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Clinical Applicable Carboxymethyl Chitosan with Gel-Forming and Stabilizing Properties Based on Terminal Sterilization Methods of Electron Beam Irradiation

Huili Yuan, Wenjing Liu, Yu Fu, Jiangjie Wu, Si Chen,* and Xu Wang**

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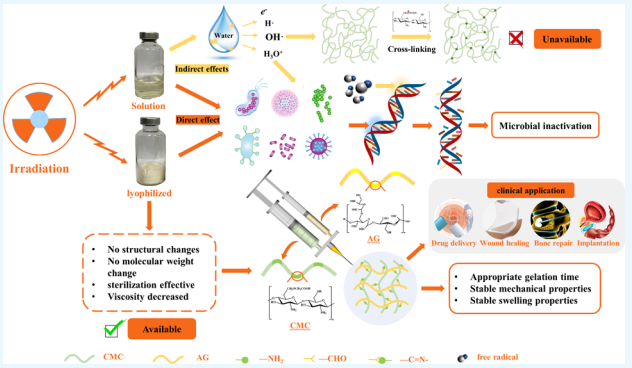
ABSTRACT: Carboxymethyl chitosan (CMC)-based hydrogels have great potential for clinical applications, but a critical sterilization process must be addressed to bring them to market. Compared to ethylene oxide sterilization or heat sterilization, irradiation sterilization avoids alkylation and heat damage, while available studies on γ-irradiated other polysaccharides show that solution polysaccharides are susceptible to degradation or cross-linking. Aiming at the challenges brought by the γ-irradiation process of polysaccharide aqueous solution, this paper innovatively proposes the lyophilized CMC using electron beam (EB) irradiation, which not only avoids the generation of free radicals in the irradiated water leading to the degradation and cross-linking of polysaccharides but also retains the properties of CMC in terms of gel formation, stabilization, and clinical application. We used FTIR, TG, GPC, and microbial load tests to demonstrate that lyophilized CMC did not have significant changes in structure and molecular weight after EB irradiation, complied with the requirements for sterilization, and still had gel stability, thus proving that lyophilized CMC could be used for EB irradiation and met the requirements for clinical application. Therefore, this work is expected to further advance CMC injectable hydrogels toward clinical applications and provide a new direction for the sterilization processes of other polysaccharide hydrogels.



1. INTRODUCTION

Hydrogels, which can be categorized into natural and synthetic hydrogels,[1] have gained a great deal of attention in the field of tissue engineering,[2,3] wound dressings,[4] implants,[5] and other medical materials because of their good biocompatibility and unique three-dimensional cross-linked network structure.[6] Compared with synthetic hydrogels, natural hydrogels are composed of chitosan,[7] sodium alginate,[8] hyaluronic acid,[9] cellulose,[10] and other natural polysaccharides that have the advantages of high stability, nontoxicity, good biocompatibility, and biodegradability.[11] Among them, chitosan is the only alkaline polysaccharide with an amino group on its monomer in nature, which has excellent biocompatibility and biodegradability, making it widely researched in the biomedical field.[12,13] While chitosan is only soluble in moderately acidic environments due to the protonation of amino groups at pH < 6.5, it thus limits many of its potential applications.[14] Carboxymethyl chitosan (CMC) is obtained from the carboxymethylation modification of chitosan, which not only possesses a wide pH solubility range, but also has higher antimicrobial activity and the ability to interact with other substances.[15] Therefore, CMC can be further applied in areas where chitosan is deficient due to its limited solubility, such as wound healing, anticancer/anti-inflammatory/antimicrobial drug delivery systems, and implantable biomaterials.[16−19]

Despite a large number of potential clinical applications for CMC-based materials published in recent years, only individual wound dressings can be found on the medical market, as the critical step of a clinically referenced sterilization process that meets clinical references has to be overcome to bring such material to the clinical market,[20] which is clearly defined in the European Medical Devices Regulation (EMDR)[21] or the Chinese Medical Devices Regulation (CMDR).[22] Sterilization refers to the use of physical or chemical methods to kill or eliminate all microorganisms (live cells, live spores, viruses, etc.) from the material so that the material must meet the sterility assurance levels (SAL = 10^{-6}) specified by international companies. SAL can be achieved through aseptic production or terminal sterilization (ethylene

