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Synthesis and Characterization of a Hydroxyethyl Derivative of Chitosan and Evaluation of Its Biosafety

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Abstract Hydroxyethyl chitosan (HE-chitosan) is a water-soluble derivative of chitosan with many apparent biological properties. For example, it is non-toxic and rapidly biodegradable. Moreover, HE-chitosan has advantages in water-solubility, moisture retention and gelling property due to its hydroxyethyl group. However, the biocompatibility and biodegradability of this multifunctional derivative have rarely been documented although they are critical for its application in biomedical and clinical treatments. The purpose of this work was to evaluate the biosafety of HE-chitosan, and draw important clues for its diverse applications. HE-chitosan was synthesized and characterized its chemical structure with FTIR. Its molecular weight (M_W) was determined by gel permeation chromatography (GPC), and its deacetylation degree (DD) was investigated through potentiometric analysis. The cytotoxicity of HE-chitosan on mouse fibroblast cell L929 was tested. The biocompatibility and biodegradability of HE-chitosan in rat and rabbit were evaluated. The FTIR results indicated that the hydroxyethyl groups were linked to C^6 of chitosan. The GPC analysis confirmed that its Mw was about 90.01 kDa. It was also demonstrated that HE-chitosan had excellent biocompatibility and biodegradability *in vivo* and had no cytotoxicity on L929. These findings indicated that HE-chitosan can potentially be applied as a biomaterial in tissue engineering, drug delivery, and other biomedical fields.

Key words hydroxyethyl chitosan; biomaterial; cytotoxicity; biocompatibility; biodegradability

1 Introduction

Chitosan is a naturally occurred polysaccharide composed of D-glucosamine and N-acetyl D-glucosamine units (Anitha *et al*., 2009). It can be found in crustacean shell, cartilage of mollusk, fungi and cell wall of plants. Chitosan is naturally non-toxic (Carreira *et al*., 2010), non-antigenic and antibacterial (Liu *et al*., 2004a), and biocompatible and biodegradable (Abdull Rasad *et al*., 2010; Baldrick, 2010; Muzzarelli, 2010), and has good performance in hemostasis and wound healing (Ishihara *et al*., 2002; Jayakumar *et al*., 2011). These properties make chitosan as an absorbable biomaterial widely be applied in medicine, food and agriculture. A broad range of studies have been reported in the above areas (Ravi Kumar, 2000; Rinaudo, 2006; Dutta *et al*., 2009; Gomaa *et al*., 2010). However, the poor solubility of chitosan in neutral solution has greatly limited its application, because the primary amino groups are protonated only in acidic solution (pH < 6.5) (Takei *et al*., 2012). Thus, it is necessary to improve the water solubility of chitosan and endow it with new bioactivities by chemical modifications. Until now, many chemical groups, especially the hydrophilic groups, have been introduced into chitosan by chemical modifications, which include carboxyl, hydroxyl, sulfonic acid, alkyl, acetyl groups and among others (Dash *et al*., 2011).

HE-chitosan is a multifunctional derivative of chitosan with good water-solubility (Liu *et al*., 2007; Zhao *et al*., 2008; Nanaki *et al*., 2012). The introduction of hydroxyethyl group improves the spatial structure, weakens the intermolecular forces and increases the water-solubility thus improving the moisturizing property of HE-chitosan (Wan *et al*., 2004; Xie *et al*., 2007; Yu *et al*., 2010). The excellent physical and chemical properties of HE-chitosan make it to be applied as antioxidant (Huang *et al*., 2005), and tissue engineering material, medicine carrier, medical dressings and other biomaterials (Hang *et al*., 2003; Liu *et al*., 2004b; Xu *et al*., 2010). Particularly, HE-chitosan is a newly appeared carrier candidate in the short list of modified chitosan of interest in drug delivery (Zhang *et al*., 2005). HE-chitosan derivatives have been employed as an implant for glaucoma filtering surgery (Li *et al*., 2011), an effective potential drug candidate for the treatment of chronic renal disease (Yuan *et al*., 2011; He *et al*., 2012) and the oral deliverer of paclitaxel (Zhang *et al*., 2005). In addition, HE-chitosan derivatives have been employed as a composite hydrogel for corneal endothelium reconstruction in tissue engineering (Liang *et al*., 2011). However, the biosafety of HE-chitosan has not

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been evaluated appropriately, which is critical for its application.

In this study, HE-chitosan was synthesized by linking hydroxyethyl group to C^6, and the basic physical and chemical properties of HE-chitosan were characterized. Moreover, cytotoxicity of HE-chitosan was evaluated on mouse fibroblast cell L929. By implantation of HE-chitosan gel into subcutaneous tissues and muscles of rats and the percutaneous irritation in rabbits, we determined its biocompatibility and biodegradability *in vivo*. The results will serve as an important reference for the application of HE-chitosan in drug delivery, tissue engineering and other fields.

