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Synthesis of Carboxymethylated and Quaternized Chitosans and Their Therapeutic Effect on Nonalcoholic Fatty Liver Disease

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ABSTRACT: *O*-Carboxymethyl chitosan (O-CMCs) and *N*-((2-hydroxy-3-*N*,*N*-dimethylhexadecylammonium)propyl)chitosan chloride (N-CQCs) were synthesized for nonalcoholic fatty liver disease (NAFLD) treatment. The weight-average weight and substitution degree of O-CMCs and N-CQCs were 6.5×10^4 and 0.72 and 7.9×10^4 and 0.21, respectively. O-CMCs was negatively charged with a zeta-potential value of −31.82 mV, whereas that of N-CQCs was +36.1 mV, and both showed low cytotoxcity. Serum lipid level and liver fat accumulation were reduced with chitosan and its two derivatives. Furthermore, mRNA and protein expression assay of hepatic lipid metabolism enzymes and low-density lipoprotein receptor (LDL-R) were observed by RT-PCR and Western blot. Results showed that N-CQCs exhibited a more evident desired effect than chitosan and O-CMCs, indicating that amphiphilicity, solubility, and surface charge of chitosan and its two derivatives played roles in the expression of hepatic lipid metabolism enzymes and LDL-R. Therefore, dietary supplementation of O-CMCs and N-CQCs can alleviate the high fat diet induced aberrations related to NAFLD by their antilipidemic property.

KEYWORDS: chitosan, derivatives, NAFLD, hepatic lipid metabolism enzymes, low-density lipoprotein receptor

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of disorders characterized by predominantly macrovesicular hepatic steatosis occurring regardless of the individual's alcohol consumption.[1] The prevalence of NAFLD is around 20−30%, and with a rapid increase in the metabolic risk factors in the general population, NAFLD has become the most common cause of liver disease worldwide.[2] At the moment, obesity,[3,4] insulin resistance,[5] oxidative stress,[6] and cytokine/adipokine interplay have been identified as the major factors involved in NAFLD pathogenesis. Being overweight or obese is clearly associated with NAFLD. High obesity degree is accompanied with a high likelihood of developing NAFLD. In addition, numerous papers have documented resolution of a fatty liver following gradual weight loss.[7,8]

Among the various biomaterials proposed for the fashioning of obesity therapy, chitosan has recently attracted much attention because of its biocompatibility, biodegradability, low cytotoxicity, low cost,[9] and good lowering of cholesterol[10] and NAFLD-controlling effects.[11] However, applications of chitosan in medicine and the food industry are limited because of its poor water solubility. Chemical modifications have been attempted to overcome the limited solubility, such as acylation, etherification, alkylation, esterification, sulfonation, carboxylation, and quaternization due to the existence of living amidos and hydroxys.[12,13] Alternatively, much attention has been focused on chitosan derivatives that may be beneficial for the prevention or treatment of obesity to improve NAFLD. However, up to now, there is no apparent mechanism for chitosan or its derivatives' effect on obesity or NAFLD.

One of the apparent symptoms of NAFLD is liver fat accumulation, associated strongly with lipid metabolism disturbance. It has been known that there are many components affecting lipid metabolism in the liver, such as lipid metabolism enzymes and lipid receptors.[2] Thus, to preliminarily explore the mechanism of chitosan derivatives on NAFLD, we attempt to observe mRNA and protein expression of the prime enzymes and lipid receptors with chitosan and its derivatives at the molecular level.

In this study, two chitosan derivatives, *O*-carboxymethylchitosan (O-CMCs) and *N*-((2-hydroxy-3-*N*,*N*-dimethylhexadecylammonium)propyl)chitosan chloride (N-CQCs), were prepared via the introduction of carboxyl and *N*,*N*-dimethylhexadecyl quaternary ammonium salt groups onto a chitosan backbone. The experiment investigated the effect of chitosan derivatives on the serum lipid level, the expression of low-density lipoprotein receptor (LDL-R), 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, hepatic lipase (HL), and lecithin cholesterol acyltransferase (LCAT) and then led to a proposed mechanism of therapeutic effects on NAFLD with chitosan and its derivatives.

MATERIALS AND METHODS

Materials. Chitosan, with a weight-average molecular weight (M_w) of 5×10^4 and a deacetylation degree (DD) of 0.85, was supplied by Qingdao Medicine Institute, Shandong, China. *N*-(2-Hydroxyethyl)-piperazine-*N*-(2-ethanesulfonic acid) (HEPES), dimethyl sulfoxide (DMSO), MTT assay, and diethyl pyrocarbonate (DEPC) were obtained from Sigma Chemical Co. Fetal bovine serum (FBS) was obtained from Lanzhou Minhai Biotech Co., Gansu, China. Hanks'

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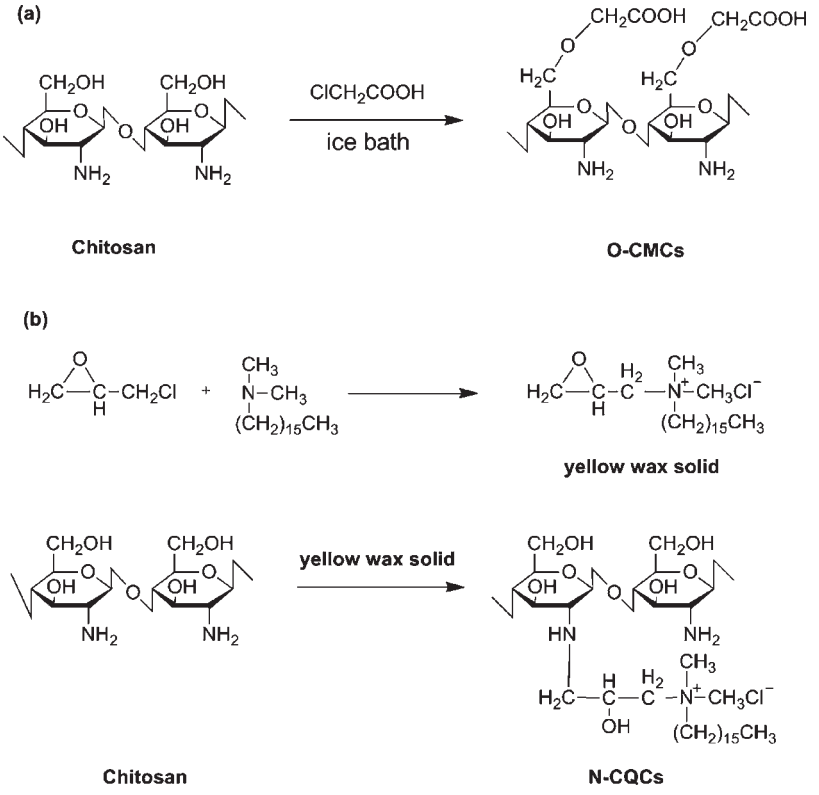

Figure 1. Chemical structures of chitosan, O-CMCs, and N-CQCs.

