Effects of Molecular Structural Parameters of Carboxymethyl Chitosan on the Growth of Fibroblasts *In Vitro*

Ye Wang, Xi Guang Chen, Ying Hui Lv, Hui Yun Zhou, Jing Zhang

College of Marine Life Science, Ocean University of China, Qingdao 266003, People's Republic of China

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ABSTRACT: A series of carboxymethyl chitosan samples (CMCHs) with different molecular structural parameters were synthesized to estimate their different influences on the growth of fibroblasts. All the CMCHs stimulated the fibroblasts proliferation and CMCHs with different molecular weight (MW) had the similar effect on fibroblasts proliferation. The least concentration for CMCHs (the degree of deacetylation, DD 70.3–79.9%, the degree of substitution, DS 1.12–1.26) exhibiting acceleratory effect on fibroblasts proliferation was 50 μg mL^{-1}. As the DD increased from 70.3 to 93.6%, CMCH's ability of stimulating fibroblasts proliferation increased significantly. CMCH possessed much higher proliferation rate with the DS increasing to 2.39. CM40 with 92.4% DD and 2.39 DS had the strongest acceleratory fibroblasts proliferation at the range tested. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 106: 3136–3142, 2007

Key words: carboxymethyl chitosan (CMCH); molecular structural parameters; acceleratory proliferation; the MTT-CVS assay; mouse embryonic fibroblasts (MEF)

INTRODUCTION

Chitin, poly-β-(1→4)-*N*-acetyl-D-glucosamine, is the second most abundant natural polysaccharide and exists largely in the shells of crustacean and insects. Chitosan (CH) is a unique polysaccharide derived from chitin by partial deacetylation with alkali. Chitin and CH are recommended as suitable functional materials, for their excellent biological properties such as nontoxicity, biodegradation, immunological, antibacterial, and wound-healing activities.[1–4] Despite of advantages above, poor solubility of chitin and CH in neutral water and in common organic solvents had prevented them from being applied widely. To improve the solubility of CH, chemical modification, such as a partial N-acetylation,[5] PEG-grafting,[6] sulfonation,[7] quaternarization,[8] N- and O-hydroxylation,[9] and carboxymethylation,[10] have been studied.

Carboxymethyl chitosan (CMCH) is one of the water soluble derivatives of CH which has been studied widely. The substitution of carboxymethyl groups on 6-O-, 3-O-, and 2*N*- sites are determined with the preparation conditions. CMCH has unique chemical, physical, and biological properties such as high viscosity, large hydrodynamic volume, low toxicity, biocompatibility, and film, gel-forming capabilities, all of which make it an attractive option in biomedical and pharmaceutical formulations. For instance, calcium-alginate-N, O-CMCH beads[11] and N, O-CMCH/alginate hydrogel[12] could serve as polymeric carriers for site-specific protein drug delivery in the intestine. *N*—CMCH has been proven to be a suitable polymer for the delivery and intestinal absorption of anionic macromolecular therapeutics.[13] N, O-CMCH is capable of stimulating the extracellular lysozyme activity of fibroblasts[14] and CMCH has twofold bioactivities, promotes the proliferation of normal human skin fibroblasts and inhibits the proliferation of keloid fibroblasts, in our previous study.[15] Other researchers[16] demonstrate that CMCH is able to stimulate the migration of fibroblasts and markedly enhanced wound healing in terms of rates of wound reduction.

It is well known that some of the structural characteristics such as DD, DS, and MW of chitin/CH and their derivatives greatly influence their various properties such as solubility, physiological activities, chemical activities, and biodegradability.[17–20] There are also reports on the relationship between molecular structure and CMCH's properties of moisture-retention ability,[21] aggregation behavior[22] and so on.

Correspondence to: X. G. Chen (xgchen@ouc.edu.cn).
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However, there are no reports on the relationship between the CMCH's ability of stimulating the growth of fibroblasts and its molecular structural parameters.

In this article, CMCHs with different molecular structural parameters were prepared. The relationship between MW, DD, and DS of CMCHs and the effect on the growth of mouse embryonic fibroblasts (MEF) was investigated. MTT assay and crystal violet staining assay (CVS) were used for seeking a fundamental understanding of the function of CMCH.

MATERIALS AND METHODS

Materials

CHs, derived from crab shell, were made in our own lab (MW90-2000 kDa, DD 80–94%). The mice were purchased from the Institute for Drug Control of Qingdao, China. Dulbecco's modification of eagle's medium (DMEM) and Newborn calf serum (NBCS) were obtained from Hyclone, New Zealand. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) and Tween-20 (Polyoxyethene-20-Sorbitan Monolaurate, MW1227.54) were obtained from Amresco. Crystal Violet ($C_{25}H_{30}N_3Cl$, Formula Weight: 407.98) was purchased from Sigma Chemicals (St. Louis, MO), DMSO (Dimethyl sulfoxide) was of reagent grade.