2 Materials and Methods

2.1 Materials and Reagents

Chitosan with DD (97%) and M_W (100–150 kDa) was supplied by Biotemed Co., Ltd., China. Sodium hydroxide (NaOH), potassium hydroxide (KOH), alcohol (C_2H_5OH), dimethyl sulfoxide (DMSO) and chloroethanol (C_2H_5ClO) were purchased from Sinopharm Chemical Reagent Co., Ltd., China. Dulbecco's modified eagle's medium (DMEM) was obtained from Gibco, USA; Dextran standards were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, China. L929 cells were obtained from the Institute of Pharmacology of Ocean University of China. Sprague-Dawley rats (220±10 g in body weight) and New Zealand white rabbits (4000±500 g in body weight) were obtained from Qingdao Institute for Drug Control, China. All other reagents used were of analytical reagent grade.

2.2 Synthesis of HE-Chitosan

HE-chitosan was prepared as described previously under alkaline condition with minor modifications (Dong *et al*., 2001). The brief processes were shown in Fig.1. Chitosan was purified with the following procedure. Chitosan (100 g) was stirred in 0.2 mol L^{-1} NaOH (1000 mL) at 65℃ for 2 h, and then the wet precipitate was washed

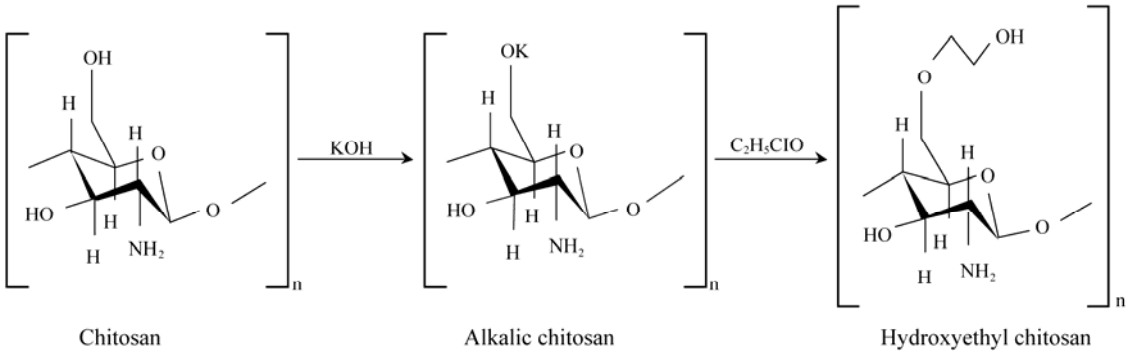

Fig.1 Synthesis of HE-chitosan from chitosan.

to neutral with distilled water and alkalized with 50% KOH. After being alkalized at −20℃ for 24 h, the wet alkaline chitosan was put into a flask and mixed with 95% ethanol. With the addition of C_2H_5ClO, the mixture was stirred at 45℃ for about 30 h until it was dissolved in water completely. Then the mixture was filtered through 1.2 μm cellulose acetate membrane. HE-chitosan product was obtained after dialysis and freeze drying under vacuum.

2.3 Characterization of HE-Chitosan

FTIR was used to verify the chemical structure of HE-chitosan. The IR spectra of chitosan and HE-chitosan were recorded on a Nicolet Nexus-470 Fourier Transform Infrared Spectrometer (Abdull Rasad *et al*., 2010). Data analysis was carried out on Jwstda-32 (Sashiwa *et al*., 1990; Shigemasa *et al*., 1996). The M_W of HE-chitosan was determined by GPC using high performance liquid chromatography (HPLC) on Breeze1525 system implemented with the refractive index. Separations were achieved with Two Shodex OHPak SB-806 M columns in series. The mobile phase was 0.02 mol L^{-1} PBS (pH 8.0), which contained 0.1 mol L^{-1} Na_2SO_4, at a flow rate of 1.0 mL min^{-1} at 35℃. Standard curve was calculated by referring the dextran standards with molecular weights of 180, 2500, 10000, 41100, 133800 and 2000000. Data analysis was carried out on GPCW32 (Dong *et al*., 2010). The DD of HE-chitosan was determined by potentiometric analysis (Muzzarelli *et al*., 1984; Wan *et al*., 1996). The water content and ash were also measured.

2.4 Cells and Cell Culture

L929 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum at 37℃ and in 5% CO_2 and serially subcultured when they reached 85% of confluence. The cells were used for cytotoxicity test at passage 5 (Zheng *et al*., 2011).

2.5 Cytotoxicity

Cytotoxicity test is often applied to detect the toxicity of materials *in vitro* because of its straightforward operation, high sensitivity and short-term. The MTT assay (Chow and Khor, 2002) was adopted to evaluate the cytotoxicity of HE-chitosan on L929 cells. L929 cells were seeded in 96-well plates with a density of 4×10^4 cells mL^{-1} and 200 μL per well. The plates were incubated at 37℃ and in 5% CO_2 for 24 h. Then the culture medium was aspirated and replaced with fresh medium containing

different concentrations of HE-chitosan, respectively. MTT assay was performed on day 2 and day 4. The optical density (OD) value was measured at 492 nm on an enzyme immunoassay instrument (Multiskan MK3, Thermo Labsystems, USA). The relative proliferation ratio (RGR) was calculated according to the formula

$$RGR = (OD_1 - OD_0)/(OD_2 - OD_0) \times 100\%,$$

where OD_0, OD_1 and OD_2 were the average OD value of the medium alone, medium for culturing L929 cells with HE-chitosan, and medium for culturing L929 cells without HE-chitosan (control), respectively.