Table 1. Detailed Composition of Experimental Diet (Grams per Kilogram)

ingredient	commercial diet	high-fat diet
flour	210	147
soybean flour	260	182
corn flour	200	140
wheat bran	160	112
fish meal	100	70
bone meal	50	35
salt	20	14
lard	0	100
reconstituted skim milk	0	120
yolk powder	0	100
casein	0	70

Table 2. Nutrition Information of Experimental Diet

	energy (kJ/100 g)	cellulose (g/100 g)	fat (g/100 g)	minerals (g/100 g)	vitamin (g/100 g)	cholesterol (mg/100 g)
commercial diet	1214.89	5.00	6.44	3.00	1.00	6.14
high-fat diet	1625.75	3.50	19.11	2.10	0.70	1044.43

balanced salt solution (HBSS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco, Grand Island, NY. Trizol was a product of Invitrogen, Carlsbad, CA. Oligo dT and AMV reverse transcriptase were products of Dalian Biotech Co., Liaoning, China. The primers used for amplification by real-time quantitative polymerase chain reaction (RT-PCR) were synthesized by Yingjun Biotech Co., Shanghai, China. All other reagents were of analytical grade provided by No. 3 Chemical Reagent Factory, Tianjin, China.

Preparation of O-CMCs and N-CQCs. The synthetic process of O-CMCs and N-CQCs was prepared as follows (Figure 1). Five grams of chitosan was placed in a 250 mL flask equipped with a mechanical stirrer, and 50 mL of NaOH aqueous solution (42% w/w) was added. The mixture was then stirred in an ice bath for 2 h to allow chitosan to swell sufficiently. A total amount of chloroacetic acid (chitosan/chloroacetic acid = 3:2.3 (w/w)) was added drop by drop in about 1 h. After 48 h, the pH was adjusted to 7 with HCl. O-CMCs was obtained by dialysis for 3 days and lyophilized.[14]

Epoxychloropropane and *N*,*N*-dimethylhexadecylamine with a molar ratio of 1:1 were added into the isopropanol aqueous solution (the volume ratio of isopropanol to water was 2:1) in a flask equipped with a mechanical stirrer, thermometer, reflux cooler, and drop funnel. After reaction at 50 °C for 7 h, isopropanol was removed under reduced pressure. A yellow waxy solid was obtained by purification with ether. Ten grams of chitosan was immersed into a NaOH aqueous solution (42% w/w) in a flask with constant stirring, followed by the addition of 15 mL of isopropanol into the solution. Then the yellow waxy solid was added slowly. The reaction proceeded at 50 °C for 24 h. After cooling, the pH was adjusted to 1 with HCl. Finally, the precipitate was washed, filtered, and dried via vacuum drying to get the final product.[15]

Characterization of Chitosan and Its Two Derivatives. The degree of substitution (DS) of O-CMCs and N-CQCs was determined by potentiometric titration.[14−19] M_w values of O-CMCs and N-CQCs were measured by a Waters 600E gel permeation chromatograghy system (GPC), in which 0.25 M $NaNO_3$ was used as the eluent with a flow rate of 0.5 mL/min at 45 °C. FT-IR spectra of chitosan and its two derivatives were obtained with a Bio-Rad FTS 6000 spectrometer. After

Table 3. Primers Used for PCR

gene	sequence	product (bp)
β-actin	F: 5′-GCACGCTGAGAGAAATCCAG-3′ R: 5′-CAGCTTGTACAGGTCCGTCTC-3′	146
HMG-CoA reductase	F: 5′-ACGCCCATGCTGCCAACATCGTCA-3′ R: 5′-CTTTGCACGCCCCTTGAACACCTA-3′	242
HL	F: 5′-AACAGCCCATTGCCCACTATGACT-3′ R: 5′-GGCCCACACTGCGCTGTTTT-3′	250
LCAT	F: 5′-ACCCCGCCAGCAGGATGAATACT-3′ R: 5′-ATCGGGATGCCCTGGTTGTCAC-3′	250
LDL-R	F: 5′-AGTGGCCGCCTCTATTGGGTTGAT-3′ R: 5′-CTCCGGGGACATGAGGTTTTTAGC-3′	237

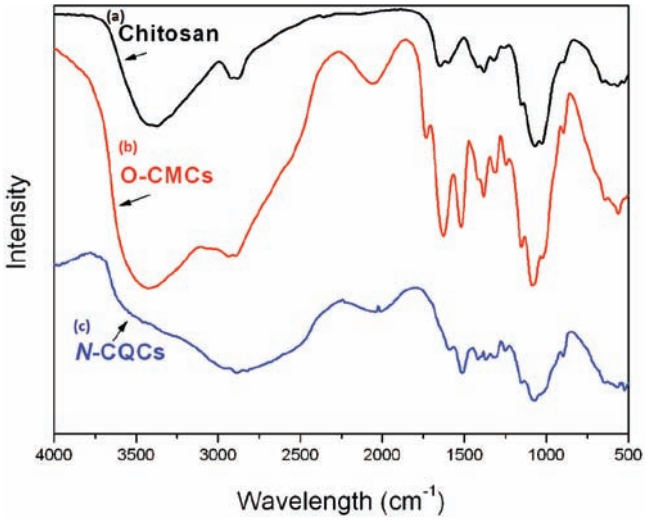

Figure 2. FT-IR spectra of (a) chitosan, (b) O-CMCs, and (c) N-CQCs.

being dried completely at 50 °C, the samples could be used for FT-IR analysis with the standard KBr pellet method. The ^{1}H NMR spectrum of O-CMCs was recorded on a Bruker ARX 300 spectrometer in D_2O at 25 °C. The measurement conditions were as follows: a spectral window of 500 MHz, 32K data points, a pulse angle of 30°, an acquisition time of 2.03 s, and 32 scans with a delay of 1 s between scans.[20,21] Zeta-potential measurements of O-CMCs and N-CQCs were carried out by a zeta-potential analyzer (Brookhaven Instruments Corp.). Zeta-potential analysis was prepared with a constant concentration 1% (w/v). Three measurements were made for each sample, and the mean was reported as the zeta-potential value of the sample.

Evaluation of Cytotoxicity. Cytotoxicity of hepatocytes was measured using the MTT dye reduction assay.[22−26] Percentage cell viability was calculated according to the following equation: percentage cell viability = $OD_{490(sample)}/OD_{490(control)} \times 100$, where $OD_{490(sample)}$ represents a measurement from a well treated with chitosan, O-CMCs , and N-CQCs and $OD_{490(control)}$ represents a well without any sample treatment.

Animal Experimental Design. Fifty male clean Wistar rats, with a mean mass of 65 ± 10 g, were provided by the Experimental Animal Center of Tianjin Medical University, Tianjin, China. All rats were kept in cages with stainless steel bottoms in a room controlled at 25 ± 1 °C and 55 ± 5% humidity under a 12 h light−dark cycle with lighting from 8:00 a.m. to 8:00 p.m. All animal protocols were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University.

