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A water-soluble photocrosslinkable chitosan derivative prepared by Michael-addition reaction as a precursor for injectable hydrogel

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ABSTRACT

With the goal of obtaining photopolymerized hydrogels for use as tissue engineering scaffolds, a water-soluble (methacryloyloxy) ethyl carboxyethyl chitosan was prepared as a photopolymerizable prepolymer through Michael-addition reaction between chitosan and ethylene glycol acrylate methacrylate. N-substitution of chitosan was verified by both ^{1}H NMR and ^{13}C NMR The degree of N-substitution, measured via ^{1}H NMR, was easily varied from 0.10 to 0.35 by varying the molar ratio of ethylene glycol acrylate methacrylate to chitosan. Using a Vero cell line, the water-soluble photocrosslinkable chitosan derivative was found to be noncytotoxic up to a concentration of 1.0 mg/mL. The precursor was blended with D-2959 photoinitiator in solution, and UV-irradiated to create hydrogels. FTIR verified the nearly complete conversion of the double bonds in the gel. Indirect cytotoxicity assessment of the hydrogel indicated that the hydrogel was non-toxic to Vero cells.

1. Introduction

From a clinical perspective, the use of injectable hydrogels is more attractive than the implantation of a preformed hydrogel as it can minimize patient's discomfort, risk of infection, scar formation, and the cost of treatment (Hou, Bank, & Shakesheff, 2004). Injectable hydrogel can be used as tissue regeneration scaffolds for cartilage and soft tissues (Fellah et al., 2006; Shin, Ruhe, Mikos, & Jansen, 2003). For effective clinical application, injectable systems should be able to gel at mild physiological conditions in a clinically relevant time period. The gel should show good biocompatibility and biodegrade into non-toxic degradation products (Gutowska, Jeong, & Jasionowski, 2001; Hatefi & Amsden, 2002). Several synthetic and natural polymers have been investigated for developing in situ gelling systems induced by stimuli such as temperature, light or pH (Jeong, Bae, Lee, & Kim, 1997; Kim & Chu, 2000; Martens & Anseth, 2000; Nair, Starnes, Ko, & Laurencin, 2007; Paige, Cima, Yaremchuk, Vacanti, & Vacaoti, 1995; Sawhney, Pathak, & Hubbell, 1993). Among these, photopolymerization is one of the most promising ways of making injectable hydrogels, due to fast curing rates at room or physiological temperatures, the ability to place the gel in vivo without surgical intervention and minimal heat production (Poon, Zhu, Shen, Chan-Park, & Ng, 2007).

Chitosan has excellent biological properties such as biodegradability, biocompatibility, antibacterial and wound-healing activity (Khor & Lim, 2003; No, Park, Lee, & Meyers, 2002; Ueno, Mori, & Fujinaga, 2001). Additionally, chitosan could achieve hemostasis and promote normal tissue regeneration (Malette & Quigley, 1985). For these reasons, chitosan has been considered to be one of the most promising biomacromolecules for tissue engineered scaffolds.

Photopolymerizable chitosan derivatives have been prepared previously through styrenation (Matsuda & Magoshi, 2002) and methacrylation (Renbutsu et al., 2005) using reaction aldehyde intermediates. However, these photopolymerizable chitosan derivatives are only soluble at acidic pH, while solubility at physiological pH values is required for the synthesis of useful biocompatible hydrogels for biological applications. Thus, a useful chitosan-based photopolymerizable precursor would be solubility in aqueous solution at physiological pH. A water-soluble photopolymerizable chitosan has previously been prepared by grafting 4-azidobenzoic acid to available free amine groups of lactose modified chitosan (Ono et al., 2000). However, this approach has a possible disadvantage that is exposure to UV irradiation may result in protein denaturation when protein drug was capsulated (Amsden, Sukarto, Knight, & Shapka, 2007).