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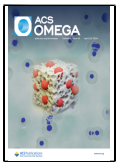
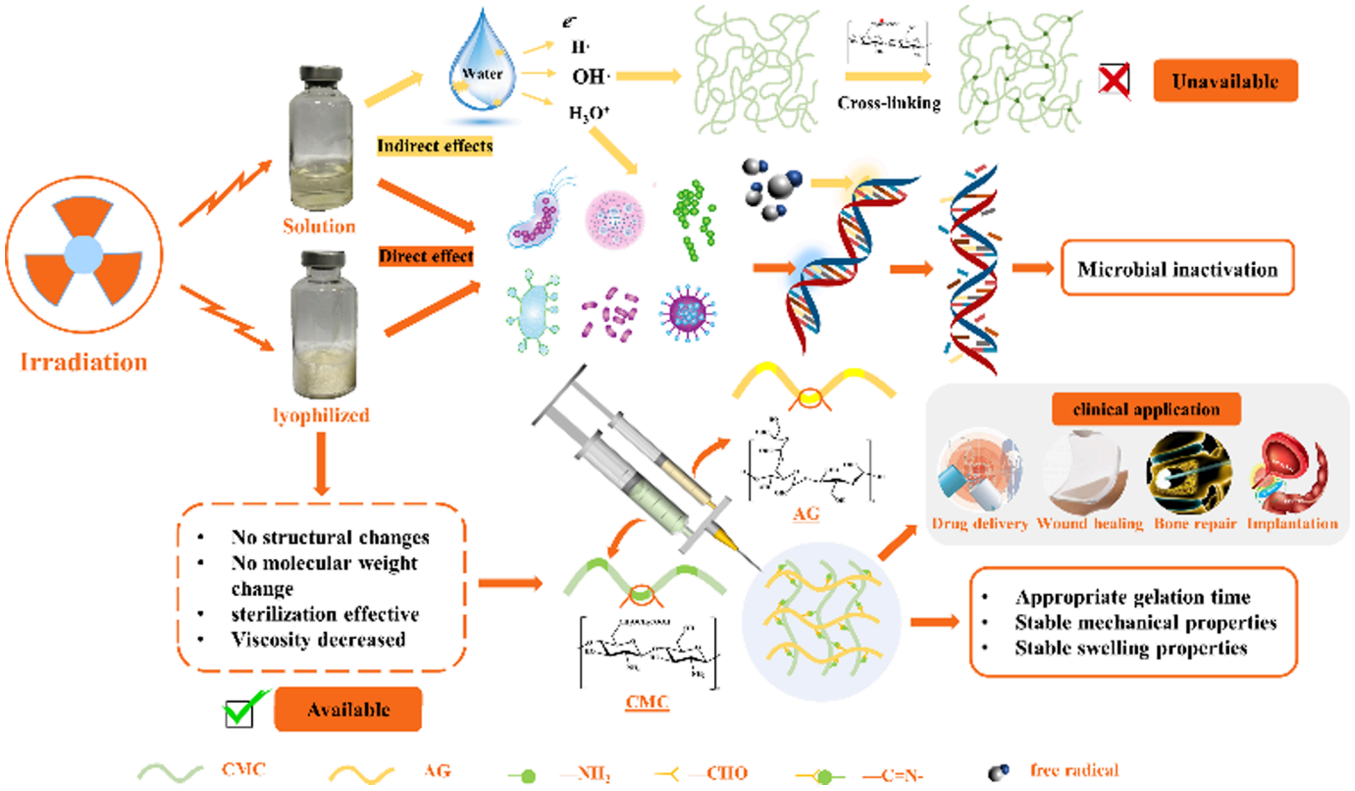


Scheme 1. Schematic Diagram of the Irradiation Sterilization Mechanism and the Different Manifestations of Different Forms after Irradiation



oxide, dry/moist heat, radiation sterilization),[23] but terminal sterilization is often preferred over aseptic production because it does not require aseptic production, class 100 or higher clean environments, and stringent process controls, and it is simple and inexpensive to operate.

However, there are still limitations in using terminal sterilization methods for CMC-based materials, especially in injectable implantable materials.[24] CMC has groups (amino, hydroxyl) that can alkylate with ethylene oxide in Eto sterilization;[25] second, CMC is very sensitive to heat, with maximum weight loss occurring at 65 °C, and heat sterilization leads to a significant decrease in viscosity and molecular weight,[26] both of which have a direct impact on the formation of hydrogels when mixed with another component for injection. Irradiation sterilization, there have been related to other polysaccharides (pectin, sodium alginate) and other research shows that irradiation will make polysaccharides depolymerization or chain breaking, resulting in lower molecular weight or cross-linking to form a new structure, which will affect the performance and even lead to the failure of the intended use..[27] Therefore, it is crucial to find a suitable sterilization method for use with CMC-based materials.

In this study, we found that the method of electron beam (EB) irradiation using lyophilized CMC can be used for terminal sterilization of CMC-based materials because it prevents water molecules from being irradiated to produce water radicals, thereby preventing water radicals from attacking the active site of the CMC, which can lead to chain breakage or cross-linking. In this paper, solution and lyophilized CMC were selected as raw materials and irradiated with EB at the same time to study and analyze the effect of EB irradiation on the physicochemical properties of CMC as well as the mechanism of the influence of the presence of water on its irradiation, and at the same time to validate the effect of the EB sterilizing dose, which proved that the lyophilized CMC was suitable for EB irradiation. The effect of EB irradiation on CMC and the sterilization mechanism are displayed in Scheme 1. Meanwhile, we compared the physicochemical property studies of CMC with an aldehyde-containing cross-linking agent to form water-for-injection gels before and after irradiation, which proved that the method does not affect the next application of CMC. In addition, we hope that our work can further promote the clinical application of carboxymethyl chitosan-based materials in biomedical fields.

2. MATERIALS AND METHODS

2.1. Materials. In this paper, solution and lyophilized CMC were sterilized by EB irradiation, while hydrogels formed by irradiated CMC in aldehyde-containing cross-linking agents were investigated.