After acclimation for 7 days, rats were randomly divided into five groups: group NF (normal fat control), group HF (high fat diet induced NAFLD model), group HFC (high-fat diet + chitosan), group HFO (high-fat diet + O-CMCs), and group HFN (high-fat diet + N-CQCs). The final concentration of each treatment group was 1000 mg/kg in the mass of diet. The detailed composition and nutrition information of the experimental diet (provided by Tianjin Laboratory Animal Co. Ltd., Tianjin, China) are shown in Tables 1 and 2, respectively. Rats in groups NF and HF were fed the commercial diet and the high-fat diet for continuous experimental 12 weeks, respectively. The treatment groups (groups HFC, HFO, and HFN) were fed the same diet as group HF in the first experimental period of 6 weeks to establish the NAFLD rat model, and then chitosan, O-CMCs, and N-CQCs, respectively, were added for another experimental period of 6 weeks. All groups were given corresponding diets. At the end of the 12 week continuous feeding experimental period, rats were deprived of food overnight and blood was collected from the femoral artery puncture under ether anesthesia. The liver was removed and rinsed in cold saline, and a portion of each liver was excised and fixed in a 10% formalin solution. The livers were stored in a −20 °C freezer until used for further analysis.[11]

Serum Lipid Analysis. Serum triglycercide (TG) and total cholesterol (TC) concentrations were measured enzymatically with commercial assay kits (BioSino Biotechnology and Science Inc., Beijing, China).[11]

Histopathologic Examination. For light microscope analysis, the liver tissue was fixed in Saint-Marie fixative,[27] dehydrated in graded ethanol series, cleared in xylene, and embedded in paraffin. Thin sections (5 μm) cut by means of a rotary microtome were dehydrated and stained with Mayer's hematoxylin-eosin (H-E) for examination under an Olympus BX 51 microscope.

BRL Cell Culture. The rat hepatocyte line (BRL cells) under passage 30 (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) was cultured in DMEM supplemented with 10% FBS and was maintained at 37 °C in a humidified atmosphere with 5% CO_2. BRL cells were seeded at a density of 1×10^6/mL in 6-well plates and divided into four groups as follows: DMEM (control) and DMEM with 100 mg/L of chitosan, O-CMCs, and N-CQCs, respectively. After treatment with chitosan and its two derivatives for 48 h, the cells were detached with a cell scraper and

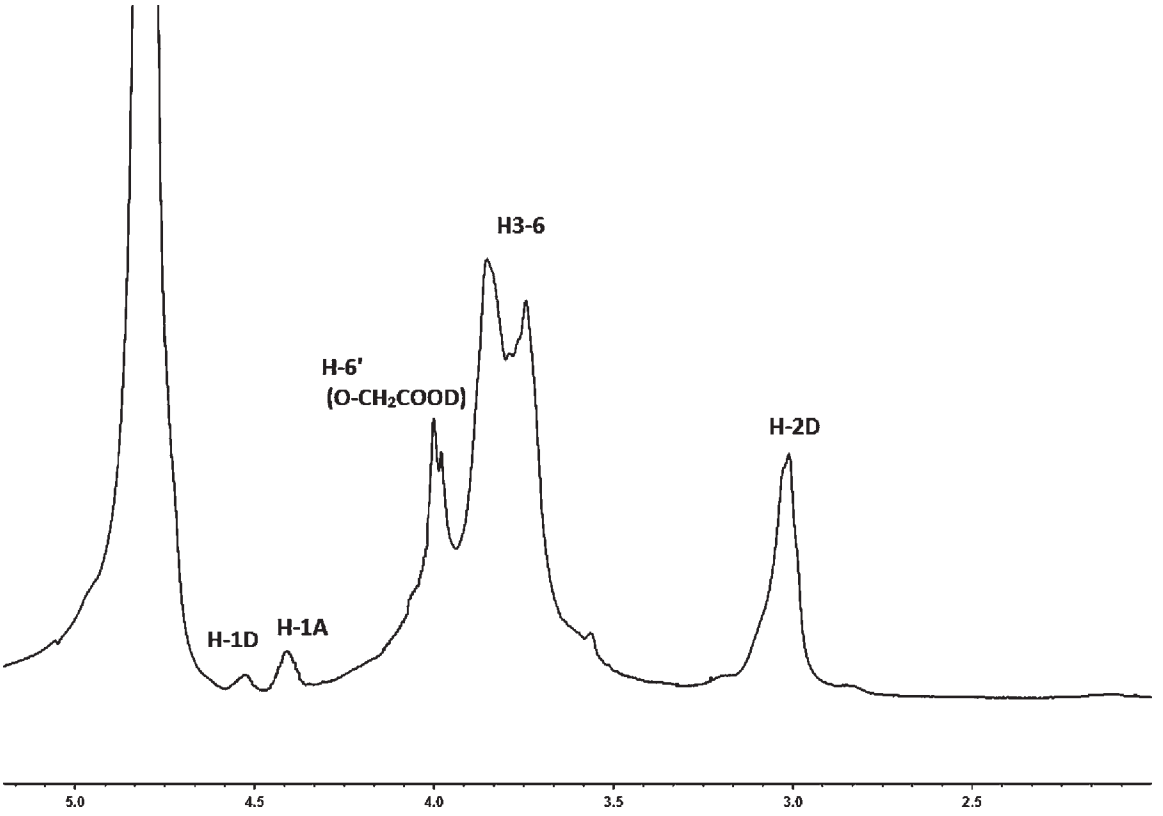

Figure 3. ^{1}H NMR spectrum of O-CMCs in D_2O at 25 °C (500 MHz; 300 K).

Table 4. Characteristics of Chitosan, O-CMCs, and N-CQCs

	chitosan	O-CMCs	N-CQCs
molecular weight (M_w)	5×10^4	6.5×10^4	7.9×10^4
degree of substitution (DS)		0.72	0.21
zeta potential/mV	+8.9	−31.82	+36.1

collected. The samples were then stored at −80 °C until RT-PCR and Western blot analysis.[28]

RT-PCR Analysis. High-quality RNA was isolated using Trizol according to a protocol modified from the method previously described by Kingsley and Richards.[29,30] Briefly, the hepatocytes were placed into Trizon reagent, and total RNA was extracted according to the manufacturer's instructions. The final RNA pellet was resolved by 0.1% DEPC-treated water. The concentration and purity of the RNA were determined spectrophotometrically by the absorbance ratio 260/280 nm. Reverse transcription was performed with the First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. The mRNA fragments were reverse transcribed by RT-PCR using the specific primer sets listed in Table 3 and under the following conditions: initial hold at 42 °C for 50 min, 99 °C for 5 min, and then 5 °C for 5 min. Reverse transcription products were stored at −20 °C.

