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Synthesis, characterization and antibacterial activity of salicyloyl chitosan

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ABSTRACT

Salicyloyl chitosan (SCS) with different degrees of substitution (DS) and molecular weight (MW) were synthesized from chitosan and salicylic acid. Their structures were characterized by UV, FTIR, ^{1}H NMR and XRD, which showed that the acylate reaction took place at the N-position of chitosan. The DS value of SCS ranged from 0.10 to 0.71. The antibacterial activities were assessed according to an inhibition zone method against *Escherichia coli* and *Staphylococcus aureus*. The antibacterial results indicated that the antibacterial activity of SCS was strengthened than chitosan with the increase of DS for both *E. coli* and *S. aureus*. As a SCS solvent, the antibacterial activity of the formic acid was most effective followed by acetic acid and lactic acid. In addition, the SCS with higher MW was more effective in inhibiting bacterial growth.

1. Introduction

During the past few decades, increasing attention has been paid to the potential uses of macromolecule in the field of antibacterial materials, including tissue regeneration, antibacterial agents and food preservation (Duan et al., 2006). Also the studies have been made on the utilization of macromolecule materials to antibacterial ones. One of them is immobilization of water-soluble, emulsible, or suspendible disinfectants onto macromolecular material surfaces. Another is the introduction of antibacterial active groups to monomers, followed by their polymerization (Aider, 2010; Rhoades & Roller, 2000).

Chitosan, a linear polysaccharide consists of β-(1 → 4)-2-amino-2-deoxy-D-glucan, is a deacetylated derivative of chitin, which is the second most abundant polysaccharide found in nature after cellulose (Zhang & Neau, 2001). Chitosan has been found to be non-toxic, biodegradable, biofunctional, biocompatible in addition to having antimicrobial characteristics (Ben-Shalom, Ardi, Pinto, Aki, & Fallik, 2003; Jayakumar, Nwe, Tokura, & Tamura, 2007; Roller & Covill, 1999). Chitosan has a bacterial effect on both Gram-positive bacteria and Gram-negative bacteria (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Therefore, it has been used as film-forming agent and additive to improve the shelf life of food product (Devlieghere, Vermeulen, & Debevere, 2004). However, the antibacterial activity of chitosan requires further improvement by chemical modification.

Salicylic acid is a kind of effective antibacterial agent with low toxicity, which is widely used in medical field, etc. The antibacterial activity of salicylic acid may be attributed to its phenolic structure. It is reported that the effect of phenolic compounds are dependent on the concentration (Juven, Kanner, Schved, & Weisslowicz, 1994). At low concentrations, phenols affect enzyme activity, while they can make protein denaturation at high concentrations. The antimicrobial effect may be due to their abilities to alter microbial cell permeability, thereby permitting the loss of macromolecules from the interior such as ribose and Na glutamate (Bajpai, Rahman, Dung, Huh, & Kang, 2008). They can also disturb membrane function (electron transport, nutrient uptake and nucleic acid synthesis, etc.) and interact with membrane proteins, leading to deformation in structure and functionality (Helander et al., 1998; Tiwari et al., 2009).

Chemical modification of chitosan by introducing salicylic acid into the polymer backbone may render excellent antibacterial activity. At present, there is little literature reported about salicyloyl chitosan (SCS). In this study, SCS was synthesized and characterized by UV, FTIR, ^{1}H NMR and XRD. The antibacterial activities against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) were investigated by an inhibition zone method. The structure activity relationship of SCS for the antibacterial effect was also discussed.

2. Experimental

2.1. Materials

Chitosan (deacetylation degree of 90.0%) with different molecular weight were purchased from Golden-shell Biochemical Corp. Ltd. (Zhejiang, China). Salicylic acid was purchased from Sinopharm

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Table 1
Parameters of chitosan and salicyloyl chitosan samples.