Methods of Preparation. CMC was synthesized by literature methods with slight modifications (Figure S1).[28] The cross-linker pyranose aldehyde (AG), which forms hydrogels with CMC, was synthesized according to the literature method with slight modifications (Figure S2).[29] Details of the preparation process are shown in the Supporting Information.

2.2. Material Processing. Water was used as solvent to dissolve the CMC powder to obtain 5 wt % solution sample, 10 mL was taken into 30 mL syringes, and the solution sample was directly capped and vacuum sealed and stored under 4 °C. Lyophilized samples should be freeze-dried and then capped and vacuum-sealed and stored at 4 °C for use. The lyophilization process setup is shown in Table S1.

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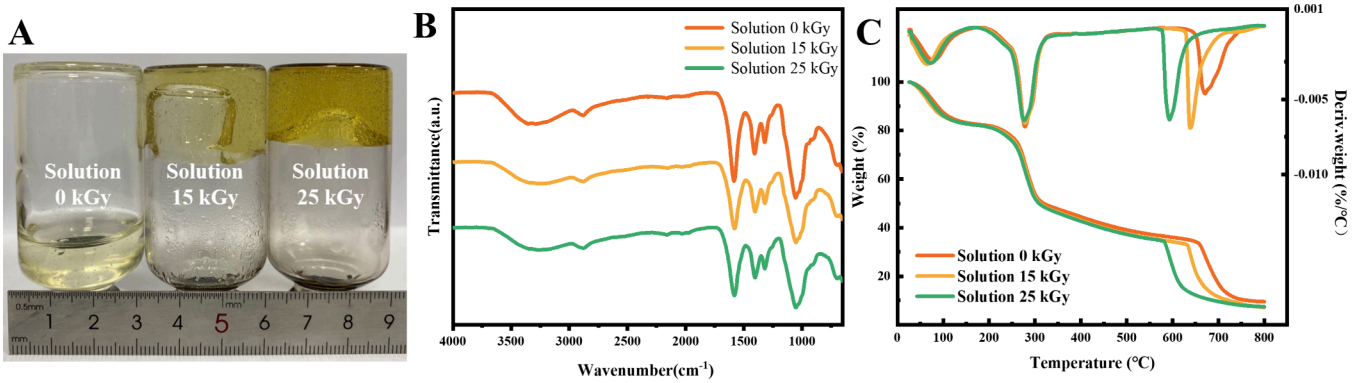

Figure 1. (A) Picture of CMC solution samples before and after irradiation. (B) FTIR of CMC solution samples before and after irradiation. (C) TG of CMC solution samples before and after irradiation.

2.3. Sterilization Method. The 5 wt % solution(10 mL) and lyophilized CMC samples were irradiated by EB using a 10 MeV/20 kw high-energy electron gas pedal irradiator (Zhejiang Zhengshi Irradiation Technology Co., Ltd.) The conditions of the irradiation sterilization treatment were as follows: a temperature of 25 °C, sterilization doses of 15 and 25 kGy (standard dose), and simultaneous sample exposure time of 5 s at 1 m from the beam source. The sterilized samples were stored at 4 °C before subsequent characterization. The conventional radiation doses mentioned in the radiation sterilization standards and guidelines issued by the International Organization for Standardization (ISO) and the Chinese National Committee for Standardization (CNCS) are 15 and 25 kGy, respectively.[30]

2.4. Viscosity Analysis by a Viscometer. The change in viscosity of CMC samples before and after irradiation was measured using an NDJ-1B rotational viscometer. The viscosity was measured directly for solution samples, and after, lyophilized samples were dissolved in water to form a 5 wt % solution. Samples were measured at 25 °C.

2.5. Structural Analysis by Fourier Transform Infrared Spectroscopy (FTIR). Structural characterization of the CMC before and after irradiation was performed using a Nicolet 6700 Fourier transform infrared spectrometer (FTIR) from Thermo Fisher, USA. The structure of the samples was examined using the attenuated total reflection mode to analyze the changes in molecular functional groups before and after irradiation. The solution samples were lyophilized before testing.

2.6. Thermal Degradation Behavior by Thermogravimetry. Thermogravimetric analyzer (TG, 209 F1, Netzsch Germany) was used to test the thermal degradation behavior of the samples with a temperature increase rate of 10 °C/min and a temperature increase range of 25−800 °C under an air atmosphere.

2.7. Molecular Weight by Gel Permeation Chromatography. The molecular weights of unirradiated and irradiated lyophilized CMC were measured using a GPC-gel permeation chromatography system (Elite Dalian) model P230. The chromatographic column was PL aqua gel−OH MIXED-H 8 μm (7.3 mml D × 30 cm) with an injection rate of 1.0 mL/min, a column temperature of 40 °C, and a mobile phase of 0.4 M NaAc (pH = 4.5).