The expression of hepatocyte genes was determined by real-time fluorescence PCR using a Platinum SYBR Green qPCR Kit (Invitrogen Co., Carlsbad, CA). To quantify RNA transcripts, cDNA fragments were amplified by RT-PCR using the primers sets listed in Table 3. β-Actin was used as an endogenous control to normalize differences in the amount of total RNA in each sample. Amplification and detection were done with an FTC-2000 detection system (Funglyn Biotech). Thermocycler conditions included an initial hold at 95 °C for 3 min; this was followed by a PCR program: 35 cycles of 94 °C for 30 s, 55 °C for 20 s, and 68 °C for 45 s. The fluorescence value of each sample was measured at 72 °C. The mean value of the triplicates for each sample was calculated and expressed as cycle threshold (Ct). The amount of gene expression was calculated as the difference (ΔCt) between the mean Ct value of the sample for the hepatic differentiation makers and the mean Ct value of that sample for β-actin. Relative expression of differentiation markers in hepatocytes cultured on various samples was expressed as $2^{\Delta Ct}$. This method has been mentioned in some other works.[30,31]

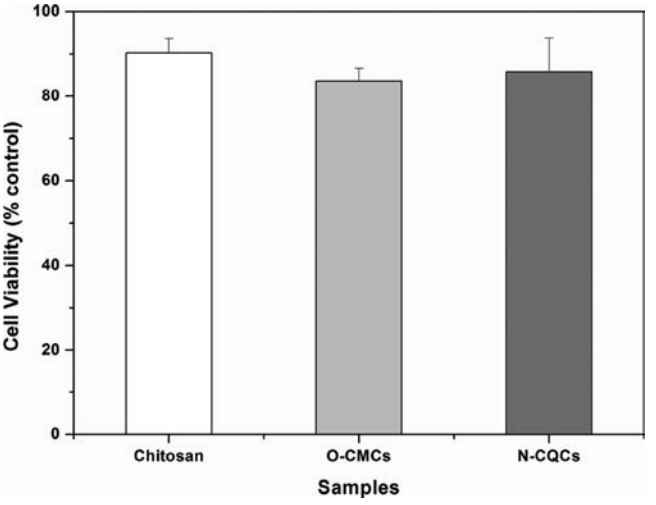

Figure 4. Cell viability of hepatocytes after treatment with chitosan, O-CMCs, and N-CQCs. Data are presented as the mean ± SD, $n = 3$.

Table 5. Body Weight and Body Fat Mass of Each Group[a]

	group NF (n = 10)		group HF (n = 10)		group HFC (n = 10)		group HFO (n = 10)		group HFN (n = 10)	
	6th week	12th week	6th week	12th week	6th week	12th week	6th week	12th week	6th week	12th week
body weight (g)	306.3 ± 13.3	419.0 ± 23.7	334.2 ± 4.0*	460.0 ± 24.7*	352.6 ± 15.6*	422.3 ± 45.9[b]	333.7 ± 15.6*	410.7 ± 27.3[b]	338.4 ± 11.7*	408.0 ± 23.8[b]
body fat mass[c] (%)	1.54 ± 0.08	3.22 ± 0.11	2.10 ± 0.23*	4.89 ± 0.50*	2.61 ± 0.06**	3.28 ± 1.00[b]	2.60 ± 0.09*	3.20 ± 1.01[b]	2.64 ± 0.24**	3.17 ± 0.11[b]

[a] *, $P < 0.05$ vs group NF related to the corresponding experimental week; **, $P < 0.01$ vs group NF related to the corresponding experimental week. [b] $P < 0.05$ vs group HF related to the corresponding experimental week. [c] Body fat mass (%) = (perirenal fat pad weight + epididymal fat pad weight)/body weight × 100.

Western Blot Analysis. Proteins were extracted from hepatocytes with Radio-Immunoprecipitation Assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 1% aprotinin; 0.1% sodium dodecyl sulfate (SDS); provided by Pulilai Biotech Co., Beijing, China) and then separated by 4 and 10% gradient SDS-PAGE Tris-HCl gels and transferred to polyvinylidene difluoride (PVDF) membrane using a PVDF iBlot Gel Transfer Stacks and iBlotTM Dry Blotting System according to the manufacturer's instructions (Invitrogen). Membranes were probed with LDL-R, HMG-CoA reductase, HL, and LCAT antibody (Ab) (Santa Cruz, CA), diluted 1:200, and β-actin Ab (Pulilai Biotech Co., Beijing, China), diluted 1:400. The final productions were visualized with an Electrochemiluminescence (ECL) Advance Western Blotting Detection Kit (Amersham Bioscience, Buckinghamshire, U.K.), exposed to Kodak XAR5 film. Every image was analyzed using Labworks 4.6 Image software.

Statistical Analysis. Values were expressed as the mean ± SD. One-way analysis of variance (ANOVA) was carried out, and statistical comparisons among the groups were performed with Fisher's protected LSD test using a statistical package program (SPSS 10.0 for Windows). Statistical significance was accepted at a value of $P < 0.05$.

RESULTS AND DISCUSSION

Preparation and Characterization of O-CMCs and N-CQCs. The FT-IR spectra of chitosan, O-CMCs, and N-CQCs are demonstrated in Figure 2. Figure 2a shows the basic characteristics of chitosan at 3455 cm^{-1} (O−H stretch), 2867 cm^{-1} (C−H stretch), 1654 cm^{-1} (N−H bend), 1154 cm^{-1} (bridge−O stretch), and 1094 cm^{-1} (C−O stretch).[32,33] The FT-IR spectrum of O-CMCs (Figure 2b) showed characteristic absorptions due to the −COOH group at 1737 cm^{-1}, confirming a successful carboxymethylation.[34,35] The absorption at 1519 cm^{-1} was assigned to $-NH_3^+$, no apparent vibration bends were assigned to amide II or III, and even no apparent C−O stretching bend corresponding to the primary hydroxyl group indicated that the carboxymethyl groups are mainly substituted at the C-6 hydroxyl group of chitosan.[20,36,37] Further evidence supporting a successful carboxymethylation of chitosan is provided by the ^{1}H NMR spectrum of O-CMCs (Figure 3); 4.10 ppm was the proton of $-CH_2-COO^-$ at the O-position at C-6 of the O-CMCs. This also indicated that the carboxymethyl substituent was observed on C-6 primary hydroxyl sites of the modified chitosan structure.[20,21,38] In Figure 2c, the peaks at 2920 and 2870 cm^{-1} were broadened in N-CQCs, compared with that of chitosan, which were attributed to the methyl groups and long carbon chain of the quaternary ammonium salt. Characteristic peaks of the hydroxyl and second hydroxyl groups of chitosan between 1160 and 1080 cm^{-1} were unchanged in N-CQCs, which indicated that no groups were introduced to the C-3 and C-6 during experiment. The N−H bending (1590 cm^{-1}) of the primary amine disappeared due to the change of the primary amine in N-CQCs. The peak at 1500 cm^{-1} was assigned to NH_3^+. Therefore, it can be concluded that the substitution mainly occurred at the amino groups of chitosan. All of these results proved that the synthesis of carboxymethylated and quaternized chitosan was successful. The molecular weight, degree of substitution, and zeta potential of O-CMCs and N-CQCs are shown in Table 4. Both chitosan and N-CQCs were positively charged, and the charge value of N-CQCs was much higher than that of chitosan due to the stronger positive charges on the quaternary ammonium salt group. O-CMCs was negatively charged due to the introduction of carboxymethyl.

