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Novel quaternized chitosan containing β-cyclodextrin moiety: Synthesis, characterization and antimicrobial activity

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ABSTRACT

A novel water-soluble β-cyclodextrin grafted with chitosan (CD-*g*-CS) was prepared by reacting chitosan with *O*-p-toluenesulfonyl-β-cyclodextrin (Ts-CD) under acidic condition. It was found that the degree of *N*-substitution (DS) was in the range of 5 ± 2% to 23 ± 2%. The DS increased with an increasing in molar ratio of Ts-CD. Quaternization of CD-*g*-CS was performed by using glycidyltrimethyl ammonium chloride (GTMAC) under mild acidic condition yielding the quaternary ammonium CD-*g*-CS derivative (QCD-*g*-CS). The degree of quaternization (DQ) was determined by ^{1}H NMR spectroscopy and potentiometric titration. It was found that the DQ was in the range of 60 ± 1% to 80 ± 1% and depended on the DS. The DQ increased with decreasing DS. Antimicrobial activity of these QCD-*g*-CSs were determined by using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods against *Candida albicans* (*C. albicans*), *Streptococcus mutans* (*S. mutans*) and *Streptococcus oralis* (*S. oralis*) in accordance with the methods of the National Committee for Clinical Laboratory Standards (NCCLS). The results revealed that the antimicrobial activity of all QCD-*g*-CSs was dependent on the DS/DQ ratio and type of microorganisms. The QCD-*g*-CSs and QCS showed higher antimicrobial activity against *C. albicans* fungus than against *S. mutans* and *S. oralis* bacteria. Moreover, the QCD-*g*-CS with DS/DQ ratio of 0.38 showed higher antimicrobial activity than DS/DQ ratios of 0.06 and 0.15, and also QCS against *C. albicans* and *S. oralis*, while all DS/DQ ratios did not enhance any antimicrobial activity against the *S. mutans*.

1. Introduction

Cyclodextrins (CDs) or cyclomaltoheptaoses are well-known as a series of cyclic oligosaccharides composed of 6–8 D-glucose units and named α-, β-, and γ-CD, respectively. The D-glucose units are covalently bonded together via α-1,4-linkages to form torus-like structure. Every D-glucose unit has three free hydroxyl groups, which differ both in their functions and reactivities. All the secondary hydroxyl groups at the 2- and 3-positions of the D-glucose units are on one side of the torus, and all the primary hydroxyl groups at the 6-positions of the D-glucose units are on the other side of the ring (Bender & Komiyama, 1978; Szejtli, 2004). The CDs have a hydrophilic outer surface and a lipophilic central cavity that can accommodate a variety of lipophilic drugs due to hydrophobic interactions (Loftsson & Duchêne, 2007). Furthermore, the guest molecule must be able to fit inside the cavity of the CD. Chitosan (CS) is a natural cationic polysaccharide, normally obtained by alkaline deacetylation of chitin. It is composed of *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN) residues. The primary amino groups of CS can be protonated at acidic pH, leading to a cationic charged CS backbone. Therefore, CS has been shown to be mucoadhesive (Sogias, Williams, & Khutoryanskiy, 2008) and antimicrobial (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Because of the advantages of both CD and CS, CD grafted with CS (CD-*g*-CS) was synthesized because of potential applications in many fields (Prabaharan & Mano, 2006; Manakker, Vermonden, Nostrum, & Hennink, 2009). However, the applications of the CD-*g*-CS can only be investigated in acidic media because of its poor solubility in water.

In order to improve the water solubility, novel quaternized CD-*g*-CS (QCD-*g*-CS) was synthesized by using glycidyltrimethyl ammonium chloride (GTMAC) as a quaternizing agent. It is well-known that the quaternized CS derivatives showed antimicrobial properties at all pH ranges (Rabea et al., 2003). In this study, antimicrobial activity of the QCD-*g*-CS was evaluated in order to be used as an antimicrobial material or antimicrobial carrier in the human oral cavity. Therefore, *Streptococcus oralis* ATCC 35037T (*S. oralis*), *Streptococcus mutans* ATCC 25175T (*S. mutans*) and *Candida albi-*

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cans ATCC 10231 (*C. albicans*) were used as bacteria and fungus models, respectively. *S. oralis* and *S. mutans* are Gram-positive bacteria, which are found in high numbers in the human oral cavity, and they are a significant contributor to tooth decay. *C. albicans* is a diploid fungus (a form of yeast) and a causal agent of opportunistic oral and genital infections in humans. The effects of the degree of *N*-substitution (DS) and the degree of quaternization (DQ) on physicochemical properties and antimicrobial activities were investigated.