2.8. Preparation of Hydrogels. Frozen CMC samples and AG powder were dissolved in phosphate buffer solution (PBS) to obtain 5 wt % CMC solution and AG solution. Hydrogels were formed from the CMC solution and AG solution by mixing them well with a duplex syringe. The absence of flow when the tubes were inverted was taken as a sign of gel completion, and the time taken from the start of mixing to the cessation of flow was recorded and noted as the gel time

2.9. Swelling and In Vitro Degradation. The remaining weight percentage was used to characterize the swelling and degradation properties of the hydrogels. The above-prepared hydrogels were packed in tea bags and weighed as the initial weight W_0. The hydrogels were placed in PBS solution to simulate the in vivo degradation process. The hydrogel was placed in PBS solution to simulate the in vivo degradation process and then taken out every 24 h. The water on the surface of the teabag was dried with filter paper and weighed as W_t. The percentage of residual weight was recorded as W_t/W_0, and the percentage of residual weight was plotted at each time to obtain the in vitro degradation curve of the hydrogel. The maximum residual weight percentage is used to represent the swelling rate; the stage of rising residual mass is regarded as the swelling process.

2.10. Dynamic Mechanical Properties. The mechanical properties of hydrogels were tested using an advanced expanded rheometer model MCR 302 from Anton Paar, Austria. Hydrogel samples were injected into the instrument's sample stage via a duplex syringe and molded in situ. When strain scanning was performed, the fixed angular frequency was 1 rad/s, and the strain scanning range was set from 0.1% to 500%. The storage modulus (G') represents the elasticity of the hydrogel, the loss modulus (G'') represents the viscosity of the hydrogel, and the intersection of G' and G'' is the sign of destruction of the hydrogel. The results of the energy storage modulus and maximum strain of the gel can be obtained by the rehomechanics test.

2.11. Surface Morphology of Hydrogels. The surface network morphology of the hydrogels was observed by a field emission scanning electron microscope (SEM) model S-4700 from Hitachi, Japan. The hydrogel samples were first immobilized with liquid nitrogen to fix the morphology and then dried under vacuum in a freeze-dryer to obtain a dry gel. After platinum was sprayed on the surface of the dry gel, the samples were observed and photographed under a 10 kV electron microscope.

2.12. Biological Load Test. Bioburden test according to GB/T 19973.1−2015:[31] 1 mL of solution sample was transferred to 9 mL of sterile diluent (0.9% NaCl) in a sterile

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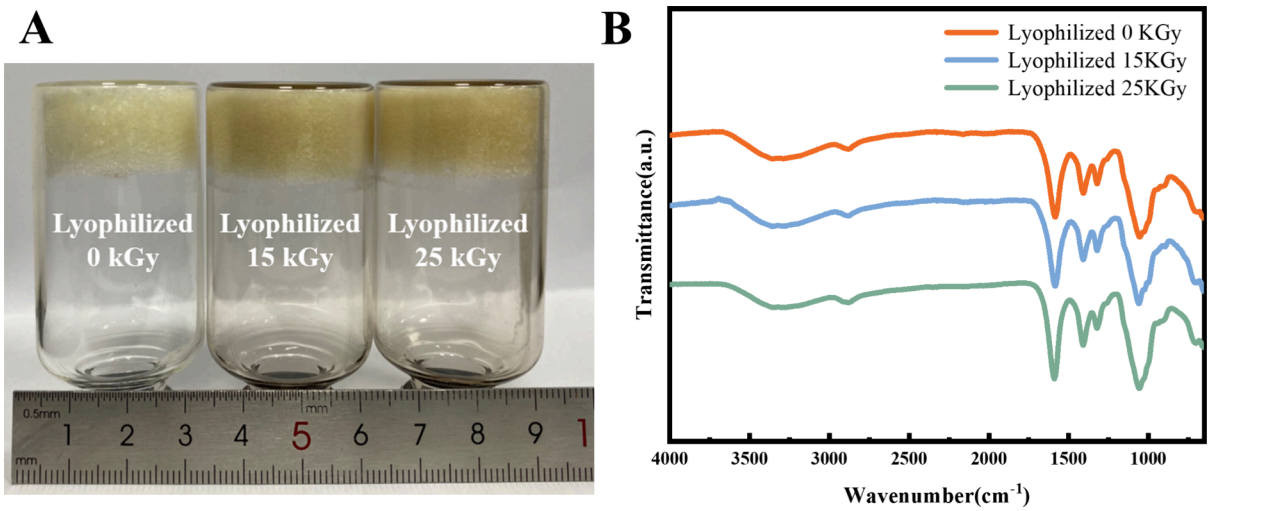

Figure 2. (A) Picture of CMC lyophilized samples before and after irradiation. (B) FTIR spectra of CMC lyophilized samples before and after irradiation.

environment; 5 mL of sterile diluent was then transferred to the lyophilized sample, and then both were shaken with a vortex mixer and then subjected to a 10-fold gradient dilution. A total of 2 mL each of the test solution and dilution solution was taken and inoculated into two plates, 1 mL per plate for use. Aerobic bacteria culture was injected with about 20 mL of TSA per plate and incubated at 32.5 °C for 7 days. For mold and yeast culture, about 20 mL of SDA per plate was injected and incubated at 22.5 °C for 7 days. For the negative control group, sterile eluent was taken instead of test eluent and incubated as above. At the end of incubation, the colonies were counted according to the colony counting principle, and finally, the dilution times were multiplied to calculate the number of colonies of each product.