Synthesis and characterization of CMCHs

CMCHs were prepared by the method of Liu et al.[23] CH (10 g), sodium hydroxide (13.5 g), and isopropanol (100 mL) were added into a flask (500 mL) to swell and alkalize at 50°C for 1 h. The temperature was maintained in a water bath (Thermocontroller, Comabiotech, Korea). The monochloroacetic acid (15 g) was dissolved in isopropanol (20 mL), and added into the reaction mixture dropwise for 30 min. The mixture reacted for 4 h at the same temperature, then stopped by adding 70% ethanol (200 mL). The solid was filtered and rinsed in 70–90% ethanol to desalt and dewater, and vacuum dried at room temperature. By changing the alkali and monochloroacetic acid concentration, CMCHs with different DS were prepared.

DD and DS of CMCHs were estimated by the method of potentiometric titration. 0.1 g of each CMCHs vacuum dried at 60°C dissolved in 0.1*M* HCl (20 mL) and was titrated with a standard solution of 0.1*M* NaOH using a pH meter (DELTA-320-S pH meter). V_1, V_2, and V_3 were the inflections (seen in Fig. 1). The differential volume ($\triangle V$) of alkali between V_1 and V_2 or V_2 and V_3 corresponded to the acid consumed by carboxymethyl groups and amino groups presented in the CMCH, respectively.

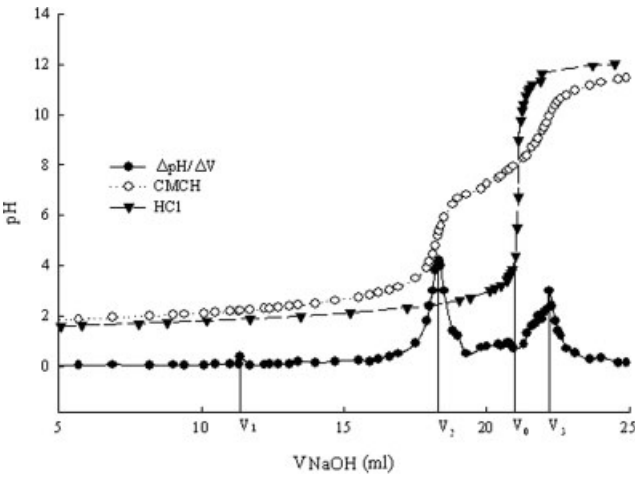

Figure 1 The integral and differential titration curves of HCl and CMCH.

DD and DS of CMCH were calculated using following equations.[24]

$$DS = \frac{0.203 \times (V_2 - V_1) \times C(\mathrm{NaOH})}{m - m_1 + m_2} \quad (1)$$

$$DD(\%) = \frac{(\mathrm{V_3} - \mathrm{V_2}) \times 0.1 \times 240.3 \times 100}{\mathrm{m} \times 1000} \quad (2)$$

$m_1 = [(V_2 - V_1) \times \mathrm{C\ (NaOH)} \times 0.080] - [(V_3 - V_0) \times \mathrm{C\ (NaOH)} \times 0.022]$; $m_2 = (V_3 - V_2) \times \mathrm{C\ (NaOH)} \times 0.042$; V_0 was the volume of alkali consumed by standard HCl. m_1 and m_2 were the weight of carboxymethyl groups and acetyl groups deviated from $-NH_2$ of CMCH in the total sample while m was the initial weight of CMCH sample.

MW of CMCH was calculated from the MW of corresponding CH according to its DS.

The FTIR spectra of CMCHs were recorded on an FT/IR-430 Fourier Transform Infrared Spectrometer (Jasco, Tokyo, Japan) based on the method of Shigemasa et al.[25] Pellets were formed from 2 mg of each sample and 100 mg of KBr.

MEF cultures with CMCHs and morphological observation

MEF were harvested using a primary explant technique' on the 13th day of pregnancy, the Kunming female mice was sacrificed according to institutional guidelines. Details of cell isolation had been published previously.[26] MEF were cultured in standard fibroblasts growth medium, consisting of DMEM supplemented with 10% (v/v) NBCS, 100 U mL^{-1} penicillin and 100 μg mL^{-1}streptomycin, and incubated at 37°C, 5% CO_2, in a humidified cell culture incubator. Cells between passages 3 and 6 were cultured in 96-well

plates (Costar, USA) for experimental work. The optimum cell seeding concentration as determined with the growth profile of the MEF was 3×10^4 cells/mL^{-1}.