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Hence, the objective of this study is the synthesis and characterization of photopolymerizable water-soluble (methacryloyloxy) ethyl carboxyethyl chitosan (MAOECECS). Briefly, chitosan was reacted with ethylene glycol acrylate methacrylate by Michael-addition reaction between $-NH_2$ of chitosan and C=C of acrylate to produce a photocrosslinkable MAOECECS hydrogel precursor. The MAOECECS was blended with a photoinitiator and water, and UV-irradiated to create hydrogels. A series of characterization of MAOECECS including crystalline and thermal stability was studied. The cytocompatibility and biodegradability behaviors of MAOECECS hydrogel also were investigated.

2. Materials and methods

2.1. Materials

Chitosan (CS, MW = 1.2×10^5, degree of deacetylation = 84.4%) was purchased from Zhejiang Golden-Shell Biochemical Co., Ltd., China. 2-Hydroxyethyl Methacrylate (HEMA) was from Beijing Chemical Reagents Company, distilled under reduced pressure in the presence of hydroquinone, and stored at 4 °C until use. Acryloyl chloride was purchased from Shanghai Chemical Reagents Company, China. Triethylamine and toluene were obtained from Beijing Chemical Reagents Company, distilled under reduced pressure and stored until use. Cytocompatible UV photoinitiator Darocur 2959 (2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone) was obtained from Ciba-Geigy Chemical Co. (Tom River, NJ). Vero cells were obtained from Department of Microbiology, Peking University Health Science Center.

2.2. Synthesis of ethylene glycol acrylate methacrylate (EGAMA) (Muh, Marquardt, Klee, Frey, & Mulhaupt, 2001)

A mixture of 83.81 g of HEMA and 105 mL of triethylamine dissolved in 250 mL of toluene was added into a three-necked flask equipped with stirrer, thermometer, and dropping funnel. Under cooling (0–5 °C), 79.79 g of acryloyl chloride dissolved in 50 mL of toluene was added over 4 h. Then, the mixture was allowed to stand overnight, the precipitate filtered off and washed twice with 20 mL of toluene. Then, the reaction mixture was extracted twice with 75 mL of water, 50 mL of 1 mol/L HCl and 50 mL of 1 mol/L $NaHCO_3$ and dried overnight with Na_2SO_4. Subsequently, the toluene was removed by rotary evaporation. The yellow crude product was purified by silica gel column chromatography. Yield: 80%. IR: 1725 cm^{-1} (s, C=O), 1637 cm^{-1} (m, C=C), 811 cm^{-1} (m, C=C). ^1H NMR: δ = 6.4 (d, $=CH_2$), 6.1 (1b, 8, m), 5.8 (9b, d), 5.6(1a, s), 4.3 (5, 6, s), 1.9 (3, s).

2.3. Synthesis of water-soluble (methacryloyloxy) ethyl carboxyethyl chitosan (MAOECECS)

Briefly, 1.5 g of chitosan (corresponding to 7.5 mmol NH_2) was first dissolved in 50 mL of water containing 0.48 mL of acetic acid, after which ethanol (40 mL) and a certain amount of EGAMA were added in sequence and stirred for 2 days at 60 °C. Thereafter, a saturated $NaHCO_3$ solution was added to the reaction mixture to adjust the pH to 7–8, and the mixture solution was then precipitated in acetone. The precipitated solid was collected by filtration and then dissolved in water and dialyzed (membrane molecular weight cut-off 12,000 g mol^{-1}) against water for 2 days and lyophilized to obtain pure MAOECECS. ^1H NMR and ^{13}C NMR spectrum was performed to confirm structure of the MAOECECS. The solubility of the product in water was evaluated from the visual observation method where 50 mg of product was suspended in water (5 mL) for 1 day.

2.4. Photocrosslinking of MAOECECS

A concentrated solution of MAOECECS in deionized water (6 w/v%) containing the photoinitiator D-2959 (0.1 w/v%) was poured into a flat mold consisting of two glass plates separated by a 2 mm-thick spacer frame. D-2959 was used in this study, as it has been demonstrated to be the least cytotoxic to various cells (Williams, Malik, Kim, Manson, & Elisseeff, 2005). The solution was then exposed to long wave ultraviolet light (320–480 nm, EXFO lite, EFOS Corpration, Mississauga, Canada) at intensity of 10 mW/cm^2 for up to 15 min to yield the photopolymerized hydrogel.

