antibacterial activity and *E. coli* (8099) was selected as the test bacterium: A representative colony was picked off with a wire loop and placed in a nutrient broth, which was then incubated at 30°C overnight. Then, a culture where *E. coli* grew in a logarithmic growth phase was prepared for an antibacterial test. Two percent of them were inoculated in the medium containing the blend films with different O-CMCh content, under shaken cultivation at 30°C for 24 h. During incubation, the turbidity value of the medium was measured at 610 nm by a 756MC UV-Vis spectrophotometer (Shanghai, China) several times during the process.

RESULTS AND DISCUSSION

Irradiation Degradation Kinetics

The irradiation with γ -rays made chitosan degrade significantly both in air and in vacuum, as shown in Fig. 1. The MW of chitosans decreases sharply with increase of the irradiation dose (D). When the irradiation dose increased as high as 250 kGy, M_v decreased from 2.7×10^5 to 2.4×10^4 and 2.0×10^4 in air and in vacuum, respectively.

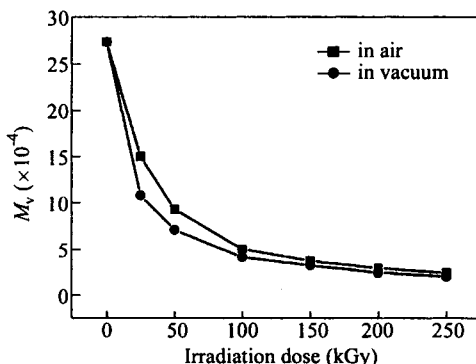


Fig. 1 Relationship between MW of irradiated chitosan and irradiation dose in different environments

In general, irradiation degradation of polymers obeys the kinetics of random degradation, which means that

identical bonds may break with equal possibility at any position along the polymer chain. During degradation, one more macromolecules will be generated in the system per scission of the main chain. Therefore, the changing rate of total number of macromolecules in the system relates to the quantity of reactive bonds connecting two repeating units:

$$\frac{d \sum_{i=1}^m p(i)}{dt} = k \sum_{i=1}^m (i-1)p(i) \quad (1)$$

where $p(i)$ is the total number of macromolecules with the degree of polymerization (DP) of i , m is the maximum DP in the system and k is the reaction rate constant.

Suppose N is the initial number of macromolecules before degradation, and then the sum total of macromolecules after breaking for R times can be described as follows:

$$\sum_{i=1}^m p(i) = N + R \quad (2)$$

Let L be the sum of DP in the system:

$$L = \sum_{i=1}^m ip(i) \quad (3)$$

Therefore, the degree of degradation (α) can be defined as the quotient of the number of broken bonds (R) and the sum total of bonds before degradation ($L - N$):

$$\alpha = \frac{R}{L - N} \quad (4)$$

By combining these four equations one can get:

$$\frac{d\alpha}{dt} = k(1 - \alpha) \quad (5)$$

Hence, the kinetic equation of random degradation is obtained after further derivation:

$$-\ln\left(\frac{\overline{X_n} - 1}{X_n}\right) = -\ln\left(\frac{\overline{X_0} - 1}{X_0}\right) + kt \quad (6)$$

where X_0 is the original number-average DP before degradation, X_n is the number-average DP of degraded chitosan.

Since a logarithmic function is difficult to manipulate mathematically, we change it into a Maclaurin series expansion:

$$\begin{aligned} -\ln\left(\frac{\overline{X_n} - 1}{X_n}\right) &= -\ln\left(1 - \frac{1}{X_n}\right) \\ &= \frac{1}{X_n} + \frac{1}{2} \times \frac{1}{X_n^2} + \frac{1}{3} \times \frac{1}{X_n^3} + \frac{1}{4} \times \frac{1}{X_n^4} + \dots \end{aligned} \quad (7)$$

Because X_n is always greater than 100 in the present experiment, an approximate process can be proposed as follows:

$$-\ln\left(\frac{\overline{X_n} - 1}{X_n}\right) \approx \frac{1}{X_n} \quad (8)$$

What is more, the viscosity-average DP (X_v) determined from a dilute polymer solution varies directly with X_n , and there is a direct proportionality between the irradiation dose (D) and time (t).

$$\overline{X_v} = k' \times \overline{X_n} \tag{9}$$

$$D = k'' \times t \tag{10}$$

After combining the equations (6), (8), (9) and (10), one can get the following result:

$$\frac{1}{\overline{X_v}} = -\frac{1}{k'} \ln \frac{\overline{X_0} - 1}{\overline{X_0}} + \frac{k}{k' k''} D \tag{11}$$

Then, equation (11) is rewritten as follows:

$$\frac{1}{\overline{X_v}} = A + kD \tag{12}$$

where A is a constant and k is the reaction rate constant of the irradiation degradation.

The relationship between X_v^{-1} and D is given in Fig. 2. The results of linear regression are satisfactory both in air and in vacuum (γ^2 is 0.998 and 0.996, respectively), which proves that the irradiation degradation of chitosan obeys the kinetics of random degradation.