3. RESULTS AND DISCUSSION

3.1. Changes in CMC in Irradiated Solution Samples.

In a large number of published studies on the formation of hydrogels by the Schiff base reaction of CMC with aldehyde-containing substances, CMC was used mostly with a solubility of 5 wt % in water,[32−34] and therefore, 5 wt % of CMC was chosen in this paper as the study object.

Table 1. Sterilization to CMC Lyophilized Dissolved as Viscosity Changes of 5 wt %

dose treatment	intrinsic viscosity (mPa·s)
lyophilized 0 kGy	5400
lyophilized 15 kGy	3980
lyophilized 15 kGy	2010

As can be seen from Figure 1A, the CMC solution sample does not flow after irradiation inverted, which may be due to the irradiation cross-linking of the solution under the irradiation energy[35] or the increase in the binding force between water molecules and CMC before irradiation.[36] At the same time, as the irradiation dose increases, the appearance of the color gradually turns yellow. According to existing research, borosilicate vials irradiated by the material will produce electronic vacancies, that is, the color center, is the physical basis for yellowing;[37] at the same time, irradiation will induce the polysaccharide molecules within the chemical bond breakage or structural rearrangement and the formation of more light-absorbing structure and thus lead to color changes.[38] Therefore, it is analyzed that the color change is the result of the joint action of irradiation on the vials and polysaccharides.

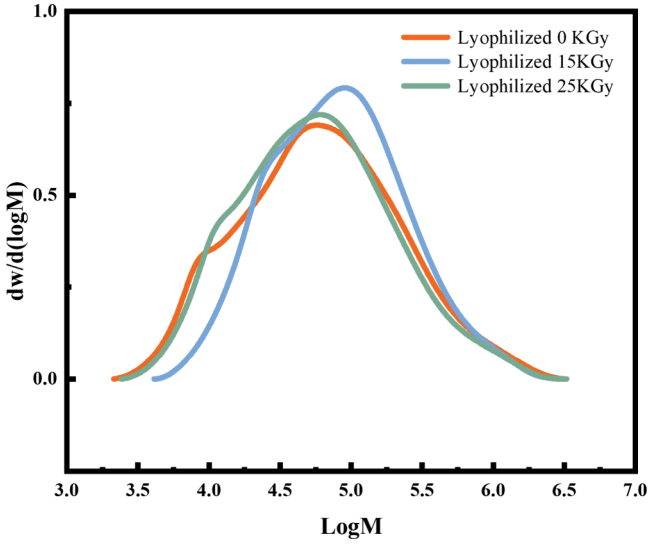

Figure 3. GPC of CMC lyophilized samples before and after irradiation.

Table 2. GPC of CMC Freeze Samples before and after Irradiation

dose treatment	M_n	M_w	M_w/M_n
lyophilized 0 kGy	2.90×10^4	1.41×10^5	4.86
lyophilized 15 kGy	4.63×10^4	1.50×10^5	3.23
lyophilized 25 kGy	2.97×10^4	1.25×10^5	4.23

As we know, the irradiation process is usually accompanied by energy input, and water as a dispersion medium in a 5 wt % CMC solution may trigger a radiochemical reaction in which 95 wt % of the water in the solution absorbs the energy and produces reactive oxygen species (e.g., hydroxyl radicals or hydrogen peroxide),[39] and these may alter the structure of the CMC, therefore leading to its cross-linking.[40] Figure 1B shows

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Table 3. Initial Contamination of CMC Solution and Lyophilized with Bacteria

	solution		lyophilized	
number	aerobic bacteria (cfu/piece)	molds and yeasts (cfu/piece)	aerobic bacteria (cfu/piece)	molds and yeasts (cfu/piece)
1	3.6×10^7	3.4×10^7	<5	<5
2	5.3×10^7	4.8×10^7	<5	<5
3	3.5×10^7	3.7×10^7	<5	<5
4	4.3×10^7	4.2×10^7	<5	<5
5	3.9×10^7	3.8×10^7	<5	<5
6	4.0×10^7	4.3×10^7	<5	<5
average	4.1×10^7	4.0×10^7	<5	<5
overall average	4.1×10^7		<5	

the FTIR-ATS spectra of CMC solution samples at different irradiation doses. There is a broader hydrophilic band at 3400 cm^{-1}, while there is a stronger hydrophilic band at 1588 cm^{-1} and a milder hydrophilic band at 1411 cm^{-1}, which is due to symmetric and asymmetric axial deformation of COO, confirming the presence of carboxymethyl.[14] After different doses of electron irradiation, although there was no significant change in the peak position and intensity of CMC, it was also found from the thermogravimetric data (Figure 1C) that the temperature of the third thermal decomposition peak of the solution CMC decreased with the increase of irradiation dose, which indicated that the polysaccharides might have changed during the irradiation process. It has been found that carboxymethyl polysaccharides in aqueous solution are susceptible to cross-linking at the carbon atoms attached to the carboxymethyl groups after irradiation,[40] so it is speculated that the irradiation of water molecules generates a large number of OH radicals to induce the generation of free radicals in the CMC sugar chains to cross-link at the point of chain entanglement (Scheme 1).[41]

Meanwhile, 5 wt % solution of CMC undergoes a change in the cohesive state after irradiation and does not have fluidity, which no longer meets the requirements for its use in injectable hydrogels,[42] and thus, we believe that solution-state irradiation is not suitable for the selection of CMC-based materials.