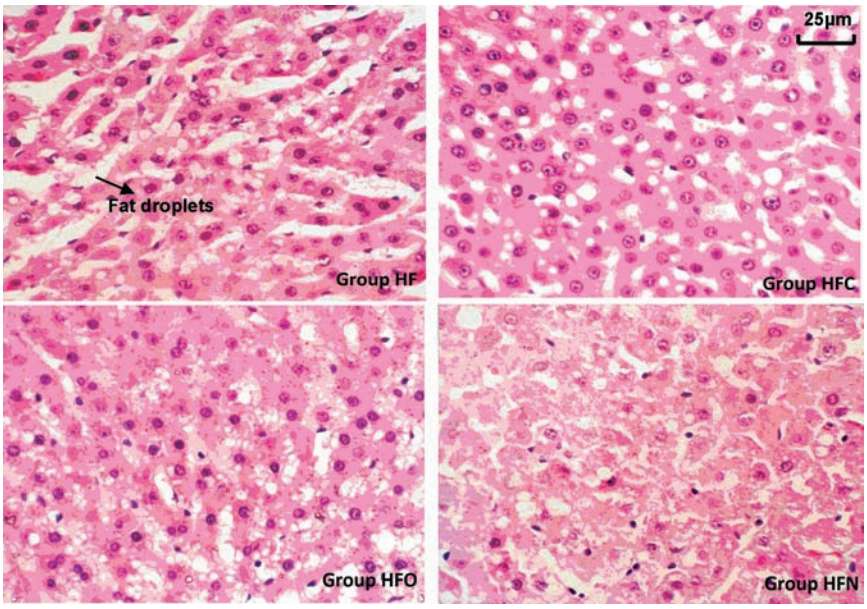

Figure 5. Histologic examination of liver tissues from groups HF, HFC, HFO, and HFN. Fat accumulation in the hepatocyte is observed with a microscope at ×400. Group HF, NAFLD model; group HFC, chitosan receiving group; group HFO, O-CMCs receiving group; group HFN, N-CQCs receiving group.

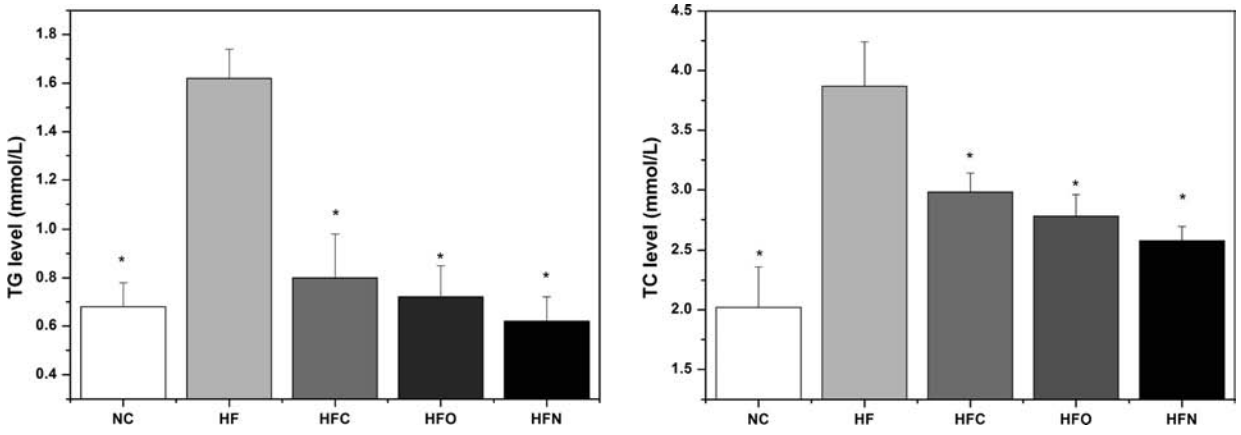

Figure 6. Serum TG and TC levels vary after treatment with chitosan, O-CMCs, and N-CQCs for 6 weeks in NALFD model rats. Data are presented as the mean ± SD, $n = 6$; (∗) $p < 0.05$ versus group HF.

Cytotoxicity Analysis. Chitosan and its two derivatives were also investigated for their possible cytotoxic effects, and the viability of cells was monitored using the MTT assay. Viability data of hepatocytes are shown in Figure 4. Chitosan, O-CMCs, and N-CQCs were confirmed to have a low cytotoxicity to hepatocytes, which was in agreement with previous results.[24−26] The cell viability of chitosan was higher than that of its two derivatives, indicating that the introduction of carboxymethyl and quaternary ammonium salt groups endowed the hepatocytes with a slightly high cytotoxicity. Some studies have shown that cationic polymers had cytotoxicity caused by polymer aggregation on cell surfaces impairing the important membrane functions.[29−31]

Establishment of NAFLD Model Rats. After the first experimental period of 6 weeks, the body weight and body fat mass of NAFLD model rats (including groups HF, HFC, HFO, and HFN) indicated that the body weight and body fat mass were significantly increased (Table 5), which proved that the establishment of the obesity rat model has been successful according to the assessment of the report.[39] Furthermore, tissues from rats were also subjected to histopathologic examination, and the results proved that livers of rats in group HF were swollen and oily. Histopathologic examination of hepatocytes revealed that rats in group HF had fat droplet accumulation, which was a symptom of NAFLD (Figure 5).[11] In hepatocytes of group HF, the fat vacuoles in the cytoplasm and the central vein were expanded. Thus, this observation combined with the successful establishment of the obesity rat model indicated that the establishment of the NAFLD model was successful. Meanwhile, fat deposition appeared to be inhibited in the livers of chitosan and its derivative groups, although some fat vacuoles were evident. The livers of group HFN were normal and had almost no fat vacuoles. This histopathologic examination was consistent with previous reports.[40−42]

Serum Lipid Analysis. After NAFLD model rats had been treated with chitosan, O-CMCs, and N-CQCs for 6 weeks, the body weight and body fat mass of each group were observed