2.5. Characterization methods

2.5.1. FTIR spectroscopy

The Fourier Transform Infrared spectra (FTIR) of CS, MAOECECS and the hydrogels were recorded at room temperature by using a Nicolet 5700 instrument (Nicolet Instrument, Thermo Company, USA) over the wave number range of 4000–400 cm^{-1}.

2.5.2. NMR spectroscopy

^1H NMR and ^{13}C NMR studies were performed by using a Bruker AMX 600 MHz instrument. ^{13}C NMR spectra were recorded using 50 mg/mL samples under ambient temperature.

2.5.3. Thermal stability

To examine the thermal properties of CS and MAOECECS, Thermal gravimetric analysis (TGA) was performed with a NETZSCH TG 209 F1 analyzer (Germany) between 20 and 500 °C with a 10 °C/min heating rate under a nitrogen flow rate of 20.0 mL/min.

2.5.4. XRD

The XRD patterns of CS and MAOECECS were performed via X-ray diffractometer (Rigaku, Damax2500) with CuKa characteristic radiation (wavelength λ = 0.154 nm at 40 kV, 50 mA, and scan speed of 1°/min in the 2θ range of 5–50°).

2.5.5. Cell culture

Vero cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, together with 1.0% penicillin–streptomycin, and 1.2% glutamine. Culture was maintained at 37 °C in a wet atmosphere containing 5% CO_2. When the cells reached 80% confluence, they were trypsinized with 0.25% trypsin containing 1 mM ethylenediamine tetraacetic acid (EDTA).

2.5.6. Cytotoxicity assays

In vitro cytotoxicity of the noncrosslinked MAOECECS in the absence of D-2959 was assessed through effect on cell proliferation in monolayer cultures of Vero cells. Lyophilized MAOECECS were dissolved in serum supplemented tissue culture medium at varying concentrations (0.5, 1, 2.5, 5 and 10 mg/mL) and sterilized by filtration (0.45 μm) prior to placement inside the wells. For these experiments, the Vero cells were seeded in wells of 96-well plate at a density of 10^3 cells per well. After incubated for another 24 h, the culture medium was removed and replaced with the test solutions and incubated for another 48 h, and then 100 μL MTT solution was added to each well. After 3 h incubation at 37 °C, 200 μL of dimethyl sulfoxide was added to dissolve the formazan crystals. The dissolved solution was swirled homogeneously about 10 min by the shaker. The optical density of the formazan solution was detected by an ELISA reader (Multiscan MK3, Labsystem Co., Finland) at 570 nm.

The cytotoxicity of the photopolymerized hydrogel was assessed by culture of Vero cells supplemented with the extractant

of the hydrogel. The hydrogel, chitosan membrane and carboxylethyl chitosan membrane were sterilized with highly compressed steam for 15 min and placed in wells of 24-well culture plate respectively. The samples were then incubated in 1 mL RPMI1640 medium at 37 °C for 24 h. The extraction ratio was 1.25 cm^{-1}/mL. At the end of this period, the hydrogel or the membranes were removed, and the so-called extracts were obtained. Vero cells were seeded in wells of 96-well plate at a density of 10^3 cells per well. After incubated for another 24, 48 and 72 h, the culture medium was removed and replaced with the as-prepared extraction medium and incubated for another 24 h, then 100 μL MTT solution was added to each well. After 3 h incubation at 37 °C, 200 μL of dimethyl sulfoxide was added to dissolve the formazan crystals. The dissolved solution was swirled homogeneously about 10 min by the shaker. The optical density of the formazan solution was detected by an ELISA reader (Multiscan MK3, Labsystem Co., Finland) at 570 nm.

