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Preparation and characterization of water-soluble *N*-alkylated chitosan

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Abstract

N-Alkylated chitosan derivative was synthesized by Michael addition reaction of chitosan and hydroxyethylacryl. The chemical structure and physical properties of the chitosan derivatives were characterized by FT-IR, ^{1}H NMR, XRD and DSC techniques. The ^{1}H NMR results indicated that the degree of substitution (DS) was from 0.18 to 1.2. The chitosan derivatives exhibited an excellent solubility in distilled water. XRD analysis showed that the derivatives were amorphous. DSC results demonstrated that thermal stability of the derivatives was lower than that of chitosan and it decomposed around 226 °C for hydroxyethylacryl-CS with DS higher than 1.05. The lysozyme enzymatic degradation results revealed that the chitosan derivatives had the ability to enzymatic degradation and the initial degradation rate of the chitosan derivatives was dependent on its molecular weight. The antimicrobial activity of the chitosan derivatives was decreased compared with that of chitosan, but it still had the ability of antimicrobial.

Keywords: Chitosan; Michael addition reaction; Enzymatic degradation; Antimicrobial activity; Hydroxyethylacrylate

1. Introduction

Chitosan is a polysaccharide consisting of β-(1,4)-2-amino-2-deoxy-D-glucopyranose units (GlcN) including a small amount of *N*-acetyl-D-glucosamine (GlcNAc) residues. Chitosan is one of the most important partially deacetylated derivatives obtained from chitin which is the second most abundant natural polysaccharide next to cellulose. It is an attractive material and utilized in a number of biomedical applications, including gene delivery (Danielsen, Varum, & Stokke, 2004; Kim et al., 2007), tissue engineering (Gobin, Froude, & Mathur, 2005), drug delivery systems (Shimono et al., 2002) and wound dressings (Peshkova & Li, 2003) because of its biological properties such as nontoxic, biocompatible (VandeVord et al., 2002) and biodegradable (Onishi & Machida, 1999). Although chitosan is an attractive biomacromolecule, it is normally insoluble in aqueous solutions above pH 7 because of its rigid crystalline structure and the deacetylation which limits its application. However, it is especially interesting in potential due to the presence of amino groups which may be modified by controlled chemical reactions. In an attempt to improve the water solubility of chitosan, many efforts have been made to introduce hydrophilic groups by covalent attachment to reactive amino groups at the C_2 position. Various kinds of modification of chitosan have been investigated in recent years and examples reported using acylation (Naoji, Tatsumoto, Sano, & Toya, 2000), alkylation (Kang et al., 2006; Lim & Hudson, 2004), carboxymethylation (Park, Cho, Chung, Kwon, & Jeong, 2003) and quaternarization (Huang, Chen, Sun, Hu, & Gao, 2007; Murata, Ouchi, & Ohya, 1996; Sashiwa et al., 2003). Many efforts to prepare functional derivatives by Michael addition reaction chemical modifications to increase the solubility in water have been reported (Sashiwa, Yamamori, Ichinose, Sunamoto, & Aiba, 2003a, 2003b).

In order to improve the solubility of chitosan in water, chemical modification of chitosan is important and necessary. In this paper, a chitosan derivative was synthesized

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by Michael addition reaction of chitosan and Hydroxyethylacrylate in moderate reaction conditions.

2. Experimental

2.1. Materials

Chitosan from crab shells was purchased from Yuhuan Ocean Biochemical Co. Ltd. (Zhejiang, China) and used as received. According to the company analysis, its molecular weight was 200 kDa and its degree of deacetylation was about 88.0%. Hydroxyethylacrylate was purified by vacuum distillation. All the other reagents and solvents were purchased from Beijing Chemical Reagent Company and used without further purification.

2.2. Synthesis of hydroxyethylacryl-chitosan

Chitosan (2.0 g) was placed into a solution of acetic acid (2.0 g) in distilled water (200 g), and hydroxyethylacrylate (3.6 g) was added with stirring. This mixture was reacted at 50 °C for 48 h then filtered. The solution was poured into 400 mL of acetone to remove the unreacted hydroxyethylacryl. The precipitate was washed three times with acetone then dried under vacuum at 30 °C overnight to obtain hydroxyethylacryl-chitosan.