Sample	Molar ratio	Temperature (°C)	DS
CS	–	–	0
SCS1	2:1	30	0.10
SCS2	2:1	40	0.18
SCS3	2:1	50	0.33
SCS4	2:1	60	0.43
SCS5	2:1	70	0.56
SCS6	2:1	90	0.71

Chemical Reagent Co., Ltd. All other reagents were of analytical grade and were used without further purification. *E. coli* and *S. aureus* were provided by the microbe laboratory of College of Chemical Engineering, Wuhan University of Technology.

2.2. Synthesis of salicyloyl chitosan

1 g of dry chitosan (MW = 650 kDa) was dispersed in DMF, and 1.71 g of salicylic acid (dissolved in DMF) was added to the reaction system, the mixture was stirred at 30–90 °C for 2 h under a nitrogen atmosphere. Finally, the whole mixture was filtered and solid products were purified by Soxhlet extraction. After dried under vacuum until a constant weight, yellowish powder product was obtained (Wu & Hu, 2006). The molar ratio of salicylic acid to chitosan was fixed (2:1), and by changing the reaction temperatures, a series of SCS with different degrees of substitution were prepared. The SCS with different MW were obtained by altering the starting MW (1350 kDa, 150 kDa, 80 kDa, 10 kDa) of the chitosan.

2.3. Characterization of salicyloyl chitosan

UV spectra were recorded on an instrument (Nicolet 5700, Thermo Company, Madison, Japan) under scan mode in the range of 200–400 nm. Salicylic acid was dissolved in deionized water, with little ethanol to help dissolve. SCS was dissolved in 0.5% (w/w) acetic acid at the concentration of 0.05 mg/mL.

Fourier transform infrared (FTIR) spectra were recorded on an instrument (Avator 360, Nicolet, Massachusetts, USA). Samples were prepared as KBr pellet and scanned against a blank KBr pellet background in the range of 500–4000 cm^{-1}.

The ^1H nuclear magnetic resonance (^1H NMR) spectra were determined on Varian 400 spectrometer (Varian, USA) at 400 MHz. Chitosan and SCS were dissolved in DCl/D_2O (1:100, v/v). Chemical shifts were given in ppm using tetramethylsilane (TMS) as an internal reference.

X-ray diffraction (XRD) patterns were recorded on a X-ray diffractometer (Rigaku Ultima III, Rigaku, Japan) with area detector operating at a voltage of 40 kV and a current of 50 mA, CuKα radiation. The scanning rate was 5°/min and the scanning scope of 2θ was from 5° to 50° at room temperature.

2.4. Determination of degrees of substitution (DS)

The weighing method was carried out to determine the DS of target product, and the formula for calculating the DS as:

$$DS = \frac{(W_1 - W_0)}{W_0} \times \frac{M_0}{M_1} \quad (1)$$

where W_0 is the weight of chitosan, W_1 is the weight of SCS product, M_0 is the molar mass of chitosan saccharide unit, and M_1 is the molar mass of salicyloyl group.

The results indicated that the degrees of substitution ranged from 0.10 to 0.71. The UV spectrophotometry method was also carried out to confirm the results (Hu, 2007). The DS of salicyloyl chitosan samples are shown in Table 1.

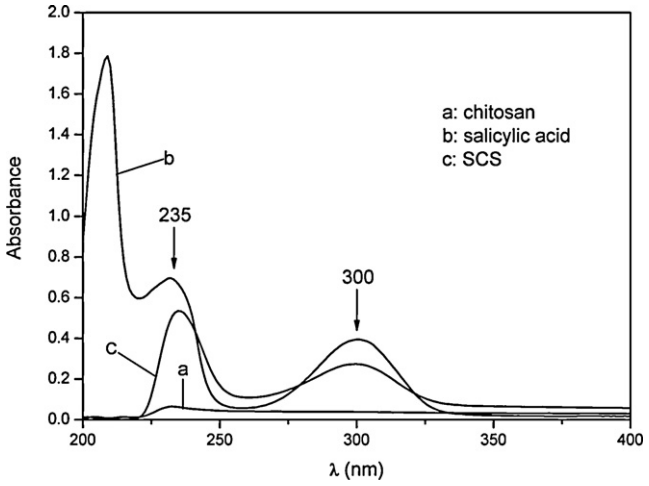

Fig. 1. UV spectra of (a) chitosan, (b) salicylic acid and (c) SCS.