2.6 Intradermal Injection Test

Intradermal injection is considered to be a direct method to observe the tissue toxicity of substances. The experiments were carried out in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The experiment group was treated with HE-chitosan solution at a concentration of 2% and the control was treated with saline. Hair of ten rabbits on both sides of the spine was completely removed before intradermal injection. The back of the rabbits was divided into two regions, one for the experimental group and the other for the control. Each region had three injection sites. The experimental group was treated with 200 μL HE-chitosan solution (2%) per site while the control group was treated with 200 μL saline per site. At 24 h, 48 h and 72 h after injection, induction of obvious inflammatory reaction (intradermal erythema or edema) in the injection regions was macroscopically judged.

2.7 Biocompatibility and Biodegradability *in Vivo*

Forty adult male rats (200 g ± 10 g in body weight) were used in muscle and subcutaneous implantation tests for the evaluation of the biocompatibility and biodegradability of HE-chitosan (Jameela and Jayakrishnan, 1995; Li *et al*., 2007). Shaved dorsum of rat was divided into two regions for subcutaneous implantation with HE-chitosan solution (2%, 2000 μL kg^{-1}) and saline (2000 μL kg^{-1}), respectively. Then gluteal muscles of the rats were used for muscle implantation with HE-chitosan solution (2%, 2000 μL kg^{-1}) in the left and saline (2000 μL kg^{-1}) in the right. Ten rats were sacrificed at 3 d, 7 d, 14 d, and 28 d after implantation, respectively. Post-mortem visual examination and hematoxylin-eosin (HE) staining were performed to observe the biocompatibility and biodegradability of HE-chitosan.

2.8 Statistical Analysis

Data were expressed as means ± SD. Difference was calculated through one-way ANOVA implemented in SPSS version 13.0 and considered to be significant if $P < 0.05$.

3 Results

3.1 Synthesis of HE-chitosan

HE-chitosan was synthesized by accessing hydroxyethyl group to the C^6 of chitosan and hydroxyethylation was achieved with chloroethanol and sodium hydroxide in alkaline alcohol water mixed phase. Temperature and pH were key factors to the reaction. The final HE-chitosan product was white with good water-solubility and moisture retention.

3.2 Chemical Structure

The FTIR spectra of HE-chitosan and chitosan were shown in Fig.2. The IR spectrum of chitosan showed basic characteristic absorption peaks at 3421 cm^{-1} (O-H stretching vibration), 2881 cm^{-1} (C-H stretching vibration), 1556 cm^{-1} (N-H bending vibration), 1153 cm^{-1} (bridge-O stretching vibration) and 1094 cm^{-1} (C-O stretching vibration) (El-Sherbiny, 2009). In the IR spectrum of HE-chitosan, the obvious -CH_2- stretching vibration and a new -CH_2- bending vibration can be detected at 2919 cm^{-1} and 1465 cm^{-1}, respectively. The hydroxyl absorption peak at 1033 cm^{-1} almost disappeared, indicating that the hydroxyethyl substitution reaction occurred at the hydroxyl group of C^6. Additionally, the new absorption peak at 3379 cm^{-1} could be assigned to the overlap of -OH and -NH_2 stretching vibrations. The intensification of C-O (-C-O-C-) stretching vibration at 1115 cm^{-1} and 1059 cm^{-1}, and C-H- vibration at 2919 cm^{-1} confirmed the existence of hydroxyethyl group.

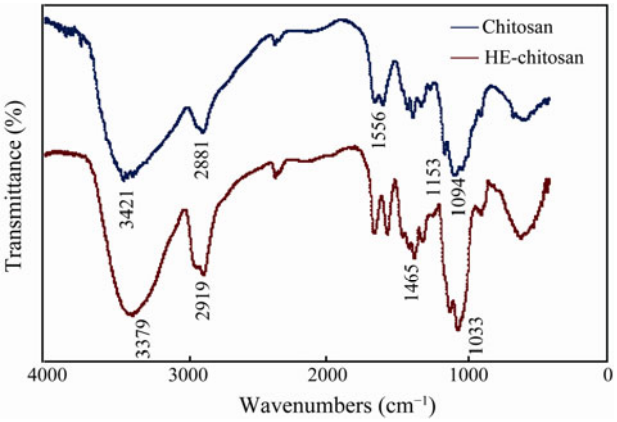

Fig.2 FTIR spectra of chitosan and HE-chitosan.

3.3 Properties of HE-chitosan

M_W, water and ash contents and DD are basic parameters which have great effects on the applications of HE-chitosan. The M_W was 90.01 kDa. The water and ash content was 1.86% and 0.08%, respectively. And the DD of HE-chitosan was 94.18%.