2.3. Solubility test

The solubility of hydroxyethyacryl-chitosan in distilled water was evaluated at a concentration of 5 mg/mL under different temperatures of 20 °C, 35 °C, 50 °C and 75 °C for 6 h.

2.4. FT-IR spectroscopy

Fourier transform infrared (FT-IR) spectrum was recorded on Nicolet 5700 instrument (Nicolet Instrument, Thermo Company, Madison, USA). Samples were prepared as KBr pellet and were scanned against a blank KBr pellet background at wavenumber range 4000–600 cm^{-1} with resolution of 4.0 cm^{-1}.

2.5. ^1H NMR spectroscopy

^1H NMR spectra were carried out on a Bruker AV600 MHz (Bruker, Rneinstetten, Germany). Chitosan was dissolved in a mixed solvent of D_3CCOOD and D_2O, and hydroxyethylacryl-chitosan was dissolved in D_2O. Degrees of substitution (DS) was calculated from the peak area at about 2.87 ppm of $–CH_{2b}–$ proton against 2.06 ppm of NHAc proton.

2.6. X-ray Diffraction (XRD)

X-ray diffraction (XRD) patterns of the sheet samples was recorded on an X-ray diffractometer (D/Max2500VB2+/Pc, Rigaku Company, Tokyo, Japan) with area detector operating at a voltage of 40 kV and a current of 50 mA using Cu Ka radiation ($\lambda = 0.154$ nm). The scanning rate was 1°/min and the scanning scope of 2θ was from 5° to 50° at room temperature.

2.7. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) measurements was performed with a TA-Q100 instrument (TA Company, Kansas, USA), heated from 25 to 100 °C, cooled to −100 °C, and then heated from −100 to 250 °C at a heating rate of 10 °C /min^{-1}. The open aluminium cell was swept with N_2 at 20 mL/min during the analysis.

2.8. Enzymatic degradation

The enzymatic degradation of the chitosan derivatives was studied by decreasing in viscosity of the polymer solution in the presence of lysozyme. The hydroxyethylacryl-chitosan dissolved at 0.1 M sodium phosphate buffer at pH 7.4 (2.5 mg/mL) and 5 mL lysozyme solution was added in. The mixed solution of weight ratio of lysozyme/hydroxyethylacryl-chitosan = 0.065 and then incubated at 37 °C in a shaker. The specific viscosity of the samples was measured at 37 °C and the degradation of hydroxyethylacryl-chitosan was expressed in terms of relative specific viscosity difference between the degraded and original sample, according to the following formula:

$$\eta_{sp} = \frac{(t - t_0)}{t_0}$$

where t and t_0 were the eluent time of hydroxyethylacryl-chitosan and 0.1 M phosphate buffer solvent solution, respectively (Hu, Jiang, Xu, Wang, & Zhu, 2005; Zhang & Neau, 2001).

2.9. Antimicrobial test

Antibacterial activity of chitosan and chitosan derivatives against *Escherichia coli* (*E. coli*) was evaluated as follows: the *Escherichia coli* (ATCC 25922) were obtained from American Type Culture Collection (ATCC) Manassas, VA, USA. Firstly, a nutrient liquid medium was prepared consisted of 2.00 g peptone, 1.80 g broth and 100.00 g distilled water. A representative bacteria colony was picked off, inoculated into it, and incubated in air bath shaker (37 °C, 130 rpm) for 12 h. Then the obtained fresh microbe suspension in which the bacteria cells grew luxuriantly was ready for antibacterial test. Lastly, 1.00 g hydroxyethylacryl-chitosan were dissolved in 100 mL acetic acid solution (0.5 wt%). A volume of 0.25 mL solution of microbe suspension and 0.25 mL solution of hydroxyethylacryl-chitosan were orderly added into the petri dishes, and both of them mixed and spread in shaker uniformly. All the petri dishes were incubated at 37 °C for 24 h and take out for photograph.