2.5. Estimation of water-solubility

The pH dependence of water solubility of SCS was assessed by measuring its turbidity. 100 mg SCS was dissolved in 100 mL acetic acid (1%, w/w). Following stepwise addition of concentrated NaOH, the transmittance of the solution was recorded on a Nicolet 5700 instrument (Thermo Company, Madison, Japan) using a quartz cell with an optical path length of 1 cm at 600 nm (Qin et al., 2003).

2.6. Determination of antibacterial activity

E. coli and *S. aureus* were used as test organisms. A representative microbe colony was picked off with a wire loop, placed in nutrient broth, and then incubated in an air-bath shaker at 37 °C for 24 h. After appropriately diluting to ~10^7 CFU/mL, the cultures of *E. coli* and *S. aureus* were prepared and used for the antibacterial test.

The inhibition zone method was used for determining the antibacterial effects of SCS against *E. coli* and *S. aureus*. Stainless steel cylinders of uniform size (outside diameter = 7.8 mm, inside diameter = 6 mm, height = 10 mm) were placed on the surface of agar-covered plates, where the bacterial had been previously seeded. The cylinders were each filled with 200 μL samples solutions (0.2%, w/w). Then the plates were incubated at 37 °C for 24 h. The test disk displayed a zone of inhibition, or region where the bacterial species refused to propagate. The intensity of this inhibitory effect was determined by measuring the mean diameter of inhibition zone. The zone diameters of the growth inhibition were measured using a caliper. By changing the solvents (formic acid, acetic acid and lactic acid) at the concentration of 0.5% (w/w), the effect of solvents of SCS on the antibacterial activity against *E. coli* and *S. aureus* were also studied.

3. Results and discussion

3.1. Characterization of SCS

UV absorbance spectra of chitosan, salicylic acid and SCS are shown in Fig. 1. In the UV spectrum of salicylic acid, obvious absorption peaks appeared at 209, 235 and 300 nm. No obvious absorption peaks appeared in UV spectrum of chitosan, while in the UV spectrum of the SCS, broad absorption bands appeared at 235 and 300 nm, indicating that salicylic acid had been substituted onto the chitosan.

The FTIR spectra of chitosan and SCS are illustrated in Fig. 2. The FTIR spectrum of chitosan showed large and intense bands

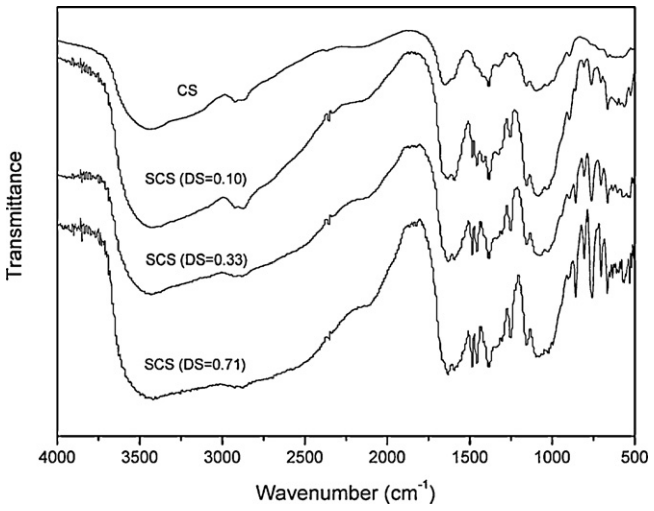

Fig. 2. FTIR spectra of chitosan and SCS with different DS.