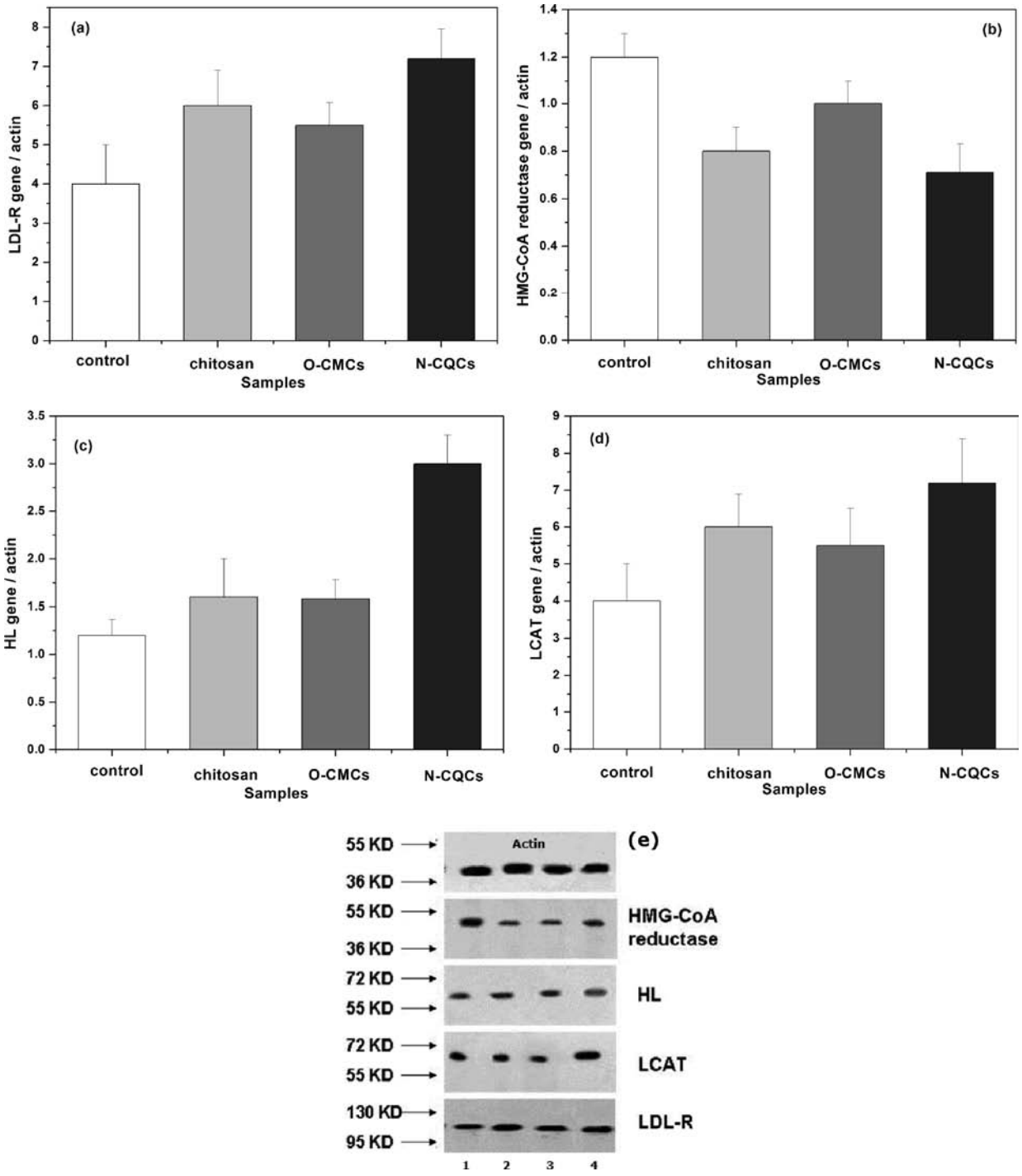

Figure 7. Effect of chitosan, O-CMCs, and N-CQCs on mRNA and protein expression: (a) LDL-R mRNA expression by RT-PCR; (b) HMG-CoA reductase mRNA expression by RT-PCR; (c) HL mRNA expression by RT-PCR; (d) LCAT mRNA expression by RT-PCR; (e) hepatic lipid metabolism enzymes and low-density lipoprotein receptor protein expression by Western blot in cultured hepatocytes after treatment with samples for 48 h. Data are presented as the mean ± SD, $n = 3$. Lanes: 1, normal control; 2, 100 mg/L of chitosan; 3, 100 mg/L of O-CMCs; 4, 100 mg/L of N-CQCs.

(Table 5). The result shows that the body weight and body fat mass were significantly decreased, reaching normal levels. Moreover, the order of down-regulated effect is N-CQCs > O-CMCs > chitosan. Therefore, to investigate the NAFLD in vivo at cellular level, serum lipid analysis was carried out. The effects of chitosan and its two derivatives on TG and TC levels are shown in Figure 6. TG and TC levels in group HF were significantly higher than those of the other four groups ($P < 0.05$). Chitosan, O-CMCs, and N-CQCs significantly decreased TG and TC levels, and N-CQCs exhibited the best down-regulated effect on TG and TC.

Expression of LDL-R and Hepatic Lipid Metabolism Enzymes Analysis. Increasing hepatic LDL-R expression can enhance cholesterol uptake from LDL particles, which has the

effect of lowering plasma LDL concentration directly. This initial correlation between mRNA expression of LDL-R and cholesterol concentration has been confirmed by some studies.[43,44] After BRL cells were incubated with 100 mg/L of chitosan, O-CMCs, and N-CQCs, respectively, for 48 h in vitro, the LDL-R expression was sample-dependently increased ($P < 0.05$), which has achieved our desired result. Moreover, N-CQCs exhibited the best enhanced effect on the LDL-R expression (Figure 7a). HMG-CoA reductase can regulate the synthesis of cholesterol, and the liver is the major place where the increase of cholesterol production leads to obesity.[45] Figure 7b shows that chitosan, O-CMCs, and N-CQCs showed a reduction role in HMG-CoA reductase expression, which has been regarded as a good way to reduce liver cholesterol. HL deficiency is a rare, autosomal recessive disorder that results in elevated high-density lipoprotein (HDL) due to a mutation in the hepatic lipase gene.[46] Figure 7c is the result of HL mRNA expression in the presence and absence of chitosan and its derivatives. It demonstrated that treatment groups can activate HL gene expression in different degrees. The order of the enhanced effect was as follows: N-CQCs > chitosan > O-CMCs. LCAT, as a key plasma enzyme, functions to maintain the cholesterol transportation and concentration in the liver. This enzyme also has a strong effect on the concentration of HDL and LDL in the liver.[47] As shown in Figure 7d, compared with the control group, chitosan, O-CMCs, and N-CQCs increased LCAT expression sample-dependently. The effect of chitosan and its two derivatives on LDL-R and hepatic lipid metabolism enzyme mRNA expression was further proved by the protein expression of LDL-R and hepatic lipid metabolism enzymes (Figure 7e), which was determined by Western blot.

The discovery of effective therapies for NAFLD is an urgent issue in the biopharmaceutical field. The study of new materials for promoting NAFLD therapy is usually carried out in the diet-induced NAFLD rat model.[11] Although NAFLD therapy using chitosan and its derivatives has been confirmed in a few studies, the therapeutic effect shows considerable differences, which are determined by complicated factors. Moreover, the mechanism of NAFLD therapy using chitosan and its derivatives is still under discussion. To cope with these remaining issues, chitosan and its carboxymethylated and quaternized derivatives were used to investigate their therapeutic effects in a diet-induced NAFLD rat model and then to project a mechanism at molecular level.

In this study, O-CMCs and N-CQCs with M_w of 6.5×10^4 and 7.9×10^4 and DS of 0.72 and 0.21, respectively, were prepared. The zeta-potential analysis showed that O-CMCs was negatively charged, whereas N-CQCs was positively charged due to the introduction of different groups onto the chitosan backbone. The therapeutic effects of the tested biomaterials in a diet-induced rat model, which was considered to be a relevant model of human NAFLD, were examined at both cellular and molecular levels.

There have been many reports on positively surface charged chitosan effectively binding negatively charged substrates such as lipids,[48] and the probable hypocholesterolemic mechanism of chitosan and its derivatives was by adsorption, electrostatic force, and entrapment.[49] The present experimental results in vivo demonstrated that chitosan and its two derivatives played an acute regulated role in serum lipid level and liver fat accumulation, and the order of improvement effect was as follows: N-CQCs > O-CMCs > chitosan. This is probably because N-CQCs and O-CMCs are amphiphilic polymers and have better solubility than chitosan. The amphiphilic property made N-CQCs and O-CMCs adsorb lipid to decrease the concentration of lipid in plasma. Moreover, the positive surface charge of N-CQCs made it easy to bind with negatively charged lipid. Therefore, the lipid-lowering effect of N-CQCs was better than that of O-CMCs. The lipid-lowering effect of chitosan was limited by its poor solubility.

