The Effect of Different Sterilization Procedures on Chitosan Dried Powder

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ABSTRACT: The aim of this study was to investigate the effects induced by three different sterilization methods [steam, γ-radiation, and ethylene oxide (EO)] with different dose or time period of sterilization by means of Fourier transform infrared spectroscopy, X-ray diffraction, and assessments of molecular weight and degree of deacetylation. The experiment results clearly showed that the steam sterilization greatly darkened the color of chitosan powder; γ-irradiation strongly depolymerized the chitosan especially at irradiation dose above 10 kGy; and exposure to EO led to minor change in crystallinity and network structure of chitosan powder. Therefore, these three methods have their respective advantages and disadvantages, suggesting that the selection of the sterilization method of chitosan should depend upon the specific requirement of the final application. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 104: 1968–1972, 2007

Key words: chitosan; sterilization; viscosity; X-ray; FTIR

INTRODUCTION

Chitosan is made up of D-glucosamine units linked by β(1–4) glycosidic bonds and derived from deacetylation of chitin, which is the second most abundant polysaccharide in nature next to cellulose and can be extracted from the exoskeleton of crustacean such as shrimps and crabs, and from some fungi cell walls.[1,2] Chitosan is a biocompatible and biodegradable polymer with a variety of useful biological properties, and has been widely used as food ingredient, drug delivery vehicle, and wound-healing agents. Its biodegradability, biocompatibility, and specific interactions with components of the extra cellular matrix and growth factors have led to its growing use in tissue engineering, such as in the repair of skin, bone, nerve, and cartilage.[3–5]

Chitosan material implanted in human body as drug delivery vehicle or medical devices must be sterilized prior to use. People often assume that existing sterilization technologies will be appropriate for chitosan, and in most instances; the more popular methods are adequate for the chitosan material. But for selecting the sterilization method, we not only focus on the efficacy of a sterilization process in terms of effect of killing microorganisms and the nature of the residuals formed, but also take into account the effect of sterilization processes on the properties of the chitosan material, which is often ignored. Obviously, the effect of sterilization processes on the properties of the chitosan material should be as minor as possible. Before the sterilization method is endorsed for chitosan, its effects on the properties and end performance of chitosan should be well investigated.

Commonly used sterilization methods for chitosan include exposure to steam, ethylene oxide (EO) and γ-ray radiation. These three sterilization processes act either chemically or physically leading to a lethal change in the structure or function of the organic macromolecules in microorganisms. Given the nature of their action, the different forms of sterilization can also attack the materials of polymers by the same mechanisms, resulting in hydrolysis, oxidation, chain scission, and depolymerization.[6] Some studies have shown that γ-radiation can modify the chemical, physical, and mechanical properties of chitosan.[7,8] Some studies have been related to the effect of different sterilization methods on the chitosan membranes and injectable chitosan.[9,10] Few studies have discussed the modification of chitosan powder properties induced by steam or EO sterilization, while the chitosan powder has been widely used as food

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ingredient, drug delivery vehicle, and wound-healing agents.[4,5] So it is difficult for people to select the most adequate sterilization method for chitosan products.

The goal of this work was to investigate the changes in the color, degree of deacetylation (DD), molecular weight (MW), and structure of chitosan produced by three different sterilization methods (steam, exposure to γ-radiation, and EO) with different dose or time period of sterilization by means of Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction, and assessments of MW and DD.

METHODS

Materials and reagents

Chitosan powder, derived from snow crab, was obtained from Nantong Xincheng Biochemical Company (Jiangsu, China). All the other chemicals used in this study were of research purity grade.

Degree of deacetylation determination

The degree of deacetylation (DD) of chitosan was determined by titration as previously described with minor modification.[11,12]

A 0.5 g dried chitosan sample was accurately weighed and dissolved in 0.1*M* HCl. Then the solution was titrated with 0.1*M* NaOH. The DD was calculated as follows:

$$NH_2\% = [(C_1V_1 - C_2V_2) \times 0.016]/G$$

$$DD\% = 203(NH_2\%)/[16 + 42(NH_2\%)] + \times 100\%$$

Where C_1 and C_2 are the concentrations of HCl (M) and NaOH (M); G is the chitosan weight (g); and 0.016 is the weight of NH_2 equivalent to 1 mL 0.1 mol/L HCl (g).