Cells were allowed to attach for 24 h before treatment with CMCHs. The stock solution of CMCHs were made in didistilled water and filtered with Minisart Filters (0.22 μm) and diluted for use with fibroblasts growth medium. Cells were treated with CMCHs over a range of concentrations from 10 to 1000 μg mL^{-1} for 1 day, 3 days, and 5 days. Blank growth medium without any CMCH was used as the control. MEF morphology was assessed by phage contrast microscope.

Cell proliferation assays

Cell proliferation properties were tested by MTT assay and CVS on the same cell plate, named the MTT–CVS assay.

MTT assay was based on the protocol described by Mossmann.[27] Briefly, cells were incubated for 4 h with 20 μL of MTT (5 mg mL^{-1}, dissolved in D-Hank's buffer: NaCl 136.9 m*M*, KCl 2.68 m*M*, Na_2HPO_4 8.1 m*M*, KH_2PO_4 1.47 m*M*, pH 7.4). Then supernatants were removed and 100 μL DMSO was added. Plates were gently shaken in 37°C water bath for 10 min to solubilize the formazan crystals and the optical density (OD) was measured at 490 nm using a multiwell plate reader (Molecular Devices).

CVS was examined using a modification of the method described earlier.[28] It was performed on the MTT test plate containing the cells. The wells were washed three times with D-Hank's contained 0.05% Tween-20. Crystal violet solution was added to the wells (50 μL/well, 0.1%). The plates were incubated for 30 min with continuous shaking and washed with tap water four times. Then the stained cells were solubilized after air-dry by addition of 33% acetic acid (100 μL/well). After shaking for 15 min, supernatants were transferred into another 96-well plate and the absorbance was determined spectrophotometrically at 595 nm.

Strong correlations between the number of viable cells and the absorbance in the independent MTT assay and CVS had been well documented. Therefore, to confirm the reliability of the MTT–CVS assay, a 96-well plate was seeded with cell dilutions of MEF (0.2, 0.7, 1, 2, 4 $\times$ 10^4 cells/well). The plate was cultured for 24 h and then the MTT–CVS assay was performed as above. The OD was correlated with the initial cell numbers.

All the CMCHs were tested in five replicates for three separate experiments with reproducible results. The percent of proliferation was expressed as the relative growth rate (RGR) as follows

$$\mathrm{RGR} = \frac{\text{the absorbance of the sample}}{\text{the absorbance of the control}} \times 100\%$$

Statistical analysis

Statistical analysis was performed using the SPSS10.0. Data from three independent experiments were quantified and analyzed for each variable. Comparisons between two treatments were made using the one paired student's *t*-test and $P < 0.05$ was considered as statistically significance.

RESULTS AND DISCUSSION

Synthesis and characterization of CMCHs

Three serials CMCHs with different MW, DD, and DS were successfully synthesized and all samples had water solubility. The molecular structural parameters of CMCHs were shown in Table I. The DD and DS of CMCH were estimated using potentiometric titration with eqs. (1) and (2). The integral titration and differential curves were shown in Figure 1. MW of CMCH was calculated from the MW of corresponding CH according to its DS.

The FTIR spectra of CMCHs and CH were shown in Figure 2. All CMCHs had large —COOH group (1741 cm^{-1}) and —NH_3^+ group (1506 cm^{-1}) peaks. The C—O stretching band at 1030 cm^{-1} corresponds to the primary hydroxyl group of CH disappeared, which verified a high carboxymethylation of OH—6. The basic characteristics peak of CH at 1654 cm^{-1} (Amide I) disappeared. The characteristic peak of second hydroxyl group at 1080 cm^{-1} was not changed. The differences among FTIR spectra of CMCHs were presented in the intensity at 1741 cm^{-1} peak, which suggested the difference of DS. These indicated that all synthesized CMCHs had similar structure. The FTIR spectra of CMCHs were in agreement with the reported spectra.[29,30]

MEF morphological observation

After 2–3 times of subculture, epithelial cells were disappeared and only fibroblasts were present, which expressed the typical spindle morphology of fibroblasts. When MEF were cultured with 10–1000 μg mL^{-1} CMCHs, respectively, MEF maintained

TABLE I
Physico-Chemical Characteristics of the Various CMCHs

Symbols	CM9	CM29	CM138	CM200	CM40
M_W (×kDa)	127.2	399.3	1965.0	3037.7	731.9
DD (%)	79.7	79.9	70.3	93.6	92.4
DS	1.23	1.12	1.26	1.49	2.39