Both animal data and human studies have shown that LDL-R, HMG-CoA reductase, HL, and LCAT are involved in the regulation of lipid metabolism.[43−47] PCR results indicated mRNA expression of LDL-R, HMG-CoA reductase, HL, and LCAT in sample-treated rats was sample-dependently improved. The desired improvement order of chitosan and its two derivatives is N-CQCs > chitosan > O-CMCs. The result was further confirmed by the protein expression of hepatic lipid metabolism enzymes and low-density lipoprotein receptor via Western blot analysis. The sample-dependent effect on the hepatic lipid metabolism enzymes mRNA expression was probably attributed to the amphiphilic, solublility, and surface charge of chitosan and its two derivatives. Thus, in consideration of the amphiphilic part, O-CMCs and N-CQCs were amphiphilic molecules. These two amphiphilic molecules probably can combine with the hepatic lipid metabolism enzymes and then regulate their expression. Moreover, due to the introduction of hydrophilicity of the carboxyl group and quaternary ammonium salt group, O-CMCs and N-CQCs were given better solubility than chitosan. Meanwhile, with surface charge taken into consideration, N-CQCs and chitosan were positively charged molecules, which made it much easier to bind with negatively charged molecules. Furthermore, amphiphilic, solublility, and surface charge could interact with each other. As a result, the observation of the chitosan, O-CMCs, and N-CQCs therapeutic effects on hepatic lipid metabolism enzyme mRNA expression is interesting, but the specific biochemical reason for the sample-dependent therapeutic effect on mRNA expression is still under discussion in our further study. Overall, chitosan, O-CMCs, and N-CQCs with different chemical structures and different surface charges can promote mRNA expression of hepatic lipid metabolism enzymes to achieve the desired therapeutic effect. Meanwhile, it is reported that the prevalence of features of insulin resistance and oxidative stress was higher in NAFLD, although the mechanisms causing insulin resistance and oxidative stress in NAFLD have not been elucidated.[50] Therefore, alleviating NAFLD with the help of chitosan and its derivatives would also help reduce the chance of inducing insulin resistance or oxidative stress.

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REFERENCES

(1) Sanyal, A. J. AGA technical review on nonalcoholic fatty liver disease. *Gastroenterology* **2002**, *123*, 1705–1725.

(2) Petta, S.; Muratore, C.; Craxi, A. Non-alcoholic fatty liver disease pathogenesis: the present and the future. *Dig. Liver Dis.* **2009**, *41*, 615–625.

(3) Phan, J.; Peterfy, M.; Reue, K. Biphasic expression of lipin suggests dual roles in adipocyte development. *Drug News Perspect.* **2005**, *18*, 5–11.

(4) Maeda, K.; Cao, H.; Kono, K.; Gorgun, C. Z.; Furuhashi, M.; Uysal, K. T. Adipocyte/macrophage fatty acid binding proteins control integrated metabolic responses in obesity and diabetes. *Cell Metab.* **2005**, *1*, 107–119.

(5) Bugianesi, E.; Marchesini, G.; Gentilcore, E.; Cua, I. H. Y.; Vanni, E.; Pizzetto, M. Fibrosis in genotype 3 chronic hepatitis C and nonalcoholic fatty liver disease: role of insulin resistance and hepatic steatosis. *Hepatology* **2006**, *44*, 1648–1655.

(6) Bergamini, C. M.; Gambetti, S.; Dondi, A.; Cervellati, C. Oxygen, reactive oxygen species and tissue damage. *Curr. Pharm. Des.* **2004**, *10*, 1611–1626.

(7) Tendler, D.; Lin, S.; Yancy, J. W. S.; Mavropoulos, J.; Sylvestre, P.; Rockey, D. C. The effect of a low-carbohydrate, ketogenic diet on nonalcoholic fatty liver disease: a pilot study. *Dig. Dis. Sci.* **2007**, *52*, 589–593.

(8) Palmer, M.; Schaffner, F. Effect of weight reduction on hepatic abnormalities in overweight patients. *Gastroenterology* **1990**, *99*, 1408–1413.

(9) Amado, S.; Simoes, M. J.; Armada, P. A. S. Use of hybrid chitosan membranes and N1E-115 cells for promoting nerve regeneration in an axonotmesis rat model. *Biomaterials* **2008**, *29*, 4409–4419.

(10) Arzneim, F. Cholesterol-lowering properties and safety of chitosan. *Drug Res.* **2002**, *52*, 1–7.

(11) Zhang, J. L.; Liu, J. N.; Li, L.; Xia, W. S. Dietary chitosan improves hypercholesterolemia in rats fed high-fat diets. *Nutr. Res. (N.Y.)* **2008**, *28*, 383–390.

(12) Mohamed, E. B. Chemical modification of chitosan: synthesis and biological activity of new heterocyclic chitosan derivatives. *Polym. Int.* **2008**, *57*, 254–261.

(13) Tae-Hee, K.; Hu-Lin, J.; Dhananjay, J. Chemical modification of chitosan as a gene carrier in vitro and in vivo. *Prog. Polym. Sci.* **2007**, *32*, 726–753.

(14) Chen, L.; Du, Y.; Zeng, X. Relationships between the molecular structure and moisture-absorption and moisture-retention abilities of carboxymethyl chitosan: II. Effect of degree of deacetylation and carboxymethylation. *Carbohydr. Res.* **2003**, *338*, 333–340.

(15) Jia, Z.; Shen, D.; Xu, W. Synthesis and antibacterial activities of quaternary ammonium salt of chitosan. *Carbohydr. Res.* **2001**, *333*, 1–6.

(16) Liu, C. X.; Chen, G. H.; Jin, Z. T.; Sun, M. K.; Gao, C. J. Modification of the formula for calculation of substitution degree of *N*, *O*-carboxymethyl chitosan. *J. Beijing Univ. Chem. Technol.* **2004**, *31*, 14–17.

(17) Lu, S. J.; Song, X.; Cao, D. Preparation of water-soluble chitosan. *J. Appl. Polym. Sci.* **2004**, *91*, 3497–3503.

(18) Sun, S. L.; Wang, A. Q. Adsorption kinetics of Cu(II) ions using *N*,*O*-carboxymethyl-chitosan. *J. Hazard. Mater. B* **2006**, *131*, 103–111.

(19) Sang-Hoon, L.; Samuel, M. H. Synthesis and antimicrobial activity of a water-soluble chitosan derivative with a fiber-reactive group. *Carbohydr. Res.* **2004**, *339*, 313–319.

(20) Chen, X. G.; Park, H. J. Chemical characteristics of *O*-carboxymethyl chitosans related to the preparation conditions. *Carbohydr. Polym.* **2003**, *53*, 355–359.

(21) Rinaudo, M.; Le Dung, P.; Gey, C.; Milas, M. Substituent distribution on *O*,*N*-carboxymethylchitosans by ^{1}H and ^{13}C NMR. *Int. J Biol. Macromol.* **1992**, *14*, 122–128.

(22) Jiang, H. L.; Kim, Y. K.; Arote, R.; Nah, J. W.; Cho, M. H.; Choi, Y. J. Chitosan-graft-polyethylenimine as a gene carrier. *J. Controlled Release* **2007**, *117*, 273–280.

(23) Kim, S.; Gaber, M. W.; Zawaski, J. A.; Zhang, F.; Richardson, M.; Zhang, X. A. The inhibition of glioma growth in vitro and in vivo by a chitosan/ellagic acid composite biomaterial. *Biomaterials* **2009**, *30*, 4743–4751.