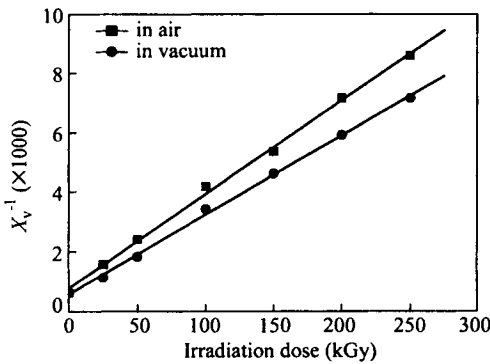


Fig. 2 X_v^{-1} versus irradiation dose during irradiation degradation

Influence of γ -Irradiation on the Degree of Deacetylation

Some degradation methods have been reported recently, including chemical degradation^[11, 18], ultrasonic degradation^[19, 20] and enzymatic degradation^[21, 22]. It is a basic requirement that the valuable properties of chitosan derived from the main chain structure and principal functional groups should be maintained during degradation. Here DD was used to illuminate the difference of amino group content in the molecular chains before and after irradiation.

The DD of degraded chitosan determined by potentiometric titration are listed in Table 1.

Table 1. Degree of deacetylation of irradiated chitosan

D (kGy)		0	25	50	100	150	200	250
DD	In vacuum	0.740	0.743	0.750	0.751	0.756	0.759	0.765
	In air	0.740	0.745	0.753	0.755	0.760	0.763	0.768

These data imply that DD is slightly raised during γ -irradiation. It is an outstanding advantage of irradiation degradation that amino groups are not destroyed during the reaction. The existence of a large amount of amino groups along the molecular chain can improve the solubility and biocompatibility of chitosan. Tomihata and Ikata reported that chitosan films with a DD of 1.0 gave a much milder tissue reaction than the partially deacetylated

ones when implanted in rats^[23]. Because of the weak basicity of the primary amine and the preferential adsorption of the anions, the zeta potential of the 100% deacetylated chitosan dropped to almost zero, which made it possible to exhibit no strong stimulation to the surrounding tissue. Moreover, some functional groups such as carboxymethyl, alkyl, aryl and acyl groups can be introduced into the amino groups through chemical reaction, which endows chitosan with many new functions leading to a much wider range of applications.

FTIR of O-CMCh

Figure 3 shows the FTIR spectra of chitosan and O-CMCh with different MW.

The FTIR spectrum of chitosan (Fig. 3a) shows that the main characteristic peaks at 3455 cm^{-1} can be assigned to the stretching vibration of OH group and 2918 cm^{-1} and 2879 cm^{-1} assigned to CH stretching. The vibration bands at 1321 cm^{-1} and 897 cm^{-1} are assigned to CN stretching and NH stretching of a primary amine, respectively. 1155 cm^{-1} can be assigned to bridge O stretching, and 1090 cm^{-1} assigned to CO stretching^[24].

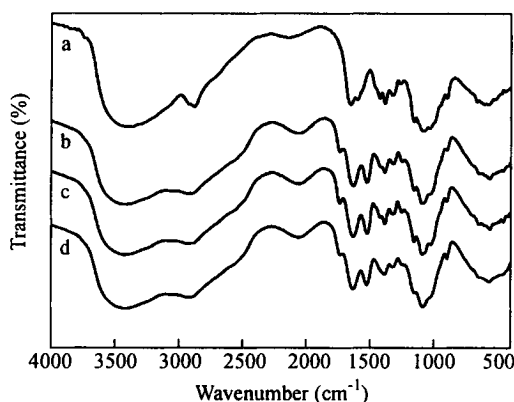


Fig. 3 FTIR spectra of chitosan a) and O-CMCh with different MW, b) $M_v = 2 \times 10^4$, c) $M_v = 1.2 \times 10^5$, d) $M_v = 2 \times 10^5$

The spectra in (Fig. 3b–3d) belong to O-CMCh with different MW. And there is no evident difference among them. The appearance of the C=O stretching vibration band at 1734 cm^{-1} and the CO stretching vibration band of the group $-\text{CH}_2-\text{COOH}$ at 1251 cm^{-1} indicates the existence of carboxymethyl group. The existence of a NH stretching vibration band at 897 cm^{-1} and the appearance of 1629 cm^{-1} and 1521 cm^{-1} assigned to $-\text{NH}_3^+$ all show that the $-\text{CH}_2-\text{COOH}$ is not connected to the $-\text{NH}_2$ group. And the CO stretching vibration band at 1090 cm^{-1} becoming stronger further proves that the reaction occurs on the $-\text{OH}$ group. Thus, the derivative obtained is the anticiated O-CMCh.