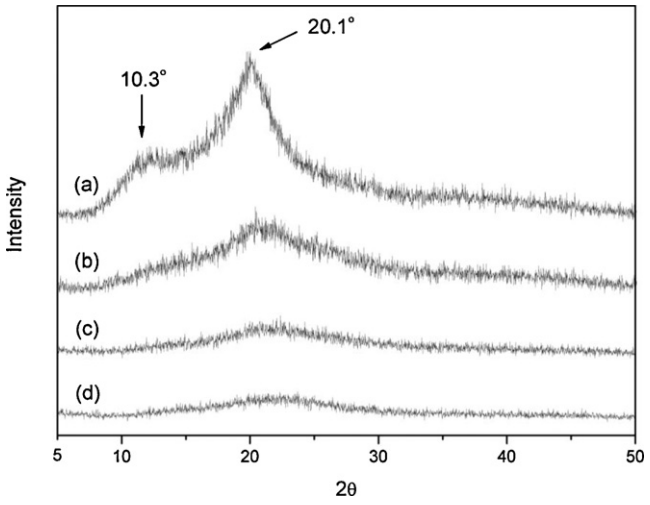

Fig. 4. XRD spectra of (a) chitosan and SCS: (b) DS = 33%, (c) DS = 56% and (d) DS = 71%.

at 3450–3200 cm^{-1} corresponding to the hydrogen-bonded O–H stretching overlapped with the several N–H stretching bands. The characteristic absorption bands appeared at 1657 and 1550 cm^{-1} attributed to the amide bands. The adsorption band at 1156 cm^{-1} was the adsorption of C–O–C stretching vibration in the glucopyranose ring and the band of at 895 cm^{-1} had been assigned to the β(1 → 4) glycoside bridge structure (Gomes, Gomes, Batista, Pinto, & Silva, 2008; Wang, Turhan, & Gunasekaran, 2004). In comparison with the chitosan FTIR spectrum, the new absorption bands at 1489 and 1450 cm^{-1} (C=C stretching vibration), 650–850 cm^{-1} (C–H deformation stretching) and 1255 cm^{-1} (C–O stretching vibration) were assigned to the phenolic structure of SCS. The peak at 1550 cm^{-1}(amide band) increased, which indicated that the acylate reaction took place at the N-position and –NH–CO– groups have been formed. The relative intensities of the absorbance of C=C, C–H, C–O and –NH–CO– groups depended upon the DS values of SCS.

The typical ^{1}H NMR spectra of chitosan and SCS in DCl/D_2O mixed solvent are shown in Fig. 3. The peak at 1.90 ppm existed because of the presence of $-CH_3$ of N-alkylated glucosamine residue. The typical signals of chitosan saccharide units at 3.06 (H2) and 3.51–3.82 (H3, H4, H5, H6) ppm did not change significantly after the reaction (Tian, Liu, Hu, & Zhao, 2004). New resonance signals appeared at 6.91–7.81 ppm represented the protons of hydroxybenzene, which confirmed the substituting of salicylic acid.

The XRD patterns of chitosan and SCS with different substituting degree are illustrated in Fig. 4. The chitosan showed two characteristic peaks around $2\theta = 10.3^\circ$ and $2\theta = 20.1^\circ$, indicating the high degree of crystalline of chitosan as the previous reports (Qin et al., 2003). The reflection appeared at $2\theta = 10.3^\circ$ was assigned to crystal forms I and strongest reflection appeared at $2\theta = 20.1^\circ$ corresponding to crystal forms II. For the SCS, the peak at $2\theta = 10.3^\circ$ disappeared and the peak at $2\theta = 20.1^\circ$ became much weaker and wider as the increase of DS. These changes suggested the N-substitution led to the decrease of the crystallization.

In view of UV, FTIR, ^{1}H NMR and XRD analysis of the chitosan derivatives, it could be concluded that the acylate reaction took place at the N-position of chitosan.