2. Experimental

2.1. Materials

Chitosan (CS) with an average molecular weight (M_w) of 22 kDa was purchased from Seafresh Chitosan (lab) Co., Ltd. in Thailand. The degree of deacetylation (DDA) of CS was determined to be 90% by ^{1}H NMR spectroscopy. β-Cyclodextrin (CD) was obtained from Wacker Chemical AG (Germany). p-Toluenesulfonyl chloride was purchased from ACROS Organic (Belgium). Glycidyltrimethylammonium chloride (GTMAC) and *N*,*N*-dimethylformamide (DMF) were purchased from Fluka (Switzerland). Hydrochloric acid, acetic acid and sodium hydroxide were purchased from Carlo Erba (Italy). Methanol was obtained from Fisher Scientific (UK). Water used for all experiments was purified water obtained from a MilliQ Plus (Millipore, Schwalbach, Germany). All other reagents used were commercially available and were of analytical grade. The following materials were used from the indicated sources without further purification procedures.

2.2. Preparation of *O*-p-toluenesulfonyl-β-cyclodextrin (Ts-CD)

O-p-toluenesulfonyl-β-cyclodextrin (Ts-CD) was synthesized by a little modification of Brady et al.'s method (Brady, Lynam, O'Sullivan, Ahern, & Darcy, 2004). Briefly, CD (50 g, 44 mmol) was dissolved in 700 mL of NaOH (10% w/v) and stored overnight in the refrigerator. The p-toluenesulfonyl chloride (TsCl 20 g, 104 mmol) was added and stirred at 0–5 °C in an ice water bath. The reaction mixture was stirred vigorously at 0–5 °C for 2 h. Subsequently, another portion of p-toluenesulfonyl chloride (30 g, 157 mmol) was added and stirred continuously at the same temperature for another 3 h. The reaction mixture was filtered through Celite® on a sintered glass funnel to separate unreacted p-toluenesulfonyl chloride. The filtrate was treated with hydrochloric acid (pH 1–2) at 0–5 °C and stored overnight in the refrigerator. The solid which precipitated was isolated by filtration. Afterwards, it was recrystallized by dissolving in a hot water. The solution was then cool down to room temperature and stored in the refrigerator overnight. The white solid was obtained 13.79 g (22.3% yields).

2.3. Preparation of β-cyclodextrin grafted with chitosan (CD-g-CS)

CS (1 g, 6.11 meq/GlcN) was dissolved in 1% (v/v) acetic acid (pH 4, 80 mL). The solution of CD-TS (2.1–5.5 mmol) in *N*,*N*-dimethylformamide (DMF, 40 mL) was added into the CS solution. The reaction mixture was refluxed at 100 °C for 24 h and dialyzed with deionized water for 3 days. The solution was then freeze-dried to give a cotton like powder of CD-*g*-CS.

2.4. Preparation of quaternized chitosan (QCS) and quaternized β-cyclodextrin grafted with chitosan (QCD-g-CS)

CS or CD-*g*-CS (1 g) was dissolved in 1% (v/v) acetic acid (80 mL). Glycidyltrimethyl ammonium chloride (GTMAC, 6 mL) was added. The mixture was stirred at 50 °C for 6 h. The clear solution was dialyzed with deionized water for 3 days and then freeze-dried to give a cottoned like powder of QCD-*g*-CS.