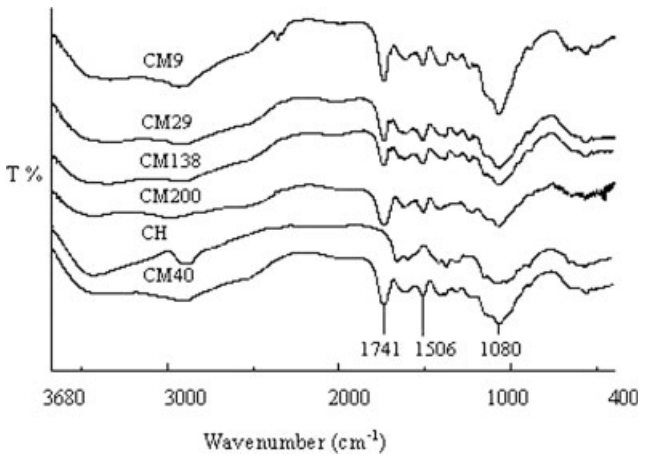

Figure 2 The FTIR spectra of CMCHs and CH.

their characteristic morphology without any morphologic alterations compared with reference. The morphology of MEF assessed by phase contrast microscope was shown in Figure 3.

The relevance of molecular structural parameters of CMCH and the growth of MEF *in vitro*

Effects of MW of CMCH on the growth of MEF

The correlation between the number of viable cells and the absorbance in the MTT–CVS assay was shown in Figure 4. The amount of the dye measured in two methods of the MTT–CVS assay was directly proportional to the number of cells in the range of $0.2 \times 10^4 - 4 \times 10^4$ cells/well and the linear regression coefficient (R^2) was 0.9957 (MTT) and 0.9936 (CVS) respectively. The MEF proliferation of CM138, CM29, and CM9 at the concentration of 500 μg mL^{-1} for 3 days was shown in Figure 5 using the MTT–CVS assay. The same trend was observed and there was no statistic difference ($P > 0.05$) between the two assays. MTT assay was based on measuring the activity of cellular mitochondrial succinic dehydrogenase and CVS was based on determining the protein level of cells, so the combination of MTT and CVS might be a more comprehensive and effective method for cell proliferation.

CM138, CM29, and CM9 with similar DD and DS but different MW were used to investigate effects of MW on the growth of MEF using the MTT–CVS assay.

In MTT assay of the MTT–CVS assay (Fig. 6), RGR of MEF was near 100% and there was no statistical difference compared with the control when the concentrations of CMCHs were 10 and 25 μg mL^{-1}. The MEF proliferation was significantly stimulated ($P < 0.05$ compared with the control) when the concentration of CMCHs increased to 50 μg mL^{-1}. Then the RGR slightly increased with the concentration of CMCHs increasing from 50 to 1000 μg mL^{-1}. So the