3. Results and discussion

3.1. DS and solubility of hydroxyethylacryl-chitosan

Table 1 listed the DS and solubility of hydroxyethylacryl-chitosan in distilled water. The results showed that increased reaction temperature increased the DS of hydroxyethylacryl-chitosan. The amino group on the monosaccharide structure of the chitosan could be alkylated, and DS would exceed one when the amino group mostly be alkylated by the Michael addition reaction of chitosan and hydroxyethylacryl. Increase the reaction time increased to the DS, as did of the hydroxyethylacryl to chitosan ratio. Although chitosan does not dissolve in water, conversion to hydroxyethylacryl-chitosan facilitates increasing solubility with increase in DS. When the DS was 0.18, hydroxyethylacryl-chitosan only dissolved in distilled water at 70 °C completely. As the DS increased to 0.38, swelled at 35 °C and dissolved in distilled water at 50 °C completely. On further increase in the DS to 0.65, hydroxyethylacryl-chitosan started to completely dissolve in distilled water at 35 °C. Hydroxyethylacryl-chitosan could be dissolved in distilled water at 20 °C completely when the DS was higher than 0.90. These phenomena could be interpreted in that the random distribution of hydroxyethylacryl residue disturbs the formation of the ordered structure and the hydrogen bonding among the amino groups of chitosan. Furthermore, the presence of hydroxyethylacryl residues which are hydrophilic groups changes the solubility of chitosan in water significantly.

3.2. FT-IR analysis

Fig. 1 showed the FT-IR spectra of chitosan and hydroxyethylacryl-chitosan with different DS. The broad band at around 3443 cm^{-1} attributed to –NH and –OH stretching vibration, as well as inter- and extra-molecular hydrogen bonding of chitosan molecules. The weak band at 2926 cm^{-1} attributed to –CH– stretch chitosan. The characteristic peaks at 1654, 1597 and 1323 cm^{-1} assigned to the amide one, the amine $–NH_2$ and amide three absorption band of chitosan, respectively. The absorption band at 1153 cm^{-1} was the asymmetric stretching of the C–O–C bridge. Bands at 1075and 1033 cm^{-1} assigned to the skeletal vibration of C–O stretching. Meanwhile, it was worth noting that the peak at 1597 cm^{-1} for primary amine $–NH_2$ bending disappeared or decreased in hydroxyethylacryl-chitosan. Compared with chitosan, hydroxyethylacryl-chitosan showed that absorption peaks became strong at 1726, 1580 and 1405 cm^{-1} due to the asymmetrical and symmetrical stretching of the COO– groups which was brought by the by Michael addition reaction of chitosan and hydroxyethylacryl. The relative intensities of the absorbance of the COO– group (1726 cm^{-1}) depended upon the DS value of hydroxyethylacryl-chitosan. The FT-IR spectrum results were in agreement with the reported result (Aoi et al., 2000).

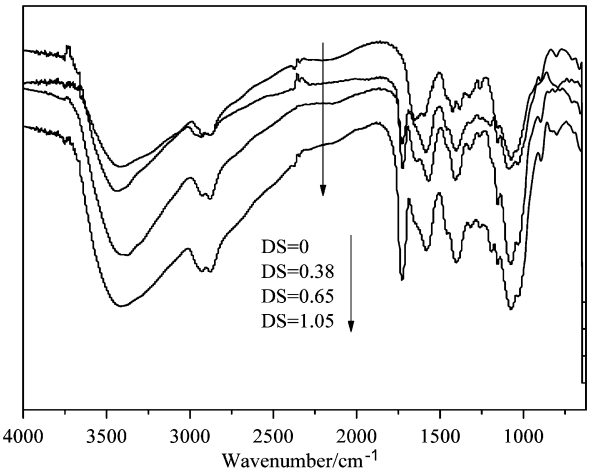

Fig. 1. FT-IR spectra of chitosan and hydroxyethylacryl-chitosan.