3.2. Changes in CMC in Irradiated Lyophilized Samples. From Figure 2A, it can be seen that the color of CMC lyophilized samples gradually turned yellow after irradiation with the increase of irradiation dose, which was consistent with the phenomenon of the change in appearance of solution samples after irradiation. Meanwhile, the FTIR diagram in Figure 2B shows that after irradiation at different doses of 15 and 25 kGy, the CMC samples did not undergo any obvious chemical changes, and the structure did not change.

The lyophilized samples were redissolved to 5 wt % in water to characterize the change in viscosity as well as the change in molecular weight of the lyophilized samples after irradiation. Table 1 shows the change in viscosity of the CMC lyophilized samples after redissolution.

From the change of viscosity in the table, we can see that the viscosity of 5 wt % solution after rethawing by irradiation decreases with the rise of dose, which may be due to the sublimation of water under vacuum, leaving the dry loose and porous CMC, which is altered by the irradiation to change the interactions between the polysaccharide molecules and water molecules to change, affecting the viscosity of the solution after the rethawing; or it may be that the irradiation energy may result in the breakage of CMC molecules, which affects the molecular weight and thus caused the viscosity decrease.

To verify this hypothesis, GPC analysis was conducted on CMC before and after irradiation. From the analysis of the GPC plot in Figure 3, it can be seen that the molecular weight distribution curve of CMC after irradiation is slightly different from that for irradiation; the peak of the molecular weight distribution curve shifted to the right; the molecular weight increased after irradiation by 15KGy; and the peak became sharper; that is, the molecular weight distribution became narrower, while the position of the peak of the molecular weight distribution curve shifted to the left slightly after irradiation by 25 kGy, and the peak shape did not change much. The values of M_n, M_w, and M_w/M_n changes of CMC after irradiation are shown in Table 2, which correspond to the changes of molecular weight distribution curve. Overall, the molecular weight of the lyophilized CMC after irradiation can be considered to have no effect.Meanwhile, the lyophilized samples of CMC without irradiation sterilization were tested for viscosity changes after rethawing. The results showed that the dissolution time of the lyophilized CMC was shorter when re-dissolved into a 5 wt % solution, and its viscosity also decreased compared to that before lyophilization, which was only 4250 mPa·s.Overall, the molecular weight of lyophilized CMC after irradiation shows almost no effect, while the lyophilization treatment induces a decrease in CMC viscosity.

The results of our performance are broadly in line with existing studies on other different natural polysaccharides, such as studies on the changes occurring in polysaccharides such as pectin, cellulose, sodium alginate, etc., as a result of exposure to ionizing radiation.[43,44] These polysaccharides in the aqueous state are all very reactive free radicals due to water emission and tend to undergo irradiation degradation in the low viscosity state[45] and irradiation cross-linking in the high viscosity state to the extent[46] that they are not suitable for the next stage of the material's use. While the solid state below 25 kGy does not change in the structure of the main chain, there will be a decrease in viscosity, as well as small changes in molecular weight, mainly due to the solid polysaccharides exposed to ionizing radiation that is mostly a result of direct action (when it is directly damaged by radiation) and also the existence of residual tiny water molecules through the

Table 4. Number of Colonies of CMC Lyophilized after Irradiation Sterilization Treatment

sample	test item	count (cfu/piece)	acceptable standards (cfu/piece)	test results(cfu/piece)	test basis
lyophilized 15 kGy	aerobic bacteria	0	N/A	<1	GB-T 19973.1−2015
	molds and yeasts	0			
lyophilized 25 kGy	aerobic bacteria	0	N/A	<1	
	molds and yeasts	0			

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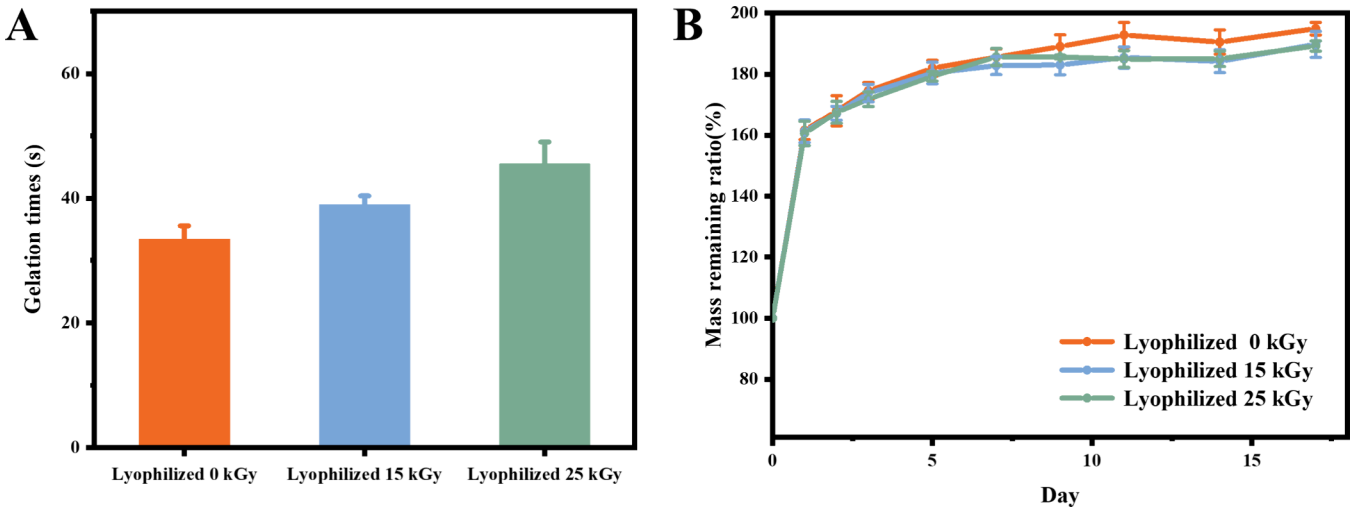