MW-dependent Antibacterial Activity of O-CMCh

At present, the antibacterial function of amino-polysaccharides is generally acknowledged to come from the $-\text{NH}_2$ group along the chains. So the content of $-\text{NH}_2$ affects their antibacterial activity for chitosan derivatives. Comparing O-CMCh with chitosan, its number of $-\text{NH}_2$ groups along its chain is not changed because the substitution occurs on the $-\text{OH}$ groups, and its $-\text{COOH}$ groups may react with $-\text{NH}_2$ groups intra- or intermolecularly and charged these $-\text{NH}_2$ groups. So, under the same conditions, the number of $-\text{NH}_3^+$ groups in O-CMCh is more than that of chitosan and the antibacterial activity of O-CMCh is stronger than that of chitosan. This has been proved in the preceding work^[25].

Figures 4 and 5 show the plots of the optical density (OD) versus the culture time for O-CMCh against *E.coli* measured by the Shaking Flask Test method. According to the theory of this test, the smaller the optical density of the medium is, the higher is the antibacterial activity of the tested material.

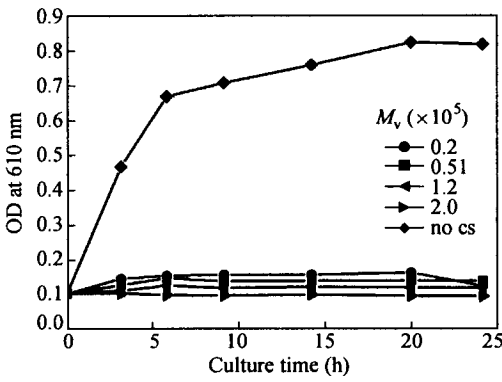


Fig. 4 Optical density versus the culture time for O-CMCh with M_v ranging from 2×10^4 to 2×10^5 against *E. coli* (“no cs” means no chitosan in the culture medium)

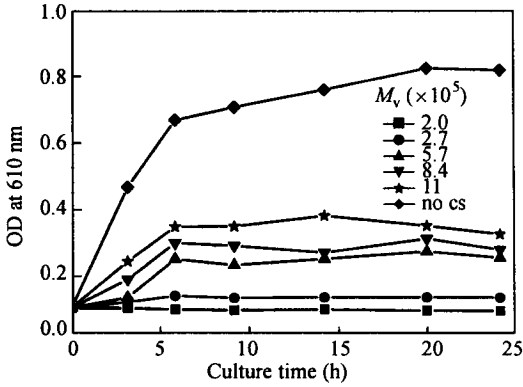


Fig. 5 Optical density versus the culture time for O-CMCh with M_v ranging from 2×10^5 to 1.1×10^6 against *E. coli*

In this experiment, O-CMCh with M_v of 2×10^4 , 5.1×10^4 , 1.2×10^5 , 2×10^5 and 2.7×10^5 was tested. To examine MW dependence comprehensively, O-CMCh with M_v of 5.7×10^5 , 8.4×10^5 , 1.1×10^6 (with the same DS) were also added. Based on the plots, all the tested derivatives have good antibacterial activity and they increase with M_v increasing from 2×10^4 to 2×10^5 , but decrease at M_v above 2×10^5 .

In our preceding work^[25], it was concluded that the antibacterial activity of chitosan increases with M_v from 5000 to 9.16×10^4 and decreases with M_v 9.16×10^4 to 1.08×10^6 . So it is clear that the two polysaccharides have similar trends in this aspect, and the second similar point is the existence of an only suppositional antibacterial activity peak where they possess the best antibacterial activity in the plots of optical density versus the culture time. However, the peak appears when M_v is 9.16×10^4 for chitosan, and 2×10^5 for O-CMCh.

Because the antibacterial function group of amino-polysaccharides is generally acknowledged as $-\text{NH}_2$, the above trend can be explained as follows: When MW increases, the content of end groups decreases, while the $-\text{NH}_2$ content of the chains increases for both polysaccharides. However, the intrinsic flexibility of chain molecule also increases with increasing MW, which in fact makes the available antibacterial groups decrease. The two opposing factors affect the antibacterial activity of the polysaccharides, and the antibacterial activity peak mentioned above appears when the two factors reach a certain balance. Based on this work, the balance point is 9.16×10^4 (M_v) for chitosan and 2×10^5 (M_v) for O-CMCh.

The enhanced balance point of MW for O-CMCh is due to the introduction of $-\text{CH}_2\text{COOH}$ which may react with the $-\text{NH}_2$ groups intra- or intermolecularly and change these $-\text{NH}_2$ groups to the cationic $-\text{NH}_3^+$ groups. The presence of the positive groups weakens the flexibility of the chains and prevents the chains from curling. Therefore, the appearance of the antibacterial peak was postponed for O-CMCh until a new balance was reached where M_v goes up to 2×10^5 .

CONCLUSIONS

This work put forward a kinetic model for the irradiation of chitosan. Results show that there is a linear relationship between the reciprocal of X_v and the irradiation dose (D) and the irradiation degradation of chitosan obeys the rule of random degradation. Also, irradiation does not destroy the amino groups along the chitosan chains in the reaction.

The antibacterial activity of O-CMCh is influenced by MW, and the trend of MW-dependence is similar to that of chitosan. An only suppositional antibacterial activity peak exists on both sides; however, the peak of O-CMCh appears at a relatively higher MW because of the introduction of carboxymethyl group.

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