Table 1
DS and solubility of hydroxyethylacryl-chitosan at different temperature

Sample	Reaction conditions	Solubility				DS
		20 °C	35 °C	50 °C	70 °C	
No. 1		–	–	–	–	0.00
No. 2	50 °C/1:4/48 h	–	+	+	+	0.65
No. 3	60 °C/1:4/48 h	+	+	+	+	1.05
No. 4	70 °C/1:4/48 h	+	+	+	+	1.12
No. 5	50 °C/1:4/48 h	+	+	+	+	0.90
No. 6	60 °C/1:4/24 h	–	±	+	+	0.38
No. 7	60 °C/1:4/72 h	+	+	+	+	1.20
No. 8	50 °C/1:2/48 h	–	–	±	+	0.18

+, Soluble; ±, partially soluble or swell; –, insoluble.
Reaction conditions: the temperature of the reaction, the ratio of $–NH_2$ of CS/HEA, and the time of the reaction.

3.3. ^1H NMR analysis

The typical ^1H NMR spectrum of the chitosan in CD_3COOD/D_2O and hydroxyethylacryl-chitosan in D_2O were shown in Figs. 2 and 3. A small peak at 2.03 ppm existed because of the presence of $–CH_3$ of *N*-alkylated GlcN residue. A singlet at 3.10 ppm assigned to H_2 of GlcN and *N*-alkylated GlcN, the multiplets from 3.5 to 3.8 ppm attributed to $N–CH_{2a}–$ and $–CH_{2c}–$ of *N*-alkyl group, H_3, H_4, H_5, H_6 of GlcN and *N*-alkylated GlcN. A small peak at 4.87 ppm was observed because of H_1 of GlcN. A signal of newly formed methylene protons adjacent to the 2-amino group of the GlcN residue was observed at 2.87 ppm ($–CH_{2b}–COO–$). A singlet at 3.10 ppm assigned to H_2 of chitosan in Fig. 2, but the H_2 proton signal was also shifted from 3.10 ppm (Fig. 2) to 3.20 ppm which attributed to H_2 of hydroxyethylacryl-chitosan (Fig. 3) after modification. Furthermore, the typical peak at 4.29 ppm newly appeared because of $–CH_{2d}$ of GlcN residue. Additionally, the H_2 proton signal was also shifted from 3.10 to 3.20 ppm, and the H_1 proton signal

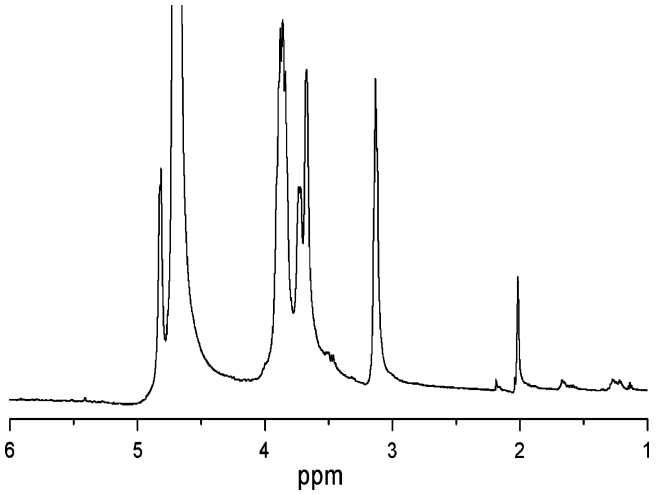

Fig. 2. Typical ^{1}H NMR spectra of chitosan.