Figure 4. (A) Gelation times of the hydrogels after sterilization. (B) Mass remaining ratios of the hydrogels.

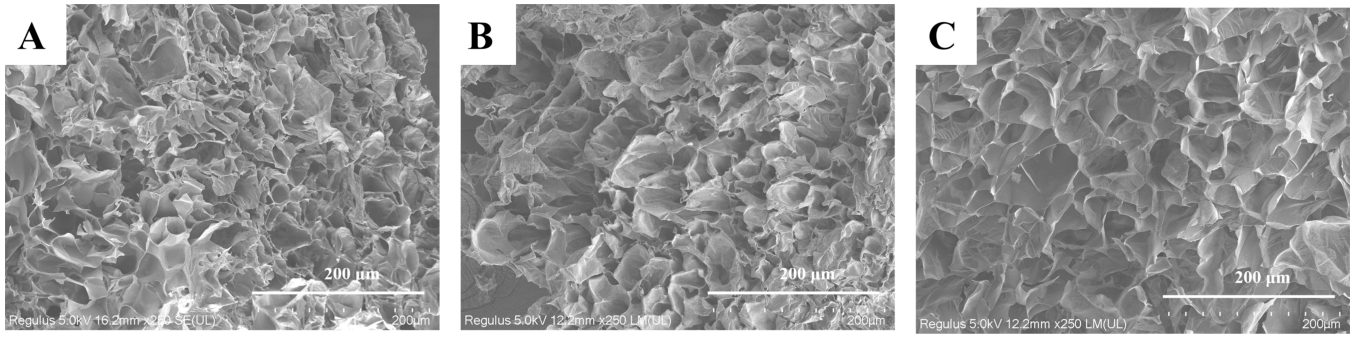

Figure 5. SEM of CMC lyophilized samples irradiated at different doses to form hydrogels with the same cross-linking agent. (A) ctrl (unirradiated). (B) Lyophilized 15 kGy. (C) Lyophilized 25 kGy.

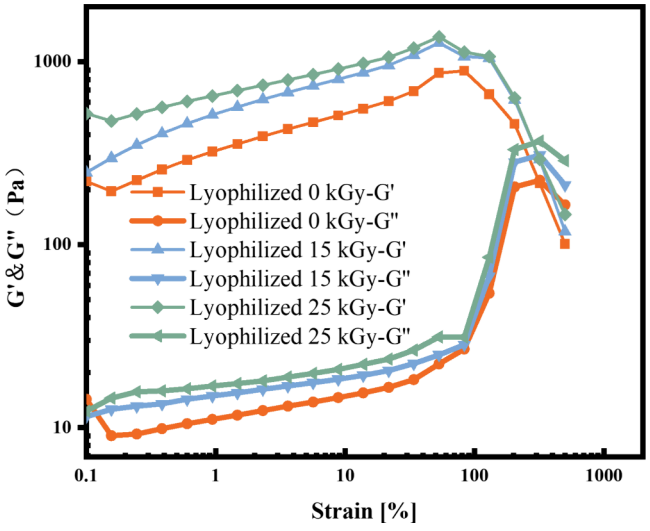

Figure 6. Strain rheology of CMC lyophilized samples irradiated at different doses to form hydrogels with the same cross-linking agent.

radioactive decomposition products caused by the results of the indirect action.[27]

For the solution and lyophilized samples of CMC after irradiation treatment of viscosity and decomposition temperature changes (see Table S2), solution samples after irradiation treatment due to cross-linking cannot measure the viscosity and decomposition temperature change is larger, compared to the lyophilized samples are more stable and resistant to irradiation.

To summarize, the present results show that CMC from lyophilized samples is more resistant to irradiation. The main reason is that the water molecules are excluded by freeze-drying, which prevents the interaction between radiation and water molecules and avoids the change of the sugar molecular structure caused by water radio-ionization radicals;[47] second, the molecular chain of CMC in a lyophilized state is more stable,[48] so the lyophilized sample state is more suitable for CMC materials to be irradiated for sterilizing treatment.