Molecular weight determination

The intrinsic viscosity describes a polymer's ability to form viscous solution and is directly proportional to the average molecular weight (MW) of the polymer. The intrinsic viscosity is a characteristic of the polymer under specific solvent and temperature conditions. It is independent of concentration. Viscosity average MW of chitosan was calculated from solution viscosity data which were determined at (30 ± 0.05)°C using a Ubbelohde capillary viscometer. The concentration of chitosan solution (in a mixed solvent of 0.2*M* acetic acid and 0.1*M* sodium acetate solvent) is 1.0 mg/mL. The intrinsic viscosity [η] was obtained from a linear plot ($r^2 \geq 0.99$) of reduced viscosity against concentration according to the Huggins equation, and MW was calculated from the resulting [η] using the Mark-Houwink equation:

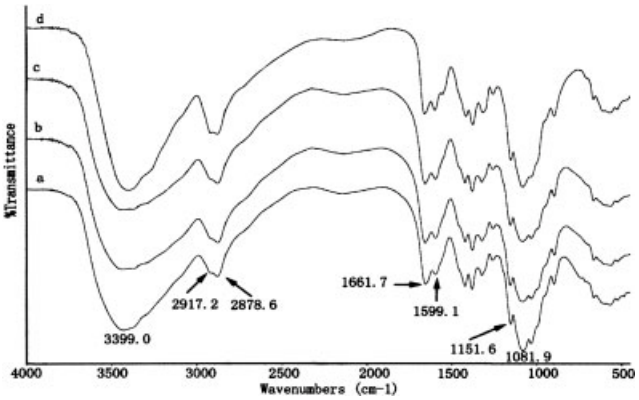

Figure 1 FTIR spectra of unsterilized chitosan (a), chitosan sterilized using steam (b), γ-irradiation (c), and ethylene oxide (d).

$$[\eta] = KM^{\alpha}$$

where constant $K = 6.589 \times 10^{-3}$; M is viscosity derived average MW; and α (0.88) is an empirical constant describing the conformation of the chitosan.[13,14]

Sterilization process

Steam sterilization

Steam sterilization is a relatively simple process, and steam sterilization kills microorganisms by destroying metabolic and structural components essential to their replication. In this study the chitosan powder was exposed to saturated steam at 121°C for 20, 30, and 45 min at a pressure of 115 kPa.

γ-Irradiation

In this study, the γ-irradiation of sample was carried out in a Nantong meikeer irradiation company (Jiangsu, China). Radiation sterilization utilizes ionizing radiation from a cobalt-60 (^{60}Co) isotope source with sterilization achieved when highly reactive free radicals induce breaks in the DNA double helix of microorganisms, preventing replication. The most commonly validated dose used to sterilize medical materials is 25 kGy. In this study, samples were exposed to 5, 10, 15, 20, and 25 kGy γ-irradiation using a ^{60}Co source, respectively.

Ethylene oxide sterilization

The ethylene oxide (EO) sterilization process utilizes EO which has bactericidal, sporicidal, and virucidal effects resulting from alkylation of amino and hydroxyl groups in nucleic acids causing cell injury or death.[13] In this study, samples were exposed to EO,