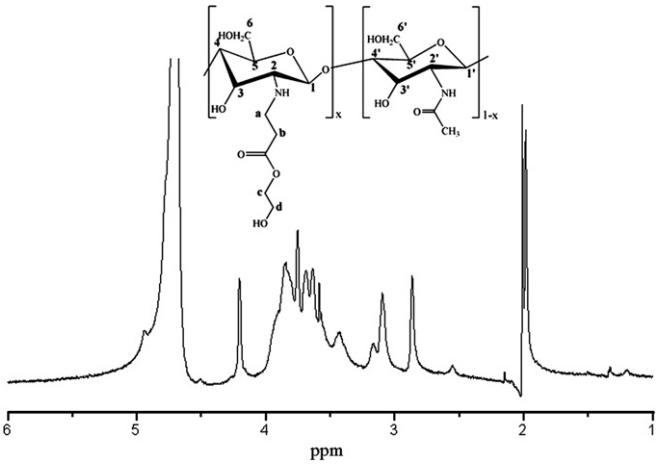

Fig. 3. Typical ^{1}H NMR spectra of hydroxyethylacryl-chitosan.

attributed to the GlcN residue was shifted from 4.87 to 4.94 ppm. These shifts were due to the substitution of the *N*-alkyl group to the amino group of the GlcN residue. The DS values based on –CH_{2b}–COO– were determined by the relative intensities of these signals and were calculated from the peak area at 2.87 ppm of CH_{2b}-proton against 2.06 ppm of NHAc proton (Sashiwa et al., 2003, 2003a, 2003b). The calculated degree of substitution data are shown in Table 1.

3.4. X-ray diffraction (XRD)

Fig. 4 illustrated the XRD patterns of chitosan and hydroxyethylacryl-chitosan with different degrees of substitution. The chitosan showed three characteristic peaks around $2\theta = 10.3°$, 15.9° and 20.1°, indicating the high degree of crystallinity of chitosan as per the previous reports (Samuels, 1981; Zhang, Du, Yu, & Zhang, 2001). The reflection fall at $2\theta = 10.3°$ assigned to crystal forms I and strongest reflection appeared at $2\theta = 20.1°$ corresponding to crystal forms II. The XRD pattern of hydroxyethylacryl-chitosan was significantly different from the crystalline structure of chitosan, hydroxyethylacryl-chitosan had only one broad peak of 2θ about 20°. The lower crystallinity of hydroxyethylacryl-chitosan ascribed to the presence of hydroxyethylacryl residue, might have hindered the formation of inter- and extra-molecular hydrogen bonds after chemical modification and the hydroxyethylacryl-chitosan could be amorphous. The results also indicated that there was no connection between the crystallinity of hydroxyethylacryl-chitosan and the value of DS.

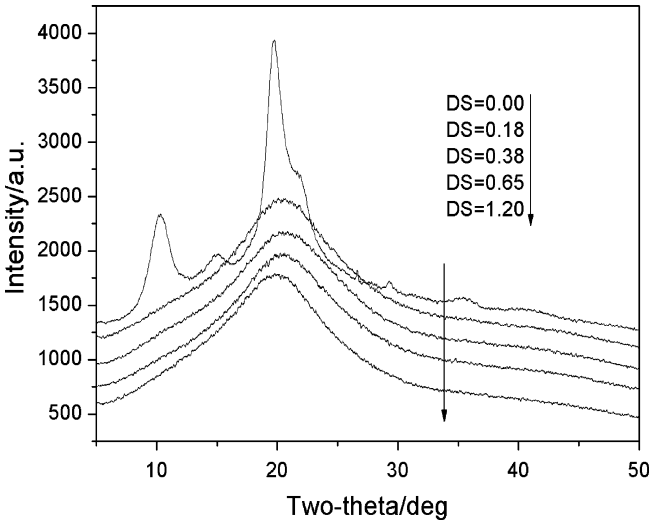

Fig. 4. XRD pattern of chitosan and hydroxyethylacryl-chitosan.