3.4 Cytotoxicity Test

The cytotoxicity of HE-chitosan on L929 was investigated by MTT assay. The results in Fig.3 showed that HE-chitosan slightly inhibited L929 cell growth at the concentration of 5000 μg mL^{-1} with a RGR reduction of 18.90% ($P < 0.05$) in 2 days whereas the inhibitory effect declined on day 4 with a RGR reduction of 8.60% ($P > 0.05$). The MTT results revealed that the RGR reduction of each tested concentration was less than 20% (Fig.4).

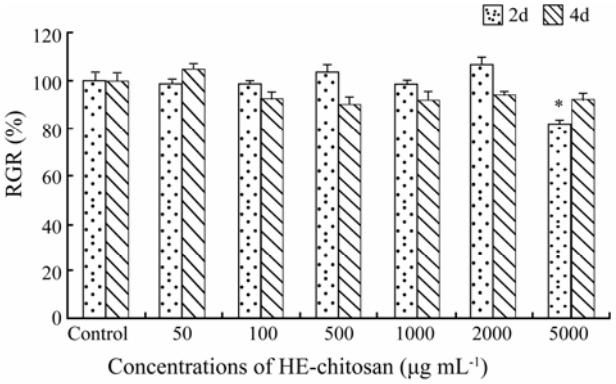

Fig.3 Cytotoxicity analysis of HE-chitosan on L929 on day 2 and day 4. RGR was expressed as means ± SD (n= 5); *, P<0.05.

Accordingly, HE-chitosan showed no significant effect on the growth of L929 cells thus was safe to them.

3.5 Intradermal Injection Test

Inflammatory reaction after intradermal injection consists of two indicators, intradermal erythema and edema. The skin stimulation was recorded according to macroscopic observation in injection regions. The result indicated that no obvious skin stimulation was observed at 24 h, 48 h and 72 h after injection in the control group treated with saline. Inflammatory reaction of the control group was at the slightest level as no intradermal erythema or edema emerged. There was also no obvious skin stimulation occurred in experimental group treated with HE-chitosan. Only a slight edema (height < 1 mm, diameter < 10 mm) was observed on the second injection site of one rabbit at 48 h. However, the edema disappeared at 72 h. No obvious intradermal erythema, edema or other irritative reaction emerged in experimental group. Inflammatory reaction of the HE-chitosan group had no signify-cant difference in comparison with the control group. These findings indicated that HE-chitosan caused no ob-