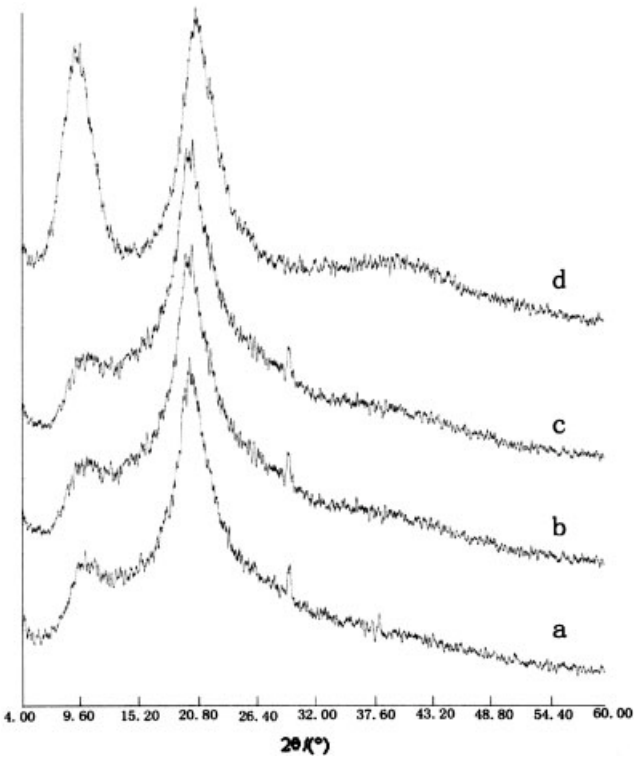

Figure 2 X-ray diffraction patterns of unsterilized chitosan (a), chitosan sterilized using steam (b), γ-irradiation (c), and ethylene oxide (d).

the concentration of which was 800g/m^3, at 50°C for 0.5, 1, 2, 4, and 8 h, respectively, and aeration for 10 days. After aeration, no EO residue was detected in chitosan sample by gas chromatography.

FTIR analysis

Fourier transform infrared (FTIR) spectra were obtained with Nexus model 870 Fourier Transform IR Spectrophotometer. Dry chitosan powder was ground with KBr powder and compressed into discs for FTIR examination. FTIR was used to study changes in the chemical structure of the chitosan samples after different sterilization procedures, which were steam heated for 45 min, γ-irradiation with 25 kGy, or exposure to EO for 8 h.

X-ray diffraction analysis

X-ray diffraction patterns in the 2θ range of 4°–60° were obtained for the samples of unsterilized and sterilized at room temperature using a scan rate of 10°(2θ)/min at 40 kV and 40 mA (x'TRA, Switzerland ARL). The samples of sterilized chitosan were steam heated 45 min, γ-irradiated with 25 kGy, or exposure to EO for 8 h.

RESULTS

Visual inspection

Visual inspection of all samples was performed before and after sterilization. A color change from light to dark yellow in chitosan samples was observed after exposure to steam, and the intensity of the color change increased with increased heating time. The yellow color darkening of the steam-sterilized chitosan may result from the Maillard reaction between NH_2 and OH groups.[7] However, no obvious color change was found in chitosan samples sterilized using EO or γ-irradiation, while it has been reported that there are color changes in chitosan membranes after γ-ray sterilization.[9] This difference may be attributed to the distinct quality of the chitosan material such as the content of protein and ash in chitosan. And more protein and ash would lead to more color change after sterilization.

FTIR analysis

Figure 1 shows FTIR spectra of unsterilized chitosan (a) and chitosan sterilized using steam (b), EO (c), and γ-irradiation (d), respectively. The characteristic absorptions in FTIR spectrum are displayed at 1661.7 and 1599.1 cm^{-1} attributable to amide bands 1 and 2, which indicated the amine groups of chitosan at 3399.0 cm^{-1} (O—H stretch), 2917.2 cm^{-1}, 2878.6 cm^{-1} (C—H stretch). No obvious difference was observed in IR spectra between unsterilized chitosan and chitosan sterilized with steam, EO and γ-irradiation, respectively.

As is known, the two main radiation induced reactions that could significantly affect sample are main chain scissions and the formation of crosslinks. Therefore the analysis by FTIR spectroscopy suggests that sterilization of chitosan by steam, EO, or γ-irridation induces neither crosslinking nor formation of new groups in chitosan. This result is in accordance with the data reported by other published article.[15] This means that no EO leaved in chitosan and no

TABLE I
The Effect of Steam Sterilization on Degree of Deacetylation and Molecular Weight of Chitosan

Time of steam sterilisation	0	20 (min)	30 (min)	45 (min)
Degree of deacetylation (%)	91.1 ± 0.4	91.9 ± 0.5	91.7 ± 0.3	92.0 ± 0.4
Molecular weight ($\times 10^{-5}$)	5.69 ± 0.11	5.61 ± 0.09	5.60 ± 0.08	5.57 ± 0.10