3.5. Differential scanning calorimetry (DSC)

DSC thermograms of chitosan and hydroxyethylacryl-chitosan were shown in Fig. 5. There were no obvious glass transition observed for chitosan and hydroxyethylacryl-chitosan. The chitosan and hydroxyethylacryl-chitosan (DS = 0.18) were thermal stable, but hydroxyethylacryl-chitosan (DS = 1.05, 1.12) were unstable above 220 °C,

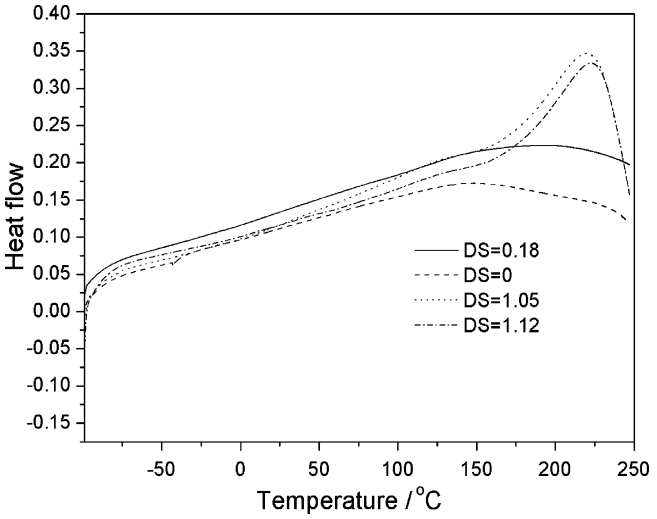

Fig. 5. DSC thermograms of chitosan and hydroxyethylacryl-chitosan.

and the exothermic peaks of hydroxyethylacryl-chitosan (DS = 1.05, 1.12) at 226 °C and 223 °C corresponded to their thermal decompositions, respectively. Compared to chitosan, the introduction of substitution groups into chitosan molecule decreased thermal stability and degree of order. This alteration caused thermal instability of the hydroxyethylacryl-chitosan because of the increasing chain mobility of hydroxyethylacryl-chitosan. This could be validated by the results that the decomposition peak of hydroxyethylacryl-chitosan (DS = 1.12) was lower than that of hydroxyethylacryl-chitosan (DS = 1.05). These evidences indicated that the thermal stability could be decreased by introduction of substituent of hydroxyethylacryl groups which could disrupt the crystalline structure of chitosan, especially through the loss of the hydrogen bonding (Zong et al., 2000).

3.6. Enzymatic degradation of hydroxyethylacryl-chitosan

Chitosan and its derivates are considered as a biodegradable polymer because of their (potential) susceptibilities to various enzymes. Lysozyme is known to be ubiquitous in the body and to produce the results correlated with in vivo degradation (Cho, Cho, Chung, Yoo, & Ko, 1999; Tomihata & Ikada, 1997). Fig. 6 shows the enzymatic degradation of hydroxyethylacryl-chitosan in aqueous neutral medium by lysozyme. According to others (Li, Du, & Liang, 2007), enzymes attack the polysaccharides by two means of apparently random splitting of interior glycosidic bonds (endo-action) and cleaving a susceptible glycosidic bond situated at a terminal residue in a chain (exo-action). The endo-action results in decrease of the molecular weight of the polysaccharide during the enzymatic degradation, but exo-action did not. The results showed that the solutions of hydroxyethylacryl-chitosan exhibited higher enzymatic degradability during the initial 12 h. This indicated that the enzymatic hydrolysis was an endo-action which brought on the decrease in the molecular weight of the polysaccharide. Whereas, no significant enzymatic degradability was observed after 15 h, this could be that the enzyme activity was inhibited by the end products. In addition, the degradation process was slowed down by more substrates generated by endo-type depolymerization.

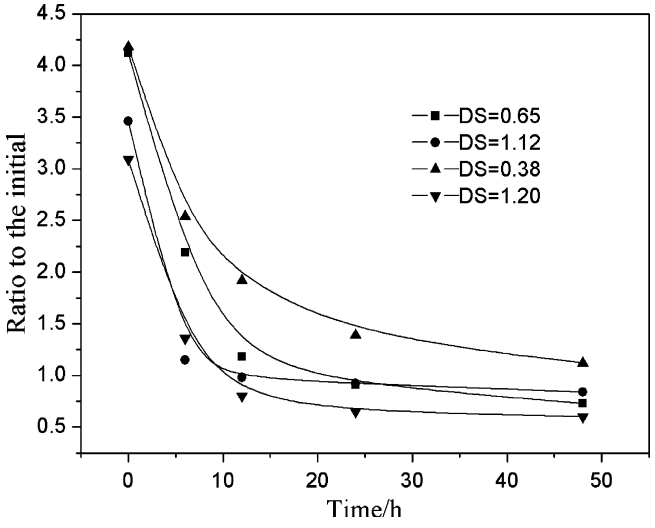

Fig. 6. Enzymatic degradation profiles of hydroxyethylacryl-chitosan in 0.1 M PBS at pH 7.4 at 37 °C by lysozyme.