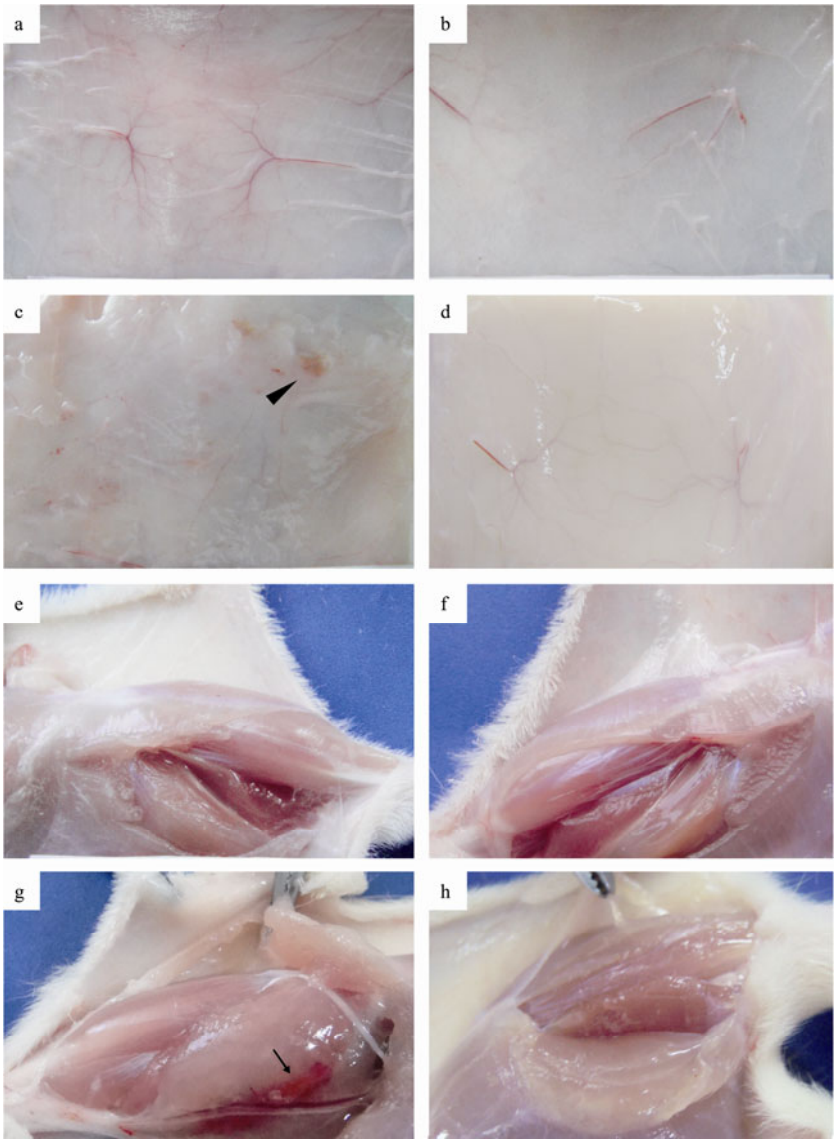

Fig.4 Tissue morphological images after subcutaneous (a-d) and muscle (e-h) implantations in rat. Subcutaneous implantation control group (a, b) and HE-chitosan group (c, d) on day 3 and day 7; muscle implantation control group (e, f) and HE-chitosan group (g, h) on day 3 and day 7. Arrows indicate the inflammatory reaction.

vious inflammatory reaction on rabbits in intradermal injection test.

3.6 Biocompatibility and Biodegradability Test

Biocompatibility and biodegradability of HE-chitosan were evaluated *in vivo* by subcutaneous and muscle implantation tests. All experimental rats survived after HE-chitosan gel implantation for 28 d with no infection or other complication. Typical post-mortem visual examination images and HE staining images on 3 d and 7 d after subcutaneous and muscle implantation were shown in Figs.4 and 5, respectively. Little non-degradable material was observed in subcutaneous tissues and muscles on day 3 (Figs.4c, g). No obvious inflammatory reaction but a pale yellow imprinting was observed in subcutaneous tissue (Fig.4c), and a slight local hyperemia occurred in muscles (Fig.4g). However, on day 7 postoperatively, no observable congestion, suppuration, cysts or other inflammatory reaction emerged in all groups (Figs.4d, h), and HE-chitosan was completely degraded and absorbed both in subcutaneous tissue and muscles. In the control group, subcutaneous tissue and muscles were normal all the time (Figs.4a–b, e–f). For HE staining, the results were consistent with those of the post-mortem visual examination (Fig.5). Small amount of neutrophils and lymphocytes infiltrated around the non-degradable HE-chitosan and the surrounding area on day 3 (Figs.5c, g). However, on day 7 after operation, the inflammatory cells disappeared in companion with the degradation and absorption of HE-chitosan both in subcutaneous tissue and muscles (Figs.5d, h). In contrast, there was no difference in control group compared with normal tissues during all the observation periods (Figs.5a–b, e–f). In summary, HE-chitosan gel was degraded and absorbed gradually