For the reference purpose, cells were seeded to medium containing 0.64% phenol (positive control) and a fresh culture medium (negative control) with same seeding condition, respectively.

2.6. Statistical analysis

Results are depicted as mean ± standard deviation. Significance between the mean values was calculated by using ANOVA one-way analysis (Origin7.0 SRO, Northampton, MA, USA). Probability values $p < 0.05$ were considered significant ($n = 6$).

Table 1
Mole ratio of reactant and characteristic properties of MAOECECS.

Sample name	EGAMA (g)	EGAMA to $-NH_2$ (mole ratio)	DS[a]	Solubility in deionized water
MAOECECS1	1.41	1.0	0.10	Full solubility
MAOECECS2	2.82	2.0	0.26	Full solubility
MAOECECS3	4.21	3.0	0.30	Full solubility
MAOECECS4	5.62	4.0	0.35	Full solubility

[a] DS was calculated from ^1H NMR studies.

3. Results and discussion

3.1. Characterization of MAOECECS and its hydrogels

In order to produce a photocrosslinkable product, methacrylation was carried out on chitosan polymer chain so that they can undergo free radical polymerization. The synthesis of MAOECECS was achieved via Michael addition of $-NH_2$ of chitosan to the acrylate group of ethylene glycol acrylate methacrylate (EGAMA). Four MAOECECS samples (denoted MAOECECS1–4) were prepared by using increasing amounts of EGAMA (1.41–5.62 g) in order to yield products with different degrees of substitution (DS), the values for which are also listed in Table 1. DS increases from MAOECECS1–4 indicating that DS is positively correlated to the ratio of EGAMA used in the reaction.

FTIR spectra of chitosan, MAOECECS and photopolymerized hydrogel are showed in Fig. 1. Fig. 1a shows the principal spectral features in chitosan: 3424 cm^{-1}(O–H stretch), 2921 cm^{-1}(C–H stretch), 1650 cm^{-1}(C═O stretch), 1597 cm^{-1}(N–H stretch bend), 1155 cm^{-1}(bridge-O stretch), 1081 cm^{-1}(O–H stretch). After Michael addition with EGAMA, the FTIR spectrum for all four MAOECECS samples showed absorptions at 1717 and 810 cm^{-1}, which are indicative of the carbonyl group and the double bond (vinyl) of the methacrylate group, respectively (Fig. 1b–e). After photopolymerization to form hydrogel, the peak originating from the vinyl bond (~810 cm^{-1}) disappeared, indicating almost full conversion and photopolymerization of the vinyl group (Fig. 1f).

^1H NMR of MAOECECSs and chitosan are provided in Fig. 2. Methacrylation by Michael addition is achieved, as evidenced by peaks arsing at 5.7 and 6.1 ppm due to protons on the vinyl carbon and a peak at 1.9 ppm due to the methyl group in methacrylate. The same groups in unreacted EGAMA appear at 6.1, 5.6 and 1.9 ppm, respectively. *N*-methacrylation can be discerned in the spectrum, as a reduction in peak area at 3.1 ppm (H-2 proton for the deacetylated residues) and the appearance of a small peak centered at 3.0 ppm corresponding to H-2 for the *N*-methacrylated residue. The DS of methacrylate groups onto the chitosan backbone was calculated from the relative integrations of the methyl protons at 1.9 ppm with respect to the methyl protons of $-NHCOCH_3$ at