3.7. Antimicrobial analysis

Fig. 7 shows the antimicrobial activity of hydroxyethylacryl-chitosan and chitosan. In order to eliminate any effect of acetic acid on antimicrobial activity, all examples containing acetic acid were referenced against a suitable blank. The results showed that the antimicrobial activity of chitosan was higher than that of hydroxyethylacryl-chitosan, and acetic acid had only a slight effect on antimicrobial activity. The hydroxyethylacryl-chitosan itself had no significant antimicrobial activity on *E. coli* and the higher of DS of the hydroxyethylacryl-chitosan the lower of its antimicrobial activity. Chitosan is electropositive because of its amino groups at the C_2 position, it adsorbed the electronegative substance in the cell, which could agglomerate them, on the other hand, it also formed a polymer membrane, which could prevent the transport of essential nutrients entering the cell (Qin et al., 2006; Zheng & Zhu, 2003). So the physiological activities of the bacteria were destroyed by it, which resulted in severe leakage of cell constituents and eventually the cell death. The concentration of amino groups at the C_2 position of hydroxyethylacryl-chitosan were reduced and so the antimicrobial activity of hydroxyethylacryl-chitosan was weak compared to chitosan.

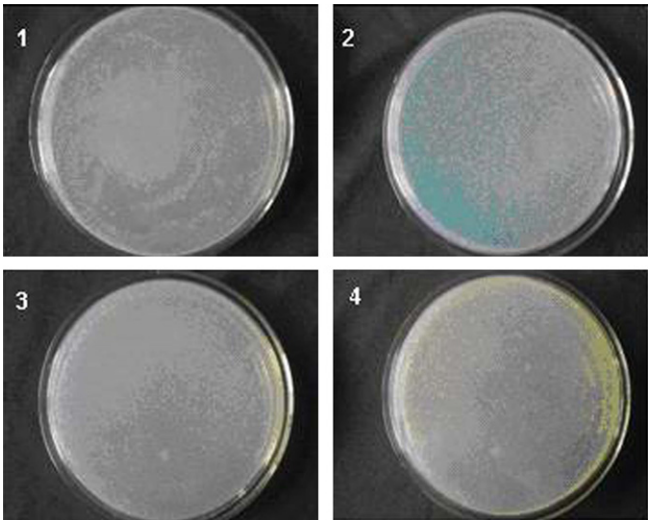

Fig. 7. Images of antimicrobial activity to *E. coli* (1) blank (2) 1% chitosan + 0.5% acetic acid (3) 1% hydroxyethylacryl-chitosan (DS = 0.38) + 0.5% acetic acid (4) 1% hydroxyethylacryl-chitosan (DS = 0.90) + 0.5% acetic acid.

4. Conclusions

Hydroxyethylacryl-chitosan with different degrees of substitution from 0.18 to 1.2 was preparable by Michael addition reaction of chitosan and hydroxyethylacrylate. The structure was defined by FT-IR, ^{1}H NMR and XRD. Hydroxyethylacryl-chitosan exhibited excellent solubility in distilled water. The thermal stability of hydroxyethylacryl-chitosan was lower than that of chitosan. Hydroxyethylacryl-chitosan showed strong lysozyme degradation and the degradation of hydroxyethylacryl-chitosan at the initial time depended on its molecular weight. The antimicrobial activity of hydroxyethylacryl-chitosan was low. This derivatisation procedure would be useful for the novel chemical modification to prepare water-soluble and biodegradable chitosan derivatives.

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