(24) Thanou, M.; Florea, B. I.; Geldof, M.; Junginger, H. E.; Borchard, G. Quaternized chitosan oligomers as novel gene delivery vectors in epithelial cell lines. *Biomaterials* **2007**, *23*, 153–159.

(25) Anitha, A.; Divya Rani, V. V.; Krishna, R.; Sreeja, V.; Selvamurugan, N.; Nair, S. V. Synthesis, characterization, cytotoxicity and antibacterial studies of chitosan, *O*-carboxymethyl and *N*,*O*-carboxymethyl chitosan nanoparticles. *Carbohydr. Polym.* **2009**, *78*, 672–677.

(26) Zhang, Y. F.; Yin, P.; Zhao, X. Q.; Wang, J.; Wang, J.; Wang, C. D. *O*-Carboxymethyl-chitosan/organosilica hybrid nanoparticles as non-viral vectors for gene delivery. *Mater. Sci. Eng.* **2009**, *29*, 2045–2049.

(27) Yildiz, S.; Gurcu, B.; Koca, Y. B.; Koca, S. Histopathological and genotoxic effects of pollution on *Anguilla anguilla* in the Gediz River (Turkey). *J. Anim. Vet. Adv.* **2010**, *9*, 2890–2899.

(28) Sun, F.; Xie, M. L.; Xue, J.; Wang, H. B. Osthol regulates hepatic PPARα-mediated lipogenic gene expression in alcoholic fatty liver murine. *Phytomed.* **2010**, *17*, 669–673.

(29) Kingsley, D. H.; Richards, G. P. Rapid and efficient extraction method for reverse transcription-PCR detection of hepatitis A and Norwalk-like viruses in shellfish. *Appl. Environ. Microbiol.* **2001**, *67*, 4152–4157.

(30) Kim, D.; Kim, S. R.; Kwon, K. S.; Lee, J. W.; Oh, M. J. Detection of hepatitis a virus from oyster by Nested PCR using efficient extraction and concentration method. *J. Microbiol.* **2008**, *46*, 436–440.

(31) Yoko, O.; Kim, E. C.; Lucia, P.; Ram, K.; Young, S. R. RNA degradation in human breast tissue after surgical removal: a time-course study. *Exp. Mol. Pathol.* **2004**, *77*, 98–103.

(32) Brugnerotto, J.; Lizardi, J.; Goycoolea, F. M.; Arguelles-Monal, W.; Desbrieres, J.; Rinaudo, M. An infrared investigation in relation with chitin and chitosan characterization. *Polymer* **2001**, *42*, 3569–3580.

(33) Shigemasa, Y.; Matsuura, H.; Sashiwa, H.; Saimoto, H. Evaluation of different absorbance ratios from infrared spectroscopy for analyzing the degree of deacetylation in chitin. *Int. J. Biol. Macromol.* **1996**, *18*, 237–242.

(34) Nishimura, S.; Nishi, N.; Tokura, S. Bioactive chitin derivatives. Activation of mouse-peritoneal macrophages by *O*-(carboxymethyl)chitins. *Carbohydr. Res.* **1986**, *146*, 251–258.

(35) Muzzareli, R. A. A.; Ramos, V.; Stanic, V.; Dubini, B. Osteogenesis promoted by calcium phosphate *N*,*N*-dicarboxymethyl chitosan. *Carbohydr. Polym.* **1998**, *36*, 267–276.

(36) Zhang, L.; Guo, J.; Zhou, J.; Yang, G.; Du, Y. Blend membranes from carboxymethylated chitosan/alginate in aqueous solution. *J. Appl. Polym. Sci.* **2000**, *77*, 610–616.

(37) Zhao, X.; Kato, K.; Fukumoto, Y.; Nakamae, K. Synthesis of bioadhesive hydrogels from chitin derivatives. *Int. J. Adhes. Adhes.* **2001**, *21*, 227–232.

(38) Chen, L.; Du, Y.; Wu, H.; Xiao, L. Relationship between molecular structure and moisture-retention ability of carboxymethyl chitin and chitosan. *J. Appl. Polym. Sci.* **2002**, *83*, 1233–1241.

(39) Liu, R.; Sun, C. H.; Li, Y.; Yu, X. F.; Wang, S. R. Comparation of blood hormore level between DIO-R and DIO rats. *Hyginen. Res.* **2003**, *32*, 212–214.

(40) Kanauchi, O.; Deuchi, K.; Imasato, Y.; Kobayashi, E. Increasing effect of a chitosan and ascorbic acid mixture on fecal dietary fat excretion. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 1617–1620.

(41) Deuchi, K.; Kanauchi, O.; Imasato, Y.; Kobayachi, E. Decreasing effect of chitosan on the apparent fat digestibility by rats fed on a high-fat diet. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 1613–1616.

(42) Deuchi, K.; Kanauchi, O.; Imasato, Y.; Kobayashi, E. Effect of the viscosity or deacetylation degree of chitosan on fecal fat excreted from rats fed on a high-fat diet. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 781–785.

(43) Puglisi, M. J.; Mutungi, G.; Brun, P. J.; McGrane, M. M.; Labonte, C.; Volek, J. S. Raisins and walking alter appetite hormones and plasma lipids by modifications in lipoprotein metabolism and up-regulation of the low-density lipoprotein receptor. *Metabolism* **2009**, *58*, 120–128.

(44) Stahlberg, D.; Rudling, M.; Angelin, B.; Bjorkhem, I.; Forsell, P.; Nilsell, K. Hepatic cholesterol metabolism in human obesity. *Hepatology* **1997**, *25*, 1447–1450.

(45) Angelin, B.; Backman, L.; Einarsson, K.; Eriksson, L.; Ewerth, S. Hepatic cholesterol metabolism obesity: activity of microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Lipid Res.* **1982**, *23*, 770–773.

(46) Karackattu, S. L.; Trigatti, B.; Krieger, M. Hepatic lipase deficiency delays atherosclerosis, myocardial infarction, and cardiac

dysfunction and extends lifespan in SR-BI/apolipoprotein E double knockout mice. *Arterioscler. Thromb. Vasc. Biol.* **2006**, *26*, 548–554.

(47) Dobiásová, M.; Frohlich, J. Advances in understanding of the role of lecithin cholesterol acyltransferase (LCAT) in cholesterol transport. *Clin. Chim. Acta* **1999**, *286*, 257–271.

(48) Ormrod, D. J.; Holmes, C. C.; Miller, T. E. Dietary chitosan inhibits hypercholesterolaemia and atherogenesis in the apolipoprotein E-deficient mouse model of atherosclerosis. *Atherosclerosis* **1998**, *138*, 329–334.

(49) Liu, J. N.; Zhang, J. L.; Xia, W. S. Hypocholesterolaemic effects of different chitosan samples in vitro and in vivo. *Food Chem.* **2008**, *107*, 419–425.

(50) Albano, E.; Mottaran, E.; Occhino, G.; Reale, E.; Vidali, M. Review article: role of oxidative stress in the progression of non-alcoholic steatosis. *Aliment. Pharmacol. Ther.* **2005**, *22*, 71–73.