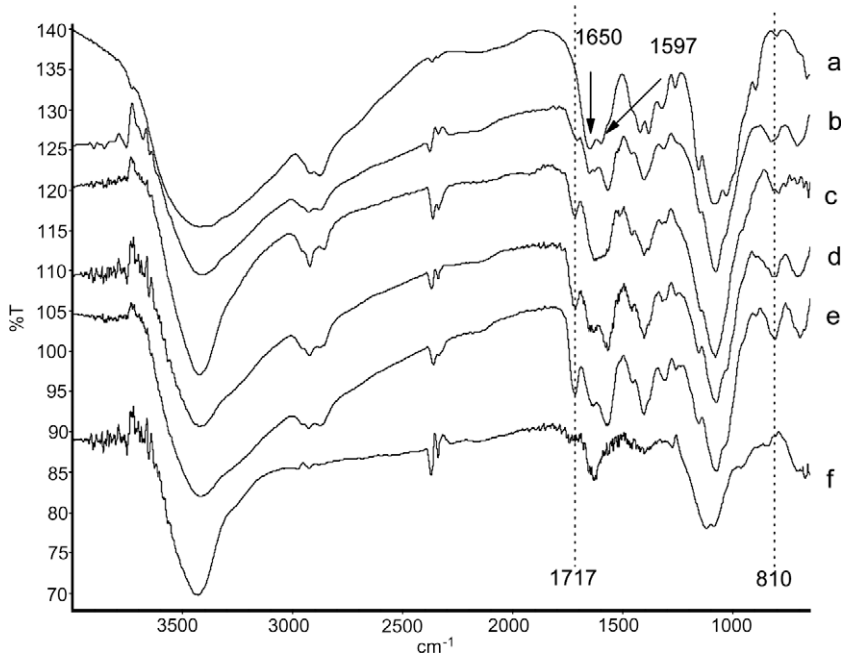

Fig. 1. FTIR spectra of (a) chitosan, (b) MAOECECS1, (c) MAOECECS2, (d) MAOECECS3, (e) MAOECECS4 and (f) MAOECECS hydrogel.

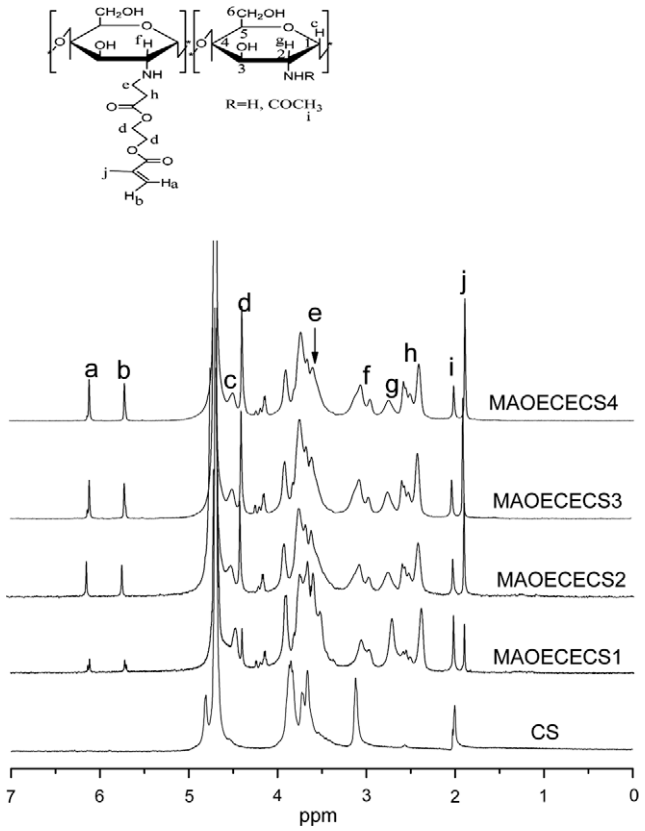

Fig. 2. ^{1}H NMR spectra of MAOECECSs in D_2O and CS in CD_3COOD/D_2O.

2.0 ppm: DS = $0.15*I_{1.9}/I_{2.0}$. By calculation, DS increased from 0.10 to 0.35 when mole ratio of acrylate group in EGAMA to $-NH_2$ of chitosan. Fig. 2 also shows there is no peak between 4.90 and 5.15 ppm, indicating substitution onto amino group of chitosan was mono-substitution (Sashiwa, Shigemasa, & Roy, 2000; Sashiwa et al., 2003). Other peaks were attributed as follows. 4.50(c, H-1 of GlcNAc), 4.37 (d, $-COO-CH_2-CH_2OCO-$), 3.6–4.0(N–CH_2– of *N*-alkyl group, H-3,4,5,6 of GlcN and GlcNAc), 3.0(f, H-2 of GlcNAc), 2.8(g, H-2 of alkylated GlcN), 2.5(h, $-CH_2COO-$), 2.0(i, $-NHCOCH_3$).