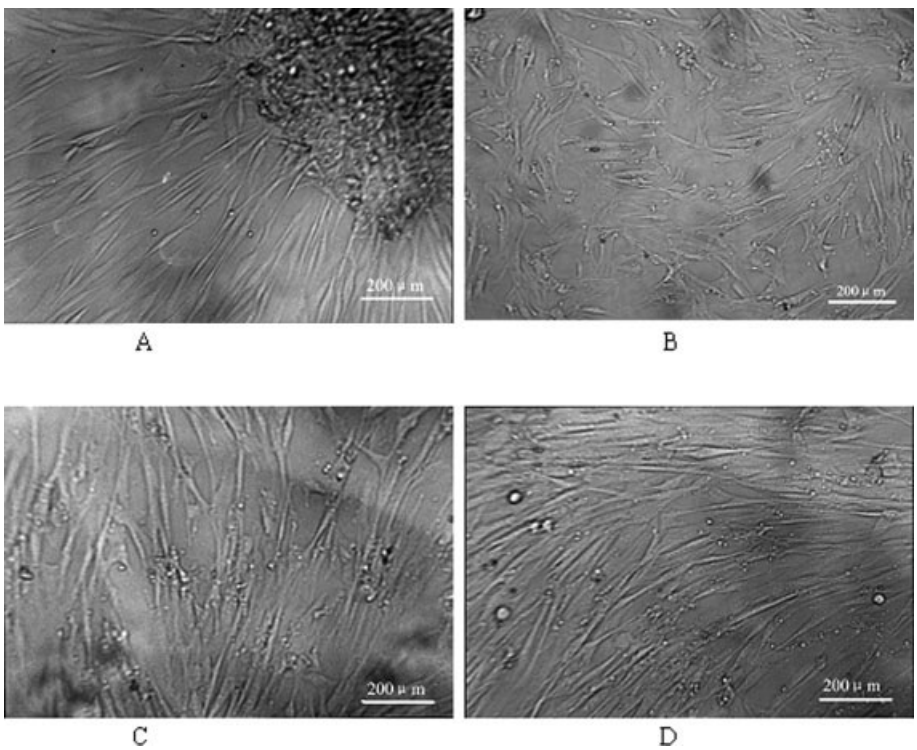

Figure 3 The morphology of MEF (magnification = ×100). (A) primary MEF, (B) MEF reference, (C) MEF cultured with 10 μg mL^{-1} CM138 for 3 days, (D) MEF cultured with 1000 μg mL^{-1} CM138 for 3 days (Bar = 200 μm).

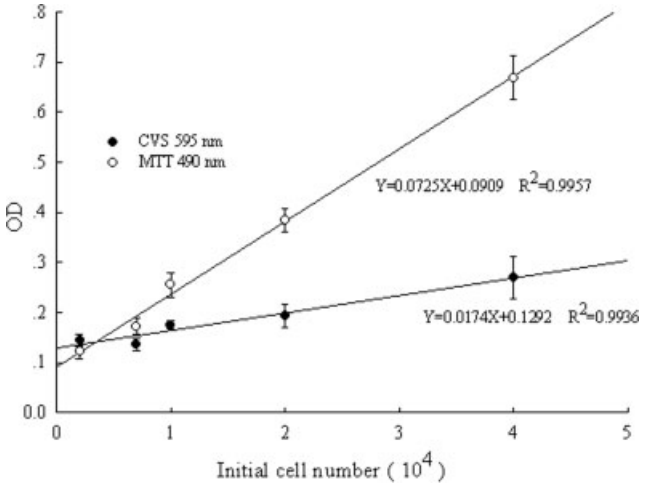

Figure 4 The correlation of the MTT-CVS assay with viable cell numbers. MEF were diluted series from 0.2×10^4 to 4×10^4 cells/well and cultured for 24 h before MTT assay and CVS were performed.

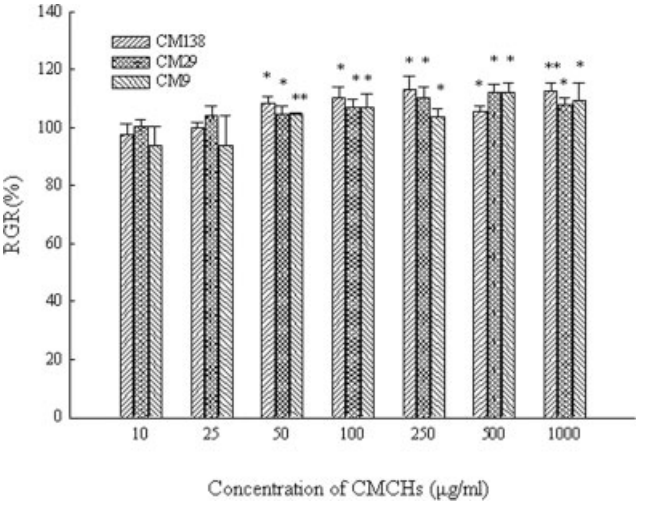

Figure 6 Effects of MW and concentrations of CMCHs on MEF proliferation estimated using MTT assay of the MTT-CVS assay. MEF were treated with various concentrations of CM138, CM29, CM9 for 3 days. (*$P < 0.05$, **$P < 0.01$, the one paired student's *t*-test).

least concentration for CMCHs (DD 70.3–79.9%, DS 1.12–1.26) exhibiting their acceleratory proliferation effect was 50 μg mL^{-1}. However, in CVS of the MTT–CVS assay (Fig. 7), all treated concentrations stimulated cell proliferation since the RGR of CMCHs were all higher than 100%, but statistical differences compared with controls were only seen at the concentration of 1000 and 250 μg mL^{-1} (CM138). Because of the less sensitive of CVS (seen in Figs. 4 and 5), only when the proliferation of fibroblasts was much enough, results with statistical difference from the CVS were exhibited. It was consistent with MTT assay.

On the other hand, all the three CMCHs with different MW stimulated the MEF proliferation and presented the similar tendency at the concentrations of CMCHs ranged between 10 and 1000 μg mL^{-1} by the method of the MTT-CVS assay.

Effects of DD of CMCH on the growth of MEF

Since CMCHs with different MW had the similar influence on MEF proliferation, CM138 (DD 70.3%) and CM200 (DD 93.6%) with similar DS were used to evaluate the effect of DD on the growth of MEF (seen in Fig. 8).