3.3. Initial Contaminating Bacteria in CMC Samples.

According to the requirements of the European Pharmacopoeia or the Chinese National Standard, an initial contaminant test of the product is required before determining the sterilizing dose.[21] We tested the initial contaminating bacteria of the lyophilized and liquid samples of CMC according to GB/T 19973.1−2005, and the results are shown in Table 3. From the table, it can be seen that the initial contaminating bacteria of the liquid sample reached 4.1×10^7, while the initial contaminating bacteria of the lyophilized sample was less than 5, which indicated that the lyophilized sample was more suitable for the sterilization of CMC.

We verified the sterilizing effect of CMC lyophilized after irradiation sterilization treatment, and from Table 4, we can see that the samples achieved sterility after 15 and 25 kGy irradiation.

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Therefore, the above results show that we can choose 15 kGy as the irradiation dose value for the CMC lyophilized sample. At this dose, the appearance morphology, viscosity, changes in the influence of M_w, and the sterilization effect of CMC samples are all excellent compared to that of 25 kGy.

3.4. Hydrogel Properties of CMC after Sterilization. While we have gained a preliminary understanding of the effects of EB sterilization on the CMC, further studies on the gel properties of irradiated CMC are needed. Therefore, we next chose a macromolecular cross-linking agent containing an aldehyde group to undergo a Schiff base cross-linking reaction with the amino group of CMC to obtain an injectable hydrogel.[49]

The irradiated 5 wt % CMC lyophilized samples of 5 wt % AG solution were mixed homogeneously to form a hydrogel at a ratio of 5:1 via a duplex syringe, and the gelation time was recorded (Figure 4A), which was found to increase slightly with increasing dosage, but this gelation time window satisfies the operation time of 20−60 s for injection implantation molding. Meanwhile, we paid attention to the swelling after gelation (Figure 4B) and found that irradiation did not affect the overall swelling of the CMC hydrogels. The morphology of the formed gel was also analyzed (Figure 5), and it was found that irradiation did not have much effect on the pore size of the hydrogel, which remained roughly the same change, matching the results of the swelling characteristics.

At the same time, we carried out tests on the effect of irradiation on the mechanical properties of the hydrogels (Figure 6). The untreated hydrogels showed softness and elasticity with an energy storage modulus of about 1000 Pa, but strains at break were as high as 300%. Compared with CMC hydrogels formed after irradiation, the energy storage modulus of the irradiated hydrogels increased slightly, while the strain at break remained almost the same, which can be summarized as irradiation does not affect the mechanical properties of CMC hydrogels.

4. CONCLUSIONS

In conclusion, we investigated the effects of EB irradiation on CMC in solution and lyophilized states and found that the group structure and molecular weight of CMC in the lyophilized state did not change significantly, and even after sterilization, CMC can be used to form stable injectable gels for clinical applications. Overall, the main point is that the use of lyophilization and drying techniques can resist the problem of degradation or cross-linking of polysaccharides by water molecules that violently generates reactive free radicals under ionizing radiation, and this result may provide interesting future advances for the development of CMC products for biomedical applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c01299.

Additional data and analyses that complement the text (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Si Chen − *College of Materials Science and Engineering and Key Laboratory of Plastic Modification and Processing Technology, Zhejiang Province, Zhejiang University of Technology, Hangzhou 310014, PR China; Hangzhou Biotech Biomedical Technology Co., LTD, Hangzhou 311103, PR China;* orcid.org/0000-0001-6371-616X; Email: chensi@zjut.edu.cn

Xu Wang − *College of Materials Science and Engineering and Key Laboratory of Plastic Modification and Processing Technology, Zhejiang Province, Zhejiang University of Technology, Hangzhou 310014, PR China;* orcid.org/0000-0003-4416-1538; Email: wangxu@zjut.edu.cn

Authors

Huili Yuan − *College of Materials Science and Engineering and Key Laboratory of Plastic Modification and Processing Technology, Zhejiang Province, Zhejiang University of Technology, Hangzhou 310014, PR China; Hangzhou Biotech Biomedical Technology Co., LTD, Hangzhou 311103, PR China*

Wenjing Liu − *College of Materials Science and Engineering and Key Laboratory of Plastic Modification and Processing Technology, Zhejiang Province, Zhejiang University of Technology, Hangzhou 310014, PR China; Hangzhou Biotech Biomedical Technology Co., LTD, Hangzhou 311103, PR China*

Yu Fu − *College of Materials Science and Engineering and Key Laboratory of Plastic Modification and Processing Technology, Zhejiang Province, Zhejiang University of Technology, Hangzhou 310014, PR China; Hangzhou Biotech Biomedical Technology Co., LTD, Hangzhou 311103, PR China*

Jiangjie Wu − *College of Materials Science and Engineering and Key Laboratory of Plastic Modification and Processing Technology, Zhejiang Province, Zhejiang University of Technology, Hangzhou 310014, PR China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.4c01299

Notes

The authors declare no competing financial interest.

H.Y. performed conceptualization, data curation, formal analysis, writing−original draft, and writing−review and editing. W.L. and Y.F. carried out visualization and supervision. J.W. performed investigation and resource gathering. S.C. and X.W. performed validation and methodology.

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