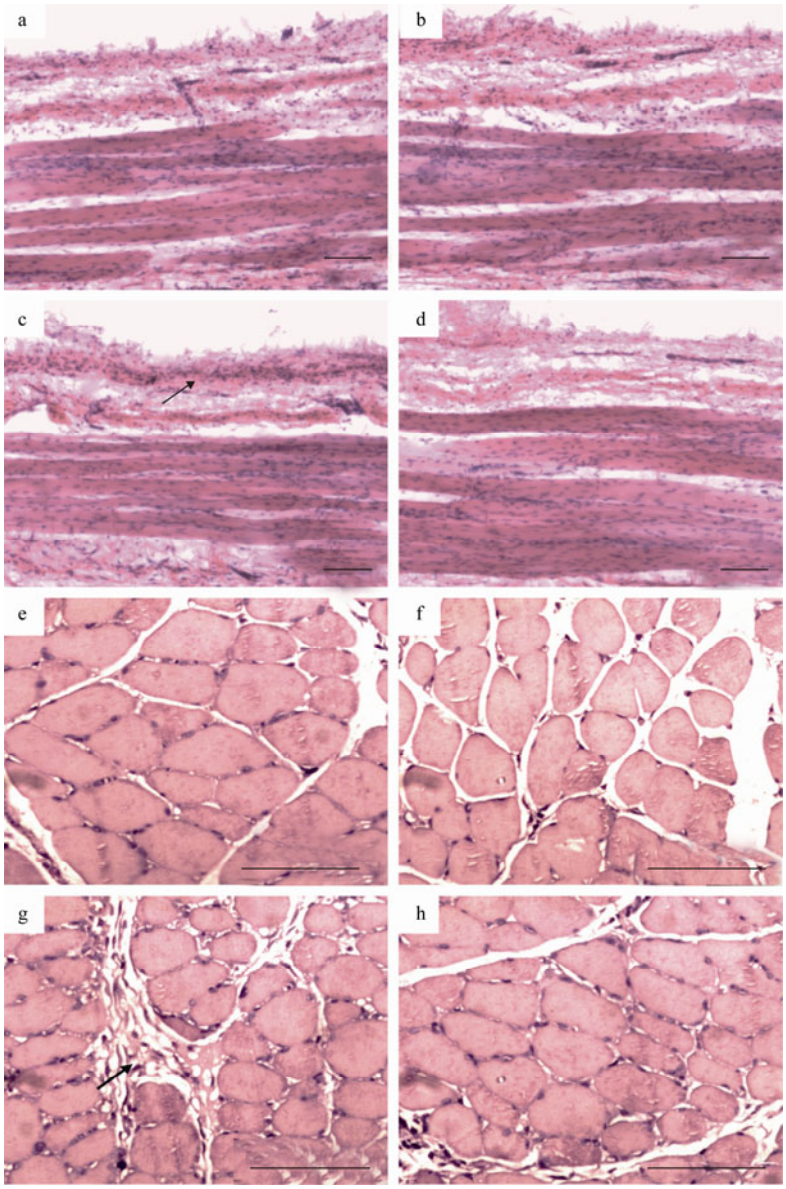

Fig.5 Inflammatory reaction and biodegradation observed by HE staining after subcutaneous (a–d, 40×) and muscle (e–h, 100×) implantation. Subcutaneous implantation control group (a, b) and HE-chitosan group (c, d) on day 3 and day 7; muscle implantation control group (e, f) and HE-chitosan group (g, h) on day 3 and day 7. Arrows indicate the inflammatory reaction and bars represent 100 μm.

with no obvious inflammatory reaction within 7 days.

4 Discussion

Due to its excellent properties, chitosan has become protagonists in a broad range of scientific areas and remains unmatched by other polysaccharides in the last a few years. Extensive previous studies showed that chitosan and its derivatives promise to be used as biomedical material in medicine, food and agriculture. It is extremely applicable to drug delivery and tissue and biomedical engineering. HE-chitosan is a newly appeared member in the list of modified chitosans carrying hydroxyethyl group at position 6. Due to the access of hydrophilic group, HE-chitosan changes its spatial structure. Its intermolecular forces are weakened, the water-solubility is increased, and moisturizing property is improved. Particularly, HE-chitosan can effectively inhibit the growth of microorganisms by combining with DNA (Liu *et al*., 2007; Zhao *et al*., 2008; Nanaki *et al*., 2012). Additionally, it is chemically and enzymatically modifiable and easy to develop into various forms (Liu *et al*., 2007; Zhao *et al*., 2008; Nanaki *et al*., 2012).

The outstanding physical, chemical and biological properties of HE-chitosan make it have potential applications in drug delivery and tissue engineering. In our previous works, a novel drug loaded membrane made of N-succinyl-hydroxyethyl chitosan and mitomycin C has been employed as an implant for glaucoma filtering surgery (Liu *et al*., 2007; Zhao *et al*., 2008; Nanaki *et al*., 2012). It was found that the drug delivery membrane was effective on permeability, swelling property, and release of mitomycin C *in vitro*. In our another early work, we developed a novel membrane consisting of HE-chitosan, gelatin and chondroitin sulfate, and found that it could be potentially used as a carrier for corneal endothelial cell transplantation (Liu *et al*., 2007; Zhao *et al*., 2008; Nanaki *et al*., 2012). However, the biosafety of HE-chitosan has rarely been studied though it is important for its applications. To gain the insights into the biocompatibility and biodegradability of HE-chitosan *in vivo* and *in vitro*, we performed the cytotoxicity test, intradermal injection test and implantation test.

Evaluation of biocompatibility on cells is critical for the biomedical application of HE-chitosan, especially for cell transplantation. In this study, HE-chitosan was found safe to cells, thus, HE-chitosan-based materials are applicable to cell transplantation in tissue engineering.

The biocompatibility and biodegradability of HE-chitosan *in vivo* was investigated by intradermal injection test in rabbit and implantation test in rat. Intradermal injection test is often basic in the safety study of absorbable material. It was demonstrated that HE-chitosan caused no obvious intradermal erythema, edema or other inflammatory reaction. Thus, HE-Chitosan has low toxicity and is safe as absorbed material *in vivo*. On the other hand, HE-chitosan showed excellent biocompatibility and biodegradability *via* the intramuscular and subcutaneous implantation in a rat model. Macroscopic observation and HE staining observation indicated that there was no obvious inflammatory reaction in muscle or subcutaneous tissue after injection of HE-chitosan gel. HE-chitosan was completely degraded and absorbed in 7 days. These findings confirm that HE-chitosan-based composite is suitable for drug delivery and tissue engineering as we have documented in our early works.

In addition, HE-chitosan is a natural polysaccharide derived from chitosan with features of the extracellular matrix (ECM), and has the potential of directing cell migration, growth and organization (Liu *et al*., 2007; Zhao *et al*., 2008; Nanaki *et al*., 2012). However, the absorption and distribution of HE-chitosan *in vivo* are also critical for its medical and clinical applications, which still need further study.

5 Conclusions

HE-chitosan was successfully synthesized and its basic physical and chemical properties were characterized. Biosafety of HE-chitosan was systematically investigated both *in vitro* and *in vivo*. Cytotoxicity test showed that HE-chitosan was not cytotoxic and had no significant effect on fibroblast cell growth. HE-chitosan caused no obvious inflammatory reaction for irritation and delayed-type hypersensitivity *in vivo*. HE-chitosan had excellent biocompatibility and biodegradability, and was suitable for biomedical and clinical applications. It is a potential material for drug delivery and tissue engineering.