CM138 stimulated MEF proliferation on 3 days with statistic differences compared with control and

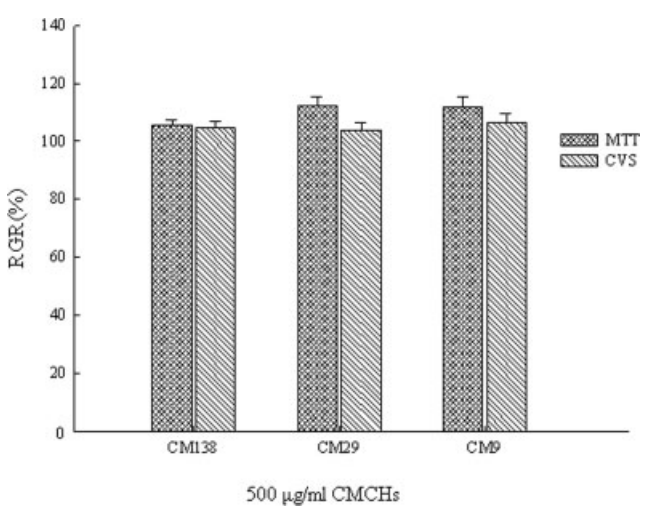

Figure 5 The MEF proliferation evaluated using the MTT-CVS assay. MEF were treated with 500 μg mL^{-1} of CM138, CM29, CM9 for 3 days.

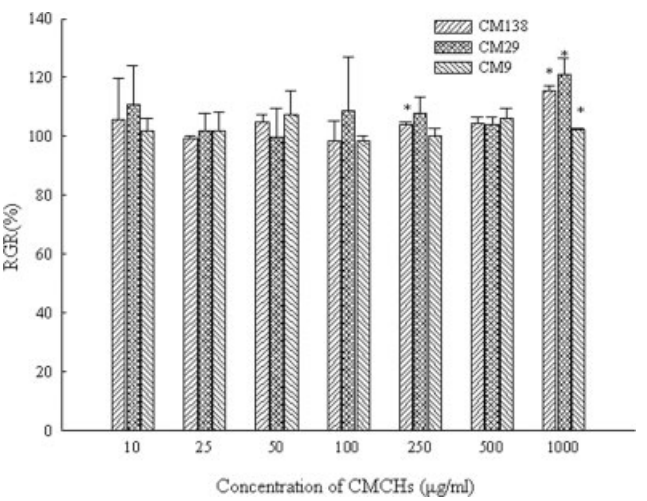

Figure 7 Effects of MW and concentrations of CMCHs on MEF proliferation estimated using CVS of the MTT-CVS assay. MEF were treated with various concentrations of CM138, CM29 and CM9 for 3 days. (*$P < 0.05$, the one paired student's *t*-test).

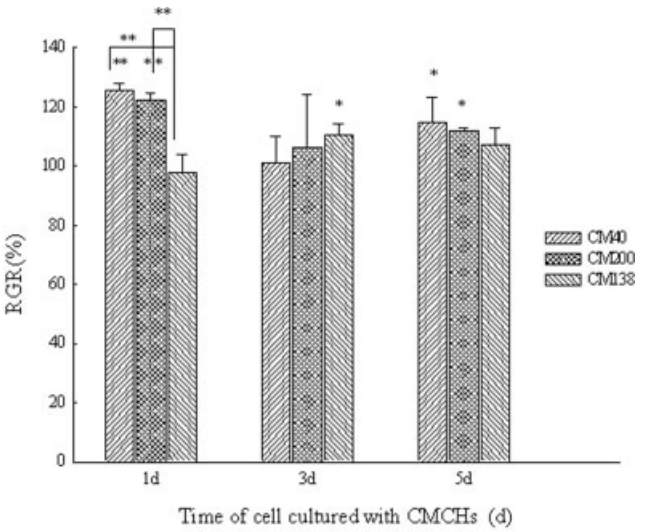

Figure 8 Effects of DD and DS of CMCHs on MEF proliferation estimated by the method of MTT assay. MEF were treated with 100 μg mL^{-1} CM40, CM200, CM138 for 1 day, 3 days and 5 days. (*$P < 0.05$, **$P < 0.01$, the one paired student's *t*-test).