3.2. Effect of pH on solubility of SCS

Fig. 5 shows the pH dependence of transmittance of SCS solution. The SCS with DS value below 50% was soluble at pH < 6, which was the same as chitosan. While with the further increasing DS values, the solubility declined. When the DS reached 0.71, the solution became very turbid. And the solubility of SCS was better than chitosan at neutral and alkali pH.

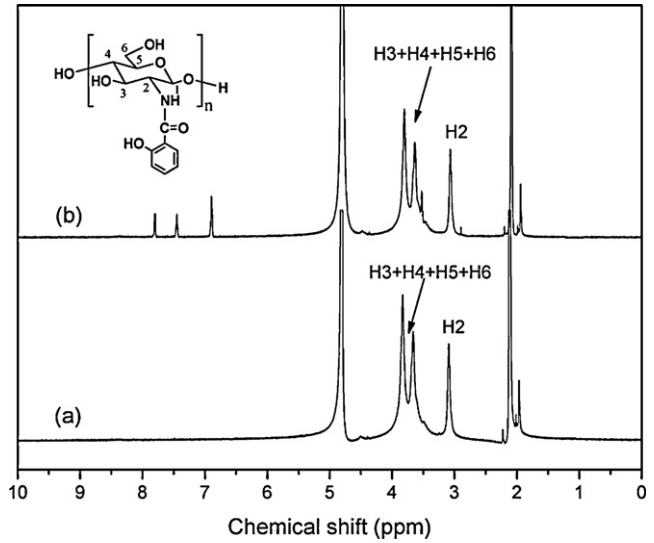

Fig. 3. ^{1}H NMR spectra of (a) chitosan and (b) SCS.

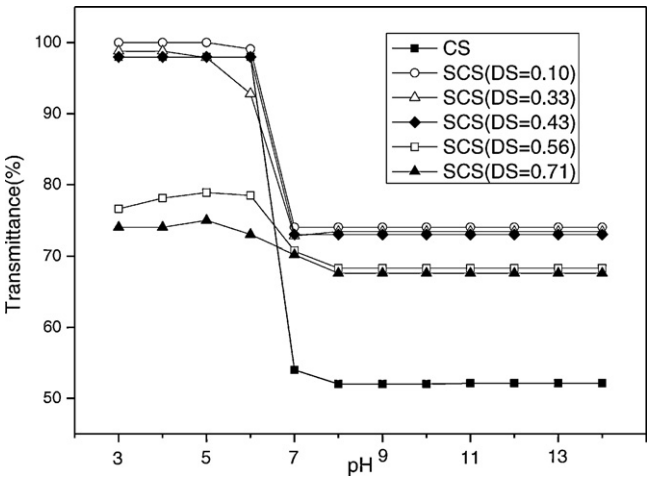

Fig. 5. pH dependence of solubility of SCS (MW = 650 kDa).

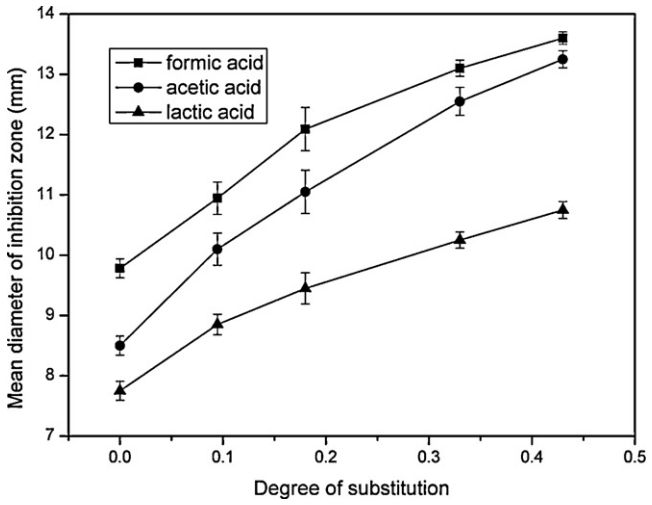

Fig. 6. The inhibitory effect of SCS with different DS and solvent against *E. coil* (MW = 650 kDa).