TABLE II
The Effect of EO on Degree of Deacetylation and Molecular Weight of Chitosan

Time of EO sterilization	0	0.5 (h)	1 (h)	2 (h)	4 (h)	8 (h)
Degree of deacetylation (%)	91.1 ± 0.5	91.1 ± 0.4	91.8 ± 0.6	91.6 ± 0.5	91.7 ± 0.3	91.5 ± 0.4
Molecular weight ($\times 10^{-5}$)	5.69 ± 0.09	5.69 ± 0.08	5.65 ± 0.06	5.62 ± 0.04	5.61 ± 0.08	5.60 ± 0.07

new chemical bond has been formed during sterilization.

X-ray diffraction patterns

The X-ray diffraction patterns of unsterilized chitosan [Fig. 2(a)] and chitosan sterilized using steam [Fig. 2(b)] or γ-irradiation [Fig. 2(c)] showed that no significant change occurred during sterilization. However, sterilization using EO was observed to induce a minor modification in crystallinity and network structure of chitosan [Fig. 2(d)], in which the diffraction peak became greatly stronger at $2\theta = 9.6^\circ$, and no small diffration peak was found at $2\theta = 30^\circ$. These observations suggest that some change have occurred in crystallinity and network structure of chitosan sterilized by EO in same diffraction directions, and crystallinity and network structure of chitosan may be related to the tensile strength and elongation of chitosan products.

Molecular weight and degree of deacetylation

Molecular weight (MW) and degree of deacetylation (DD) are key functional attributes of chitosan. Many properties of chitosan, such as degradation rate, tensile strength, elongation, bioactivity, permeability, and swelling ability, are dependent on MW and DD. Because of the chitosan source and preparation procedure, its average MW may range from 50 to 1000 kDa, and commercially available preparation has degrees of deacetylation from 50 to 95%. Sterilization processes act either chemically or physically leading to a change in MW and DD of chitosan.

After being steam sterilized for 20, 30, and 45 min (Table I) or EO sterilized for 0.5, 1, 2, 4, and 8 h (Table II), no obvious change was found in DD and MW of chitosan. While the DD of chitosan slightly increased following exposure to γ-irradiation sterilization at dose of 5, 10, 15, 20, and 25 kGy (Fig. 3), which might be attributed to that γ-irradiation could break acetylamino group. The MW of chitosan greatly decreased with increasing dose of γ-irradiation within the dose range of 5, 10, 15, 20, and 25 kGy (Fig. 4). The decreasing rate of MW is slower within the dose range of 0–10 kGy, but the MW sharply decreased within the dose range above 10 kGy. The most commonly validation dose used to sterilize medical devices is 25 kGy,[6] but at the dose of 25 kGy, the MW of sterilized chitosan is only one fifteenth of original MW. This result indicates that γ-irradiation sterilization is not good in term of effects of sterilization on MW of chitosan, and the optional dose of irradiation should not be larger than 10 kGy. In the manufacture process of chitosan powder, the deacetylation was finished by putting chitin in 50% NaOH at high temperature, and all bacteria in chitin would be killed during this process. Then in the following manufacture processes such as washing, drying, smashing, and packing, if the chitosan material were kept away from bacteria, the number of original bacteria in chitosan powder will be less. If the original bacteria can be controlled to be lesser then 500 cfu/g, the 10 kGy of γ-irradiation is strong enough to sterilize chitosan powder.

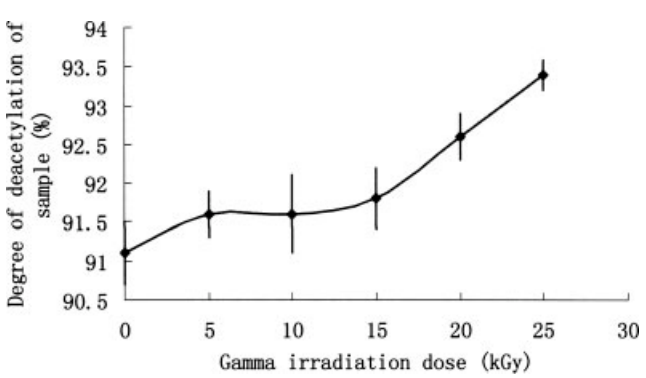

Figure 3 The effect of γ-irradiation on degree of deacetylationand of chitosan ($n = 3$).