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References

Abdull Rasad, M. S. B., Halim, A. S., Hashim, K., Rashid, A. H. A., Yusof, N., and Shamsuddin, S., 2010. *In vitro* evaluation of novel chitosan derivatives sheet and paste cytocompatibility on human dermal fibroblasts. *Carbohydrate Polymers*, **79**: 1094-1100.

Anitha, A., Divya Rani, V., Krishna, R., Sreeja, V., Selvamurugan, N., Nair, S., Tamura, H., and Jayakumar, R., 2009. Synthesis, characterization, cytotoxicity and antibacterial studies of chitosan, O-carboxymethyl and N, O- carboxymethyl chitosan nanoparticles. *Carbohydrate Polymers*, **78**: 672-677.

Baldrick, P., 2010. The safety of chitosan as a pharmaceutical excipient. *Regulatory Toxicology and Pharmacology*, **56**: 290-299.

Carreira, A., Gonçalves, F., Mendonça, P., Gil, M., and Coelho, J., 2010. Temperature and pH responsive polymers based on chitosan: Applications and new graft copolymerization strategies based on living radical polymerization. *Carbohydrate Polymers*, **80**: 618-630.

Chen, Y., C., Liu, Y. F., and Tan, H. M., 2010. Hydroxyethyl chitosan-g-poly (acrylic acid-co-sodium acrylate) superabsorbent polymers. *Journal of Applied Polymer Science*, **117**: 2233-2240.

Chow, K. S., and Khor, E., 2002. New flourinated chitin de-

rivatives: Synthesis, characterization and cytotoxicity assessment. *Carbohydrate Polymers*, **47**: 357-363.

Dash, M., Chiellini, F., Ottenbrite, R., and Chiellini, E., 2011. Chitosan–A versatile semi-synthetic polymer in biomedical applications. *Progress in Polymer Science*, **36**: 981-1014.

Dong, W., Han, B., Feng, Y., Song, F., Chang, J., Jiang, H., Tang, Y., and Liu, W., 2010. Pharmacokinetics and biodegradation mechanisms of a versatile carboxymethyl derivative of chitosan in rats: *In vivo* and *in vitro* evaluation. *Biomacromolecules*, **11**: 1527-1533.

Dong, Y., Wu, Y., and Wang, M., 2001. Studies on chitin-based liquid crystalline polymers-V. Integrated influence of number and length of substituent on hydroxyethyl chitosan. *Acta Polymerica Sinica*, **2**: 172-176.

Dutta, P., Tripathi, S., Mehrotra, G., and Dutta, J., 2009. Perspectives for chitosan based antimicrobial films in food applications. *Food Chemistry*, **114**: 1173-1182.

El-Sherbiny, I., 2009. Synthesis, characterization and metal uptake capacity of a new carboxymethyl chitosan derivative. *European Polymer Journal*, **45**: 199-210.

Gomaa, Y. A., El-Khordagui, L. K., Boraei, N. A., and Darwish, I. A., 2010. Chitosan microparticles incorporating a hydrophilic sunscreen agent. *Carbohydrate Polymers*, **81**: 234-242.

He, X. K., Yuan, Z. X., Wu, X. J., Xu, C. Q., and Li, W. Y., 2012. Low molecular weight hydroxyethyl chitosan-prednisolone conjugate for renal targeting therapy: Synthesis, characterization and *in vivo* studies. *Theranostics*, **2**: 1054.

Huang, R. H., Du, Y. M., and Yang, J. H., 2003. Preparation and anticoagulant activity of carboxybutyrylated hydroxyethyl chitosan sulfates. *Carbohydrate Polymers*, **51**: 431-438.

Huang, R., Mendis, E., and Kim, S.-K., 2005. Factors affecting the free radical scavenging behavior of chitosan sulfate. *International Journal of Biological Macromolecules*, **36**: 120-127.

Ishihara, M., Nakanishi, K., Ono, K., Sato, M., Kikuchi, M., Saito, Y., Yura, H., Matsui, T., Hattori, H., and Uenoyama, M., 2002. Photocrosslinkable chitosan as a dressing for wound occlusion and accelerator in healing process. *Biomaterials*, **23**: 833-840.

Jameela, S., and Jayakrishnan, A., 1995. Glutaraldehyde cross-linked chitosan microspheres as a long acting biodegradable drug delivery vehicle: Studies on the *in vitro* release of mitoxantrone and *in vivo* degradation of microspheres in rat muscle. *Biomaterials*, **16**: 769-775.

Jayakumar, R., Prabaharan, M., Sudheesh Kumar, P., Nair, S., and Tamura, H., 2011. Biomaterials based on chitin and chitosan in wound dressing applications. *Biotechnology Advances*, **29**: 322-337.

Li, D. H., Liu, L. M., Tian, K. L., Liu, J. C., and Fan, X. Q., 2007. Synthesis, biodegradability and cytotoxicity of water-soluble isobutylchitosan. *Carbohydrate Polymers*, **67**: 40-45.