slightly increased on 5 days. While CM200 presented very strong stimulatory effects on MEF proliferation on 1 day and 5 days with significantly statistic differences compared with control ($P < 0.01$ and $P < 0.05$, respectively). MEF proliferated at much higher rate when cultured with CM200 than CM138 with significantly statistic difference ($P < 0.01$) on 1 day, but there was no statistic difference for 5 days. These results suggested that the deacetylation level of CMCH seemed to be a key factor in the mitogenic activity on MEF. CMCH with higher DD exhibited earlier, stronger and more durable stimulating ability of MEF proliferation. The similar phenomenon about the fibroblasts proliferation influenced by the DD of CH was previously reported.[31,32]

Effects of DS of CMCH on MEF proliferation

CM40 and CM200, with the similar DD (92.4 and 93.6% respectively) but different DS (2.39 and 1.49 respectively) were employed for demonstrating the effects of DS on MEF proliferation. CM40 and CM200 both had the same tendency of MEF proliferation (Fig. 8). MEF proliferation was significantly stimulated on 1 day and 5 days by CM40 and CM200, all with statistic difference compared with control. The RGR of CM40 was higher than CM200 during the experimental time, but there was no statistic difference between the two samples. This result indicated that CMCHs exhibited more biologically activity of stimulating MEF proliferation with the increasing of DS.

In previous studies, CH showed almost no acceleratory effect on the proliferation of cultured fibroblasts. CH with high concentrations inhibited L929 fibroblasts proliferation with 10% Fetal calf serum.[33] CH exhibited both stimulation and inhibition of human skin fibroblasts proliferation.[32] We also found that CH with high concentrations inhibited MEF proliferation (data not shown). However, CMCH could promote proliferation of normal human skin fibroblasts[15] and it also stimulated the MEF proliferation with 10% NBCS.

The mechanism by which CMCH interacts with fibroblasts is hitherto poorly understood. Possible reasons may be due to the introduction of carboxymethyl groups of CMCH changes molecular structures of CH which may improve the ability of CH to accelerate fibroblasts proliferation. When CMCH was dissolved in water, the solution was a neutral system, and CMCH behaved as a relatively weak polyanionic polyelectrolyte,[34] the amino groups were not protonated and most of carboxylic groups were not dissociated in neutral aqueous solution. On the one hand, CMCH may exert its acceleratory effect on fibroblasts proliferation in the form of polyanionic polyelectrolyte. Reports suggest that CH forms polyelectrolyte complexes with serum components such as heparin,[33] or potentiating growth factors such as platelet derived growth factor,[32,35] which may protect them from enzymatic degradation or present them to the cells in an activated form. On the other hand, CH bears positive charges in the physiological environment. It may probably interact with anionic components (sialic acid) of the glycoproteins on the surface of cells and cause cytotoxic effects.[36] While the amino groups of CMCH solution were not protonated and most of carboxylic groups were not dissociated in neutral aqueous solution, these may decrease the cytotoxicity induced by positive charges and more likely to exhibit the stimulation profile on the growth of fibroblasts *in vitro*. Furthermore, CMCH may promote fibroblasts proliferation by stimulating fibroblasts secret considerable amounts cytokines, such as basic fibroblastic growth factor (b FGF), vascular endothelial growth factor (VEGF), and transforming growth factor beta (TGF-β).[16]

CONCLUSIONS

In this paper, five CMCHs with different molecular parameters were synthesized. All the CMCHs with different MW had similar effects on MEF proliferation using the MTT–CVS assay. The least concentration for CMCHs (DD 70.3–79.9%, DS 1.12–1.26) exhibiting acceleratory effect on fibroblasts proliferation was 50 μg mL^{-1}. CMCH with relatively higher DD strongly stimulated fibroblasts proliferation while samples with lower level of DD showed less activity. CMCH's ability of stimulating fibroblasts

proliferation increased significantly with the DD increasing from 70.3 to 93.6%. The DS was particularly important for CMCH's biological activity of acceleratory fibroblasts proliferation. CMCH with higher DS showed much higher proliferation rate. CM40 with 92.4% DD and 2.39 DS had the strongest acceleratory fibroblasts proliferation at the range tested. More work should be done to demonstrate the mechanism by which CMCH interacted with fibroblasts and it would be useful for the application of CMCH as wound care materials.