^{13}C NMR was also performed on MAOECECS2 to confirm the Michael-addition reaction (Fig. 3). The carbonyl carbon of the ester bond and the carbon of the methyl substituent in the methacrylate group were seen at 175 and 13 ppm while the carbons in the double bond ($C{=}CH_2$) were found at 136 and 127 ppm, respectively. Other peaks of MAOECECS2 were assigned in the region between 0 and 100 ppm by comparison with the spectrum recorded for chitosan: 100(C1), 77.7(C4), 75.1(C5), 72.0(C3), 63.0(e,f, $-OCH_2-CH_2O-$), 60.5(C6), 55.5(C2), 44.9(i, $-NHCH_2-$), 34.9(h, $-CH_2COO-$), and 17.4(j, $NHCOCH_3$). No peak was located around 65 ppm where the methacryloyl group is substituted, implying that the methacryloyl group is not substituted at the 6-*O* position of the polymer chain. However, the peak at 44.9 ppm due to the substitution of the methacryloyl groups located at the 2-*N* position implies that substitutions just occurred at the amino groups.

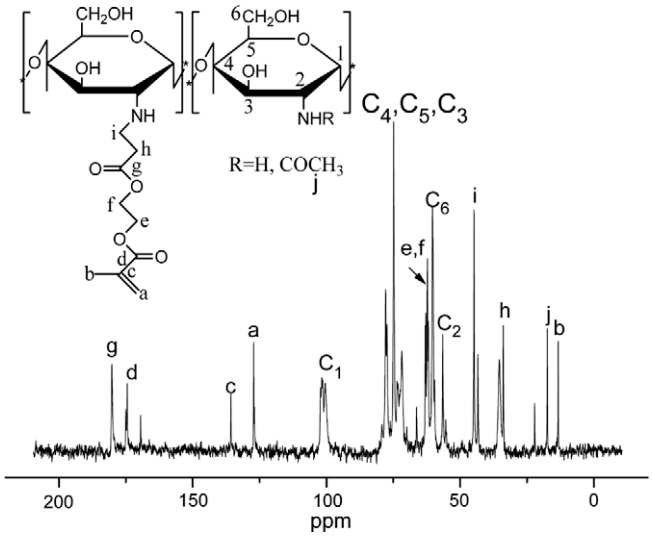

Fig. 3. ^{13}C NMR spectrum of MAOECECS2 in D_2O.

3.2. Thermogravimetric analysis (TGA)

Thermographs of chitosan and MAOECECS with different DS are shown in Fig. 4. The chitosan shows obvious loss of weight starting from 280 to 330 °C and maximum thermal decomposition at 305 °C, which could be attributed to a complex process including dehydration of the saccharide rings, depolymerization and decomposition of the acetylated and deacetylated units of the polymer (Peniche-Covas, Argüelles-Monal, & San Román, 1993). It could be seen that, a slow process of weight loss appeared in the TG spectra of MAOECECS starting from 180 to 220 °C, which was attributed to the abolition of ester groups, similar with the results of Yao (Yao, Zhang, Ping, & Yu, 2007). As observed in Fig. 5, all MAOECECSs show obvious loss of weight starting from 240 to 320 °C, lower than chitosan (280–330 °C). The initial thermal decomposition temperature of MAOECECS dropped from 271 to 249 °C with increasing of DS from 0.10 to 0.35. The results obtained from TGA curves indicate a decrease of thermal stability after reaction with EGAMA. Introduction of methacrylate groups into polysaccharide structure would disrupt the crystalline structure of chitosan, especially through the loss of the intra/intermolecular hydrogen bonding, resulting in reduced interaction forces compared to native chitosan.