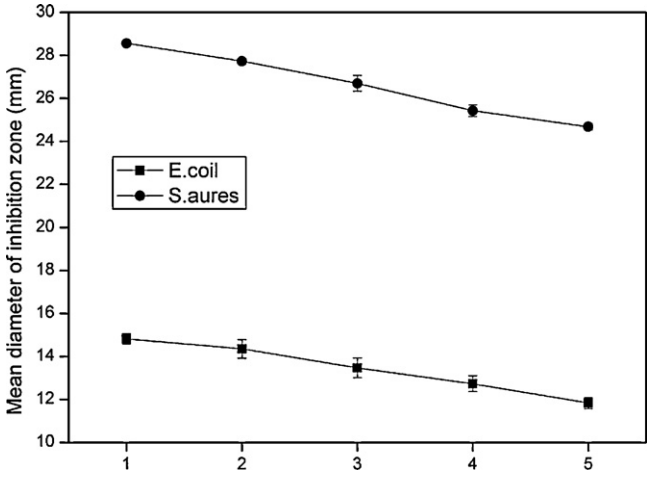

Fig. 8. The inhibitory effect of SCS with different MW against *E. coli* and *S. aureus*: (1) MW = 1350 kDa, DS = 0.43; (2) MW = 650 kDa, DS = 0.43; (3) MW = 150 kDa, DS = 0.40; (4) MW = 80 kDa, DS = 0.42; (5) MW = 10 kDa, DS = 0.39.

The results indicated that the water solubility of SCS depended on the DS. On the one hand, the substitution reaction led to destroy of the intermolecular interaction, such as van der Waals forces and hydrogen bond, which was helpful to solve. On the other hand, chitosan and SCS were dissolved in acids due to the protonated amino groups, and the higher DS at the N-position resulted in the decrease of the protonated amino groups. Therefore, the solubility of SCS declined when DS > 50%, and the SCS with DS < 50% were used for the following antibacterial experiments.

3.3. Antibacterial activity assay

3.3.1. Effect of salicyloyl chitosan DS

The effects of DS of SCS on the antibacterial activity against *E. coli* and *S. aureus* are shown in Figs. 6 and 7. The antibacterial results revealed the antibacterial activity of SCS was stronger than unsubstituted chitosan for both *E. coli* and *S. aureus*, and the samples showed more effective for *S. aureus* than *E. coli*. It was generally recognized that the chitosan was more inhibitory against Gram-positive than Gram-negative bacteria, and the possible mechanism for antimicrobial activity was that the chitosan on the surface of the cell could form a polymer membrane, which prevented nutrients from entering the cell. The presence of chitosan could make the cell membrane of *S. aureus* weakened or even broken, while the cytoplasm of *E. coli* was concentrated and the interstice of the cell enlarged clearly (Zheng & Zhu, 2003).

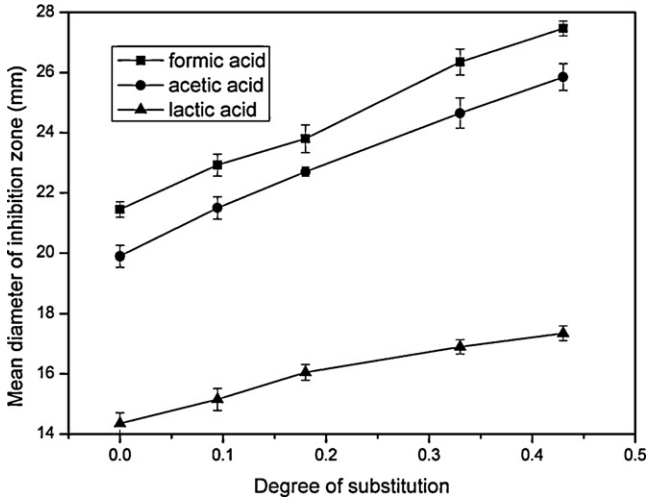

Fig. 7. The inhibitory effect of SCS with different DS and solvent against *S. aureus* (MW = 650 kDa).