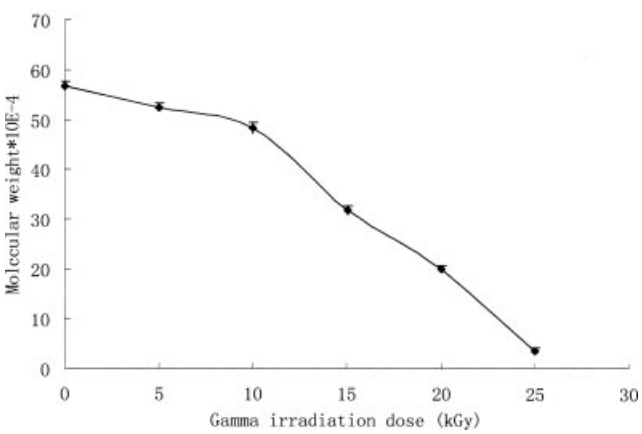

Figure 4 The effect of γ-irradiation on molecular weight of chitosan ($n = 3$).

DISCUSSION

Steam sterilization

It has been reported that the high temperature, humidity, and pressure used during steam sterilization can lead to hydrolysis, softening, and degradation of biomedical polymers.[16,17] In this study, the high temperature and moist environment created by the autoclave sterilization process were found to change the color of chitosan, and the color of chitosan darkened with increasing time period of sterilization. This phenomenon was similar to the observation in a previously published article.[18] However, no change in the chemical structure of chitosan was observed during steam sterilization as shown in FTIR and XRD spectra, and only little modification was found in the MW of chitosan after steam sterilization. It follows that little degradation of chitosan occurs in the process of steam sterilization. However, there are some limitations for steam sterilization concerning batch process scale-up.

Ethylene oxide sterilization

Ethylene oxide (EO) is one of the most widely used commercial sterilization process in the medical device and care industries. However, the alkylating reactions, which this process utilizes to achieve sterilization, have also been reported to react with the functional groups of some biomedical materials.[19] Although the primary amino groups on the chitosan molecules are reactive and provide a mechanism for side group attachment using a variety of mild reaction conditions,[20] FTIR spectra showed that EO used in sterilization process of chitosan does not affect the chemical properties and structure of chitosan. This sterilization process induces only the change in crystallinity and network structure of chitosan, as shown by X-ray diffraction patterns. Therefore, EO sterilization can be considered to be the most adequate sterilizing agent for chitosan, despite EO sterilization associated with residues, toxicity, flammability, and environmental risks. This result is in accordance with the data reported by Marreco et al.[9]

γ-Irradiation sterilization

Although γ-irradiation, as one of the most commonly employed sterilization agent, is a rapid, highly effective sterilization method for medical devices and materials, it has been reported that this form of sterilization results in a range of physical changes including embrittlement, degradation, odor generation, stiffening, softening, enhancement, or reduction of chemical resistance.[13] In this study, FTIR spectra and X-ray diffraction patterns suggest that this sterilization process does not change chemical structure and properties or crystallinity and network structure of chitosan. It has been found that the γ-irradiation slightly increases the DD of chitosan. This implies that γ-irradiation breaks acetylamino group, and results in significant degradation in chitosan, which is in agreement with other reports that the MW of chitosan decreased with increasing dose during the process of irradiation sterilization. The present study further indicates that the MW decreased sharply at the dose above 10 kGy. This is because the radiation induced radical formation can occur randomly at any C atoms of the chitosan base units. The reactions of radicals at C_1 or C_4 will lead to the splitting of the 1–4 glycosidic bonds, causing main chain scission.

CONCLUSIONS

In summary, the experiment results indicated that the steam sterilization greatly darkened the color of chitosan powder; γ-irradiation strongly depolymerized the chitosan especially at irradiation dose above 10 kGy; and exposure to EO led to minor change in crystallinity and network structure of chitosan powder. Therefore, these three methods have their respective advantages and disadvantage, and the sterilization method of chitosan should depend upon the specific requirement of the final application.

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