Li, M., Han, B., and Liu, W., 2011. Preparation and properties of a drug release membrane of mitomycin C with N-succinyl-hydroxyethyl chitosan. *Journal of Materials Science: Materials in Medicine*, **22**: 2745-2755.

Liang, Y., Liu, W., Han, B., Yang, C., Ma, Q., Zhao, W., Rong, M., and Li, H., 2011. Fabrication and characters of a corneal endothelial cells scaffold based on chitosan. *Journal of Materials Science: Materials in Medicine*, **22**: 175-183.

Liu, H., Du, Y., Wang, X., and Sun, L., 2004a. Chitosan kills bacteria through cell membrane damage. *International Journal of Food Microbiology*, **95**: 147-155.

Liu, W., Zhang, J., Cao, Z., Xu, F., and Yao, K., 2004b. A chitosan-arginine conjugate as a novel anticoagulation biomaterial. *Journal of Materials Science: Materials in Medicine*, **15**: 1199-1203.

Liu, X., Song, L., Li, L., Li, S., and Yao, K., 2007. Antibacterial effects of chitosan and its water-soluble derivatives on *E. coli*, plasmids DNA, and mRNA. *Journal of Applied Polymer Science*, **103**: 3521-3528.

Muzzarelli, R. A., 2010. Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers. *Marine Drugs*, **8**: 292-312.

Muzzarelli, R. A., Tanfani, F., Emanuelli, M., Pace, D. P., Chiurazzi, E., and Piani, M., 1984. Sulfated N-(carboxymethyl) chitosans: Novel blood anticoagulants. *Carbohydrate Research*, **126**: 225-231.

Nanaki, S. G., Koutsidis, I. A., Koutri, I., Karavas, E., and Bikiaris, D., 2012. Miscibility study of chitosan/2-hydroxyethyl starch blends and evaluation of their effectiveness as drug sustained release hydrogels. *Carbohydrate Polymers*, **87**: 1286-1294.

Ravi Kumar, M. N., 2000. A review of chitin and chitosan applications. *Reactive and Functional Polymers*, **46**: 1-27.

Rinaudo, M., 2006. Chitin and chitosan: Properties and applications. *Progress in Polymer Science*, **31**: 603-632.

Sashiwa, H., Saimoto, H., Shigemasa, Y., Ogawa, R., and Tokura, S., 1990. Lysozyme susceptibility of partially deacetylated chitin. *International Journal of Biological Macro- molecules*, **12**: 295-296.

Shigemasa, Y., Matsuura, H., Sashiwa, H., and Saimoto, H., 1996. Evaluation of different absorbance ratios from infrared spectroscopy for analyzing the degree of deacetylation in chitin. *International Journal of Biological Macromolecules*, **18**: 237-242.

Takei, T., Nakahara, H., Ijima, H., and Kawakami, K., 2012. Synthesis of a chitosan derivative soluble at neutral pH and gellable by freeze-thawing, and its application in wound care. *Acta Biomaterialia*, **8**: 686-693.

Wan, A. G., Khor, E., Wong, J. M., and Hastings, G. W., 1996. Promotion of calcification on carboxymethylchitin discs. *Biomaterials*, **17**: 1529-1534.

Wan, Y., Creber, K. A., Peppley, B., and Tam Bui, V., 2004. Ionic conductivity and tensile properties of hydroxyethyl and hydroxypropyl chitosan membranes. *Journal of Polymer Science Part B: Polymer Physics*, **42**: 1379-1397.

Xie, Y., Liu, X., and Chen, Q., 2007. Synthesis and characterization of water-soluble chitosan derivate and its antibacterial activity. *Carbohydrate Polymers*, **69**: 142-147.

Xu, X., Zhuang, X., Cheng, B., Xu, J., Long, G., and Zhang, H., 2010. Manufacture and properties of cellulose/O-hydroxyethyl chitosan blend fibers. *Carbohydrate Polymers*, **81**: 541-544.

Yuan, Z. X., Li, J. J., Zhu, D., Sun, X., Gong, T., and Zhang, Z. R., 2011. Enhanced accumulation of low-molecular-weight chitosan in kidneys: A study on the influence of N-acetylation of chitosan on the renal targeting. *Journal of Drug Targeting*, **19**: 540-551.

Zhang, C., Ping, Q., and Ding, Y., 2005. Synthesis and characterization of chitosan derivatives carrying galactose residues. *Journal of Applied Polymer Science*, **97**: 2161-2167.

Zhao, Y., Chen, J., Zeng, E., Hu, X., Liu, A., and Dong, Y., 2008. Synthesis and characterization of hydroxyethyl chitosan grafted by carboxyl ending DOVOB dendrimer: A novel liquid crystalline polymer. *Carbohydrate Polymers*, **74**: 828-833.

Zheng, M., Han, B., Yang, Y., and Liu, W., 2011. Synthesis, characterization and biological safety of O-carboxymethyl chitosan used to treat Sarcoma 180 tumor. *Carbohydrate Polymers*, **86**: 231-238.

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