References

1. Kumar, M. N. V. R. React Funct Polym 2000, 46, 1.
2. Shigemasa, Y.; Saito, K.; Sashiwa, H.; Saimoto, H. Int J Biol Macromol 1994, 16, 43.
3. Hwang, S. M.; Chen, C. Y.; Chen, S. S.; Chen, J. C. Biochem Biophys Res Commun 2000, 271, 229.
4. Khor, E.; Lim, L. Y. Biomaterials 2003, 24, 2339.
5. Kubota, N.; Tatsumoto, N.; Sano, T.; Toya, K. Carbohydr Res 2000, 324, 268.
6. Sugimoto, M.; Morimoto, M.; Sashiwa, H.; Saimoto, H. Carbohydr Polym 1998, 36, 49.
7. Holme, K. R.; Perlin, A. S. Carbohydr Res 1997, 302, 7.
8. Jia, Z.; Shen, D.; Xu, W. Carbohydr Res 2001, 333, 1.
9. Machida, Y.; Nagai, T.; Abe, M.; Sannan, T. Drug Des Deliv 1986, 1, 119.
10. Krause, T. J.; Goldsmith, N. K.; Ebner, S.; Zazanis, G. A.; McKinnon, R. D. J Invest Surg 1998, 11, 105.
11. Lin, Y. H.; Liang, H. F.; Chung, C. K.; Chen, M. C.; Sung, H. W. Biomaterials 2005, 26, 2105.
12. Chen, S. C.; Wu, Y. C.; Mi, F. L.; Lin, Y. H.; Yu, L. C.; Sung, H. W. J Controlled Release 2004, 96, 285.
13. Thanou, M.; Nihot, M. T.; Jansen, M.; Verhoef, J. C.; Junginger, H. E. J Pharm Sci 2001, 90, 38.
14. Hjerde, R. J. N.; Vårum, K. M.; Grasdalen, H.; Tokura, S.; Smidsrød, O. Carbohydr Polym 1997, 34, 131.
15. Chen, X. G.; Wang, Z.; Liu, W. S.; Park, H. J. Biomaterials 2002, 23, 4609.
16. Chen, R. N.; Wang, G. M.; Chen, C. H.; Ho, H. O.; Sheu, M. T. Biomacromolecules 2006, 7, 1058.
17. Freier, T.; Koh, H. S.; Kazazian, K.; Shoichet, M. S. Biomaterials 2005, 26, 5872.
18. Shin, Y.; Yoo, D. I.; Jang, J. J Appl Polym Sci 2001, 80, 2495.
19. Tsaih, M. L.; Chen, R. H. J Appl Polym Sci 2003, 90, 3526.
20. Xing, R.; Liu, S.; Guo, Z.; Yu, H.; Wang, P.; Li, C.; Li, P. Bioorg Med Chem 2005, 13, 1573.
21. Chen, L. Y.; Du, Y. M.; Wu, H. Q.; Xiao, L. J Appl Polym Sci 2002, 83, 1233.
22. Shi, X. W.; Du, Y. M.; Yang, J. H.; Zhang, B. Z.; Sun, L. P. J Appl Polym Sci 2006, 100, 4689.
23. Liu, X. F.; Guan, Y. L.; Yang, D. Z.; Li, Z.; Yao, K. D. J Appl Polym Sci 2001, 79, 1324.
24. Liu, C. X.; Chen, G. H.; Jin, Z. T.; Sun, M. K.; Gao, C. J. J Beijing Univ of Chem Technol 2004, 31, 14.
25. Shigemasa, Y.; Matsuura, H.; Sashiwa, H.; Saimoto, H. Int J Biol Macromol 1996, 18, 237.
26. Helgason, C. D. In Basic Cell Culture Protocols, 3rd ed.; Helgason, C. D., Miller, C. L., Eds.; Humana Press: Totowa, NJ, 2004; Chapter 1.
27. Mossmann, T. J Immunol Methods 1983, 65, 55.
28. Van Kooten, T. G.; Klein, C. L.; Kirkpatrick, C. J. J Mater Sci: Mater Med 1997, 8, 835.
29. Chen, X. G.; Park, H. J. Carbohydr Polym 2003, 53, 355.
30. Sun, L. P.; Du, Y. M.; Fan, L. H.; Chen, X.; Yang, J. H. Polymer 2006, 47, 1796.
31. Chatelet, C.; Damour, O.; Domard, A. Biomaterials 2001, 22, 261.
32. Howling, G. I.; Dettmar, P. W.; Goddard, P. A.; Hampson, F. C.; Dornish, M.; Wood, E. J. Biomaterials 2001, 22, 2959.
33. Mori, T.; Okumura, M.; Matsuura, M.; Ueno, K.; Tokura, S.; Okamoto, Y.; Minam, S.; Fujinaga, T. Biomaterials 1997, 18, 947.
34. Zhu, A. P.; Chan-Park, M. B.; Dai, S.; Li, L. Colloids Surf B 2005, 43, 143.
35. Inui, H.; Tsujikubo, M.; Hirano, S. Biosci Biotechnol Biochem 1995, 59, 2111.
36. Quinton, P. M.; Phillpot, C. W. J Cell Biol 1973, 56, 787.