3.3. XRD

Fig. 5 presents XRD patterns of CS and MAOECECS with different DS. It could be seen that CS powder exhibited three typical peaks at 2θ = 10.3°, 15.2° and 19.9°, agree to Samuels' observation (Samuels, 1981). According to Samuels, the reflection falling at 2θ = 10.3° was

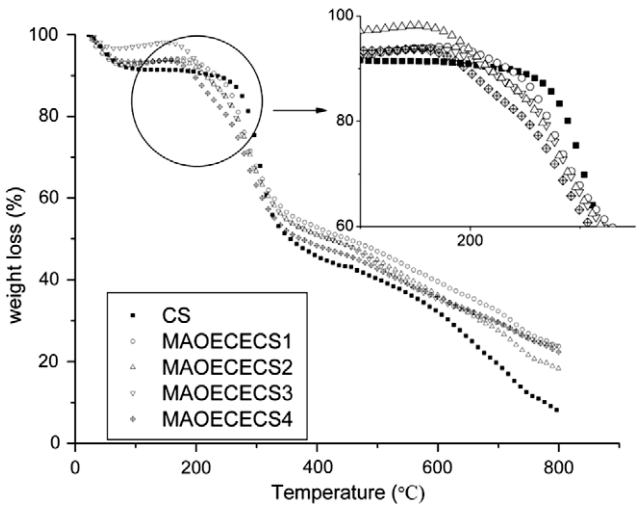

Fig. 4. TGA curves of CS and MAOECECS with different DS.

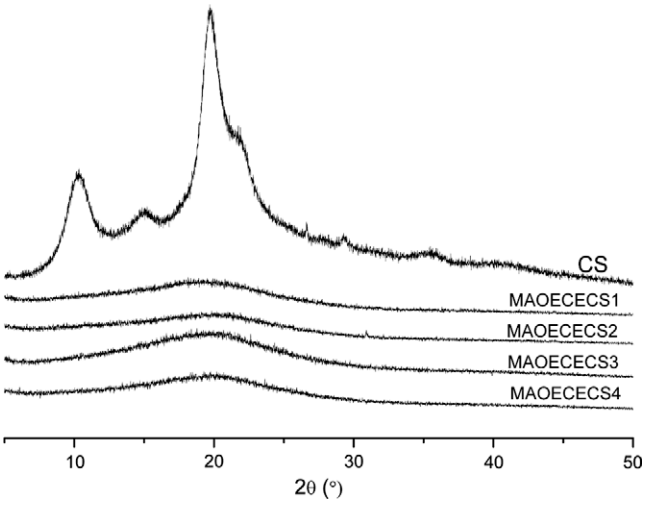

Fig. 5. XRD patterns of CS and MAOECECS with different DS.

assigned to crystal form I. The strongest falling at $2\theta = 19.9°$ corresponded to crystal form II. However, for all MAOECECSs, the crystalline peak at $2\theta = 10.3°$ and 15.2° disappeared and strong peak at $2\theta = 19.9°$ became a relative obtuse and broad. It suggested that the large number of hydrogen bonding in the CS powder was destroyed through the *N*-acylation, thus forming a smaller fraction of crystalline phase and a larger fraction of amorphous phase.

3.4. MTT assay

An ideal biomedical material should not release toxic products or produce adverse reactions, which could be evaluated through in vitro cytotoxic tests. The cytotoxicity of the MAOECECS with different concentration was assessed by using Vero cells line as reference (Fig. 6). It could be seen that, no statistically significant differences were observed in the cell activity of Vero cells culture for 48 h in the presence of MAOECECS culture solution with a concentration up to 1 mg/mL in comparison with negative control (NC), although the average absorbance values were lower than that of the control condition. However, when a concentration of MAOECECS was up to 2.5 mg/mL, statistically significant differences ($p < 0.05$) were observed in the cell activity in comparison with control. The obtained results clearly suggest that MAOECECS is non-toxic to Vero cells at the low concentration.