Compared with the unsubstituted chitosan, the substitution of salicylic acid improved the antibacterial activity of chitosan. With the increase of DS, the antibacterial activity of SCS both increased against *E. coli* and *S. aureus*. SCS dissolved in formic acid exhibited noticeable antibacterial effect, and the mean diameter of the inhibition zone increased from 9.8 to 13.6 mm (against *E. coil*), 21.5 to 27.5 mm (against *S. aureus*) as the DS increases from 0% to 43%. The antibacterial mechanism of SCS might be due to its phenolic structure, which could alter microbial cell permeability, interfere with membrane function and interact with membrane proteins, causing deformation in structure and functionality (Tiwari et al., 2009). It might also attribute to some synergies between phenolic structure and the protonated amino remained.

3.3.2. Effect of salicyloyl chitosan solvents

Acetic, lactic and formic acids are the commonly used acids as chitosan solvents. The effects of solvents of SCS on the antibacterial activity against *E. coli* and *S. aureus* were evaluated with different DS. The inhibition results are shown in Figs. 6 and 7. The inhibition effects of chitosan solvents were somewhat varying dependent on the particular bacterium and MW of chitosan (Xu, Ning, & Yu, 2002). In the present study, the SCS with MW of 650 kDa were selected, and the samples were all completely dissolved in the acids. The antibacterial activity of SCS with formic acid was most effective both against *E. coli* and *S. aureus* followed by acetic acid and lactic acid. Organic acids have been reported to have antibacterial activity differing from one to the other (No, Park, Lee, & Meyers, 2002). Therefore, it might be in relation to the antibacterial activity of the solvents themselves.

3.3.3. Effect of salicyloyl chitosan MW

The effects of MW of SCS on the antibacterial activity against *E. coli* and *S. aureus* are shown in Fig. 8. The SCS with MW of 1350 kDa appeared most effective against both *E. coli* and *S. aureus*. As the MW declined from 1350 kDa to 10 kDa, the mean diameter of the inhibition zone both decreased against *E. coil* and *S. aureus*. It meant that the antibacterial activity of SCS was strengthened with the increase of MW, which was agreed with the MW effect on chitosan (Jeon, Park, & Kim, 2001; Shin, Yoo, & Jiang, 2001). The chitosan could interfere with bacterial metabolism by stacking at the cell surface

and binding with DNA to inhibit mRNA synthesis. The possible reason might be that the SCS with higher MW contributed to more full touch and adsorption of cell surface, thereby promoting the effect of SCS with bacterial cell membrane and interference with cell function.

4. Conclusions

In this study, the salicyloyl chitosan (SCS) with different DS and MW were synthesized by acylate reaction from chitosan and salicylic acid. The results characterized by UV, FTIR, ^{1}H NMR and XRD confirmed that the amino groups of chitosan reacted with salicylic acid to form the acylate chitosan. The antibacterial results revealed that the substitution of salicylic acid strengthened the antibacterial activity of chitosan against both *E. coli* and *S. aureus*. SCS showed an increasing antibacterial activity as DS increased. SCS dissolved in formic acid exhibited noticeable antibacterial effect, and the mean diameter of inhibition zone increased from 9.8 to 13.6 mm (against *E. coil*), 21.5 to 27.5 mm (against *S. aureus*) as the DS increased from 0% to 43%. In addition, the antibacterial activity of SCS was strengthened with the increase of MW. The results suggested that the derivatives with relatively higher DS and MW exhibited stronger antibacterial activity.

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