2.5. Determination of the degree of tolylation (DT), the degree of N-substitution (DS) and the degree of quaternization (DQ)

In this study, the degree of tolylation (DT) and the degree of *N*-substitution (DS) were determined by using ^{1}H NMR spectroscopy as shown in Eqs. (1) and (2), respectively. The degree of quaternization (DQ) was determined by using Eq. (3).

$$\mathrm{DT}\,(\%) = \frac{(\mathrm{Ar}/4)}{\left\{[(\mathrm{H1–H6})/7] \times 7\right\}} \times 100 \quad (1)$$

where DT (%) is the degree of tolylation, Ar is the integral area of aromatic protons at δ 7.8–7.4 ppm, and H1–H6 is the integral areas of the CD protons at δ 5.9–3.2 ppm.

$$\mathrm{DS}\,(\%) = \frac{(\mathrm{H1CD}/7)}{\left[(\mathrm{NHAc}/3) + \mathrm{H2CS}\right]} \times 100 \quad (2)$$

where DS (%) is the degree of substitution, H1CD is the integral area of CD proton at δ 4.9 ppm, NHAc is the integral area of GlcNAc protons at 1.9 ppm, and H2CS is the integral area of CS proton at δ 3.0 ppm.

$$\mathrm{DQ}\,(\%) = \frac{(d/9)}{\left[(\mathrm{NHAc}/3) + \mathrm{H1CS} + (\mathrm{H1CD}/7)\right]} \times 100 \quad (3)$$

where DQ (%) is the degree of quaternization of CS, H1CD is the integral area of CD proton at δ 4.9 ppm, NHAc is the integral area of GlcNAc protons at 1.9 ppm, H1CS is the integral area of CS proton at 5.2 ppm, and *d* is the integral area of quaternary ammonium protons at 3.1 ppm.

2.6. FT-IR and ^{1}H NMR spectroscopy

All Fourier transform infrared (FT-IR) spectra were collected with a Nicolet 6700 spectrometer (Thermo Company, USA), and all samples were prepared as potassium bromide pellets at the ambient temperature (25 °C). The spectra were recorded by using standard spectral collection techniques and the rapid-scan software in OMNIC 7.0. In all cases spectra were collected using 32 scans with a resolution of 4 cm^{-1}. The ^{1}H NMR spectra were measured on AVANCE AV 500 MHz spectrometer (Bruker, Switzerland). All measurements were performed at 300 K, using the pulse accumulation of 64 scans and LB parameter of 0.30 Hz. D_2O/CD_3COOD was used as a solvent for dissolving the CS and CD-*g*-CS at 5 mg, while DMSO-d_6 was used as a solvent for the Ts-CD. Finally, D_2O was used as a solvent for the quaternized CS derivatives.

2.7. Estimation of water solubility

The water solubility of the QCS and its derivatives with various pHs was determined by using turbidity measurement. All samples were dissolved in deionized water. Then 0.1 or 1 M HCl solution and 0.1 or 1 M NaOH solution were slowly added. The transmittance of their solutions was recorded on a Lamda 650 UV/VIS Spectrophotometer (Perkin Elmer, USA) with an optical path length of 350 nm at 600 nm. The experiment was performed at 25 °C.

2.8. Determination of chloride ion content

The chloride ion content of the QCS and its derivatives was determined by potentiometric titration with silver nitrate ($AgNO_3$) solution. The titration curve was recorded on T50 Titrator using DM141-SC sensor (Mettler Toledo, USA). Briefly, 20 mg of each QCS

derivatives was dissolved in 50 mL of the deionized water. The sample solution was titrated by using 0.01 M silver nitrate solution. The volume of added silver nitrate solution (mL) and potential value (mV) of the solution were recorded. The DQ was determined by using Eq. (4).

$$DQ(\%) = \frac{(1 \times 10^{-5})V_1}{\left[(WQCS - (1 \times 10^{-5})V_1 MW_{GTMAC})/MW_{CS}\right] \times DDA} \times 100 \quad (4)$$

where WQCS is the weight of QCS (g) in 50 mL of deionized water, MW_{GTMAC} is the molecular weight of GTMAC (i.e. 151 g/mol), MW_{CS} is the molecular weight of CS (i.e. 165.2 g/mol), DDA is the degree of deacetylation of CS found to be 90% from the ^{1}H NMR analysis, V_1 is the volume of $AgNO_3$ added at the end point, and 1×10^{-5} corresponds to the number of moles of $AgNO_3$ in 1 mL of solution. Using the same equation described above, the WQCD-*g*-CS and $MW_{QCD\text{-}g\text{-}CS}$ were used instead of WQCS and MW_{CS}, respectively (Cho, Grant, Piquette-Miller, & Allen, 2006).

2.9. Molecular weight determination

The weight average molecular weight (M_w), number average molecular weight (M_n), and M_w/M_n of CS and its quaternized derivatives were determined by using the gel permeation chromatography (GPC). It consists of Waters 600E Series generic pump, injector, ultrahydrogel linear columns (M_w resolving range 1–20,000 kDa), guard column, pollulans as standard (M_w 5.9–788 kDa), and refractive index detector (RI). All samples were dissolved in acetate buffer pH 4 and then filtered through VertiPure nylon syringes filters 0.45 μm (Vertical Chromatography Co., Ltd., Thailand). The mobile phases, 0.5 M AcOH and 0.5 M AcONa (acetate buffer pH 4), were used at a flow rate of 0.6 mL/min at 30 °C. An injection volume 20 μL was used.

2.10. X-ray diffraction (XRD)

Crystalline characteristics of the CS and its quaternized derivatives were analyzed by powder X-ray diffraction (XRD) (JDX-3530 theta–2theta XRD, JEOL Ltd., Tokyo, Japan) using a CuKα lamp at λ = 1.5405 Å. Scan parameters were set at 5 s scan speed and increment at 0.02. Under these conditions a diffraction pattern in the range of 3–40° (2θ) was used at 25 °C.

2.11. Differential scanning calorimetry (DSC)

Thermal behavior of the CS and its quaternized derivatives were investigated using differential scanning calorimetry (DSC, Mettler Toledo DSC823e/400, USA). The powder sample was heated up at the rate of 5 °C/min from 25 °C to 600 °C. During the heating, the nitrogen gas was flowed into the furnace at a rate of 60 mL/min. An empty pan was used as a reference.

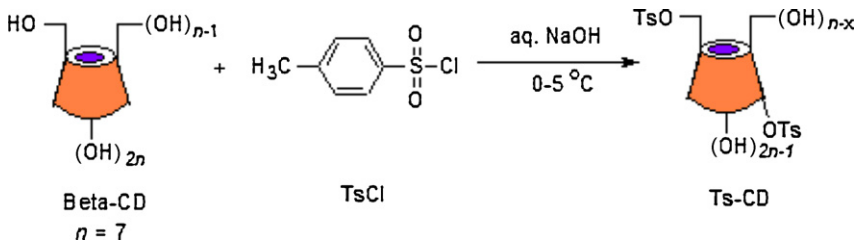
2.12. Antimicrobial assessments

The antimicrobial tests were performed in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) to determine broth microdilution minimum inhibitory concentration (MIC) values. The microorganisms used in the inhibitory test were obtained from the Department of Medical Sciences (Ministry of Public Health, Thailand). One colony of *Streptococcus oralis* ATCC 35037T (*S. oralis*) and *Streptococcus mutans* ATCC 25175T (*S. mutans*) from culture on Brain Heart infusion Agar (BHA) was inoculated into Brain Heart infusion Broth (BHB) and incubated aerobically at 35 °C for 24 h, whereas for *Candida albicans* ATCC 10231 (*C. albicans*) the colony was transferred from Saboraud Dextrose Agar (SDA) into Saboraud Dextrose Broth (SDB). These derivatives were dissolved in culture media and serial diluted. The dilution series of the extract, ranging from 20 to 0.4 mg/mL, were prepared and then transferred to the broth in 96 well-microtitre plates. The final concentrations were in the range 2000–40 μg/mL in the medium. A bacterial culture of 1×10^6 CFU/mL was added and incubated at 37 °C for 24 h. The concentration of microbial growth after at 24 h was determined by UV spectrophotometer at 600 nm. Minimal bactericidal concentration (MBC) was determined by subculturing the test dilution of minimum inhibitory concentration (MIC) value onto agar and incubating for 18–24 h. The highest dilution that yielded no single bacterial colony on a solid medium was taken as MBC (National Committee for Clinical Laboratory Standards, 2000, 2002).

3. Results and discussion

3.1. Synthesis, characterization and quaternization of the β-cyclodextrin grafted with chitosan

It is well-known that the 6-*O*-p-toluenesulfonyl-β-cyclodextrin (Ts-CD) is the most important derivative for access to modifications on the primary hydroxyl groups of the Ts-CD into other functional groups such as amino (Melton & Slessor, 1971), alkylamino (Mentzafos, Terzis, Coleman, & De Rango, 1996), thioalkyl (Nelles et al., 1996), halo (Omichi & Matsushima, 1978), formyl (Huff & Bieniarz, 1994) groups and so on. In this study, the Ts-CD was carried out by modification of Brady et al. (2004) procedure by increasing NaOH concentration from 1.6% (w/v) to 10% (w/v) in order to increase a percent yield. The Ts-CD was synthesized by tosylation of either the primary hydroxyl or secondary hydroxyl groups of the CD with p-toluenesulfonyl chloride (TsCl) under aqueous basic condition (Scheme 1). The degree of tosylation (DT) was determined by ^{1}H NMR spectroscopy (Eq. (1)). The DT was found at 1.76, whereas the yield was high up to 22%. The DT was higher than 1 due to ditosylation in the CD backbone, which can be attributed to high NaOH concentration used. Previously, various methods prepared the Ts-CD were reported in different conditions. Basically, the Ts-CD was prepared by reacting the CD with TsCl in dry pyridine, *N*,*N*-dimethylformamide (DMF) (Melton & Slessor, 1971; Takahashi, Hattori, & Toda, 1984) or in aqueous acetronitrile at alkaline condition in stead of water as a solvent (Nelles et al., 1996; Vizitiu, Walkinshaw, Gorin, & Thatcher, 1997). Normally, monotosylation of the CD is a non-selective reaction and produces a mixture of primary as well was secondary side tosylated products along with di- or tri-tosylated derivatives. Therefore, the monotosylation of the CD requires a careful control of reaction conditions and extensive purification steps. Disadvantages of this method are poor yield because the tosylates can undergo an exchange reaction with chloride ions or an elimination process to give either the 3,6-anhydro compound or an alkene, and they are difficult to separate from byproducts (Khan, Forgo, Stine, & D'Souza, 1998). Moreover, this was due to moisture intervention during the synthesis step (Tang & Ng, 2008), formation of the CD-pyridine gel (De Rango et al., 1992) and chlorination during the work-up step (Defaye, Gadelle, Guiller, Darcy, & O'Sullivan, 1989). However, the major advantage of pyridine is its ability to direct the reaction to the 6-position as compared to DMF. In order to solve these problems, the p-toluenesulfonic acid anhydride (Ts_2O) was used instead of TsCl reported by Zhong et al. (Zhong, Byun, & Bittman, 1998). They suggested that the Ts_2O can be used to large-scale synthesis, and it is easy to carry out and proceeds in high yield. However, difficulty in preparing tosic acid-free p-toluenesulfonic anhydride frequently results in a lower yield of the 6-*O*-p-toluenesulfonyl-β-cyclodextrin. Therefore, 1-(p-toluenesulfonyl)imidazole was used in stead of the Ts_2O again to synthesize the 6-*O*-p-toluenesulfonyl-



Scheme 1. Synthesis of intermediate *O*-p-toluenesulfonyl-β-cyclodextrin (Ts-CD).

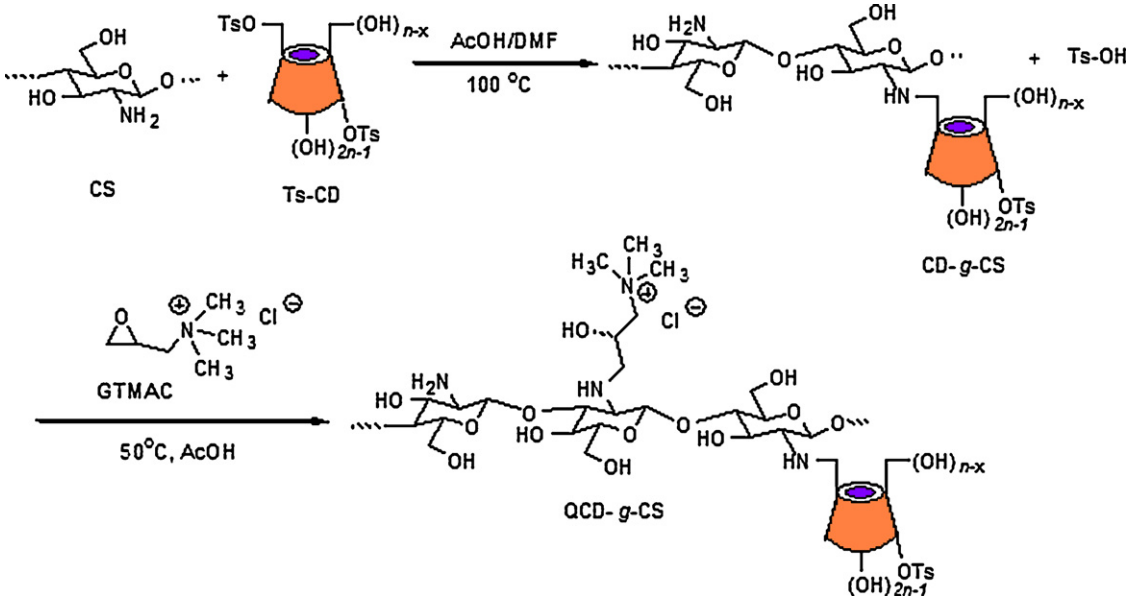
β-cyclodextrin. Utilization of the imidazolide of tosic acid as a sulfonating agent has many advantages such as the aqueous solubility of 1-(p-toluenesulfonyl)imidazole was higher than that of the Ts_2O or TsCl and the tosylimidazole was more resistant to hydrolysis at room temperature than the Ts_2O or TsCl. So less free tosic acid would be formed during the sulfonation reaction, and significant multi-tosylation of β-cyclodextrin was not observed, even though 4 equivalents of 1-(p-toluenesulfonyl)imidazole were used (Byun, Zhong, & Bittman, 2004).

Afterwards, the Ts-CD was grafted with CS (CD-*g*-CS) by nucleophilic displacement of the tosyl group under acidic condition as shown in Scheme 2. The CD-*g*-CS was carried out by reacting Ts-CD with CS under an acidic condition at 100 °C. In this study, the DMF was used to dissolve the Ts-CD prior an addition into the CS solution, leading to homogenous reaction. The degree of *N*-substitution (DS) was determined by ^{1}H NMR spectroscopy as shown in Eq. (2). The DS was in the range of 5–23% depending on the mole ratio of the Ts-CD per primary amino group of the CS (Table 1). The result revealed that the DS increased with an increase in mole ratio of Ts-CD. Because of ditosylation, therefore, the tosyl group was still remained in the CD-*g*-CS. This was confirmed by the ^{1}H NMR spectrum of the CD-*g*-CS. The obtained products were all insoluble in water. To improve the water solubility of the CD-*g*-CS, the quaternization was carried out by using glycidyltrimethylammonium chloride (GTMAC) which yielded quaternized CD-*g*-CS (QCD-*g*-CS). Under this condition, the free primary amino groups of the CD-*g*-CS reacted with the epoxide group of the GTMAC compound via a nucleophilic substitution pathway (Scheme 2) to introduce the quaternary ammonium substituent. Similar reactions with GTMAC had been reported to quaternize polysaccharides under the catalytic action of acid or base (Lang, Wendel, & Konrad, 1990; Li, Du, Wu, & Zhan, 2004). The degree of quaternization (DQ) of the QCD-*g*-CSs were determined by the ^{1}H NMR spectroscopy and potentiometric titration method as shown in Eqs. (3) and (4), respectively. The DQ was in the range of 66 ± 2% to 85 ± 3% for ^{1}H NMR spectroscopic method, while the DQs of the potentiometric titration method were in the range of 60 ± 1% to 80 ± 1% (Table 1). The low DS has higher the DQ compared to the high DS because the low DS has higher the number of free primary amino groups onto the CS backbone than the high DS.

Table 1
The percentage of the degree of *N*-substitution (DS), the degree of quaternization (DQ) and recovery of the quaternized chitosan (QCS), quaternized cyclodextrin grafted with chitosan 5% (QCD5-*g*-CS), quaternized cyclodextrin grafted with chitosan 11% (QCD11-*g*-CS) and quaternized cyclodextrin grafted with chitosan 23% (QCD23-*g*-CS).

Samples	DS (%)	DQ (%)[a]	DQ (%)[b]	Recovery (%)[c]
QCS	–	85 ± 3	80 ± 1	100 ± 1
QCD5-*g*-CS	5 ± 2	80 ± 2	74 ± 1	66 ± 1
QCD11-*g*-CS	11 ± 2	73 ± 2	65 ± 1	79 ± 1
QCD23-*g*-CS	23 ± 2	66 ± 2	60 ± 1	71 ± 1

[a] DQ determined by ^{1}H NMR method.
[b] DQ determined by potentiometric titration method.
[c] Recovery (%) is weight of product (g)/weight of chitosan (g) × 100.



Scheme 2. Quaternization of β-cyclodextrin grafted with chitosan (QCD-*g*-CS).

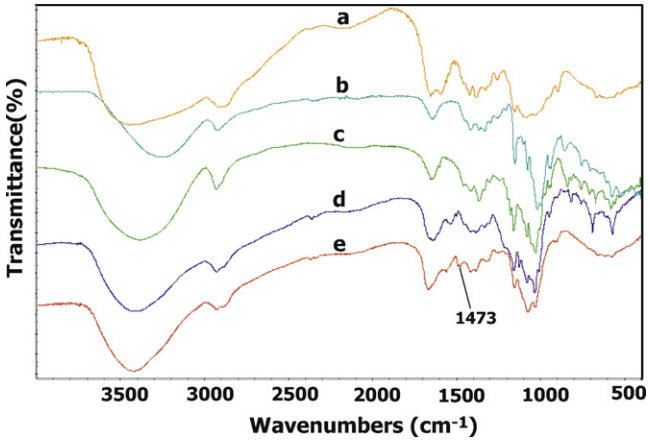


Fig. 1. FT-IR spectra of chitosan (a), β-cyclodextrin (b), *O*-p-toluenesulfonyl-β-cyclodextrin (c), cyclodextrin grafted with chitosan 11% (d) and quaternized cyclodextrin grafted with chitosan 11% (e).

3.2. Characterization of the β-cyclodextrin grafted with chitosan and its quaternized derivatives

The chemical structures of CS and its derivatives were characterized by FT-IR and ^{1}H NMR spectroscopy. Fig. 1 displays the FT-IR spectra of CS, CD, CD-TS, CD-*g*-CS, and QCD-*g*-CS. Fig. 1a displays the characteristic FT-IR pattern of the CS at wavenumber 3441 cm^{-1} due to OH and NH_2 groups. The absorption bands at wavenumbers of 1645 and 1380 cm^{-1} were corresponded to the C=O and C–O stretching of amide group, whereas the absorption band at wavenumber 1598 cm^{-1} was due to N–H deformation of amino groups. Moreover, the CS showed the absorption band at wavenumbers of 1156, 1087 and 1025 cm^{-1} corresponded to the symmetric stretching of the C–O–C and involved skeletal vibration of the C–O stretching (Brugnerotto et al., 2001). Fig. 1b exhibits the FT-IR pattern of the CD at wavenumber of 3250 cm^{-1} due to the O–H stretching. The absorption band at wavenumbers of 1149, 1074 and 1015 cm^{-1} corresponded to the symmetric stretching of the C–O–C and involved skeletal vibration of the C–O stretching, respectively. The FT-IR spectra of the CD-TS exhibit the additional absorption bands at wavenumbers of 1595 and 1157 cm^{-1} corresponded to the C=C stretching of the aromatic group and S=O stretching of sulfonyl group, respectively (Fig. 1c), while the CD-*g*-CS exhibits the characteristic absorption band of the CD and CS such as at wavenumbers of 1593 and 1121–1005 cm^{-1} corresponded to N–H deformation of amino groups and the symmetric stretching of the C–O–C and involved skeletal vibration of the C–O stretching (Fig. 1d). The QCD-*g*-CS exhibits the characteristic FT-IR spectra at wavenumbers of 1473 cm^{-1} due to C–H stretching of the methyl substituent of quaternary ammonium groups (Fig. 1e) (Loubaki, Ourevitch, & Sicsic, 1991).

Fig. 2 shows ^{1}H NMR spectra of the CD, CD-TS and CD-*g*-CS. Fig. 2a shows ^{1}H NMR spectra of the CD and CD-TS in DMSO-d_