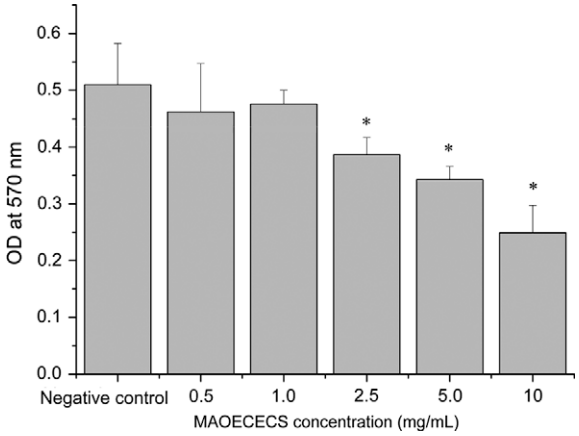

Fig. 6. In vitro cytotoxicity of MAOECECS2 towards Vero cells as a function of MAOECECS concentration. $^{*}p < 0.05$ when compared to the negative control of indirect cytotoxicity. The data represented mean and standard deviations of six samples.

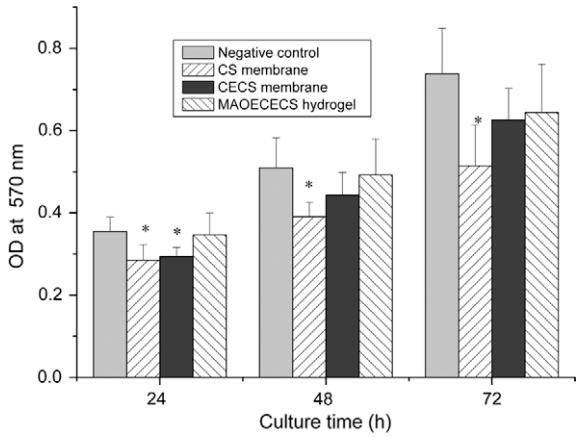

Fig. 7. Cytotoxicity test of CS, crosslinked CECS membrane, MAOECECS2 and negative controls ($p < 0.05$). $^{*}p < 0.05$ when compared to the negative control of indirect cytotoxicity. The data represented mean and standard deviations of six samples.

Injectable materials are advantageous in that they can be applied to tissue defects with irregular shapes and form tight interfaces with surrounding tissues. However, it is hard to remove unreacted residues after curing. Therefore, cytotoxicity of leachable products after cross-linking should be examined. Fig. 7 shows the absorbance obtained from an MTT assay of Vero cells which were cultured with the extraction medium from various types of specimens in comparison with control. It could be seen that, no statistically significant differences ($p < 0.05$) were observed in the cell activity of Vero culture within 72 h in the presence of MAOECECS hydrogels extracts in comparison with control, although the average absorbance values were lower than that of the control condition. However, when crosslinked carboxyethyl chitosan (CECS) membrane or CS membrane extract was used, statistically significant differences ($p < 0.05$) were observed in the cell activity in comparison with control within 72 h, but the viability of the cell still reached 80% of the negative control. This indicates that the crosslinked CECS membrane or CS membrane were less toxic to Vero cells. A similar result was observed in the CS membrane when culture time was 48, 72 h. The obtained results clearly suggest that MAOECECS hydrogel is non-toxic to Vero cells and are good candidates to be used as injectable materials.

4. Conclusions

Chitosan can be reacted with ethylene glycol acrylate methacrylate to yield a water-soluble, photopolymerizable precursor MAOECECS that can be used to prepare hydrogels. The degree of DS can be readily manipulated by adjusting the molar excess of EGAMA to chitosan reactive amine ratio and the reaction time. The MAOECECS shows noncytotoxicity toward Vero cells. Moreover, the gels prepared through photopolymerization of their precursors did not possess unreacted C=C and supported the growth of Vero cells. MAOECECS thus appears to be a promising biomaterial for use in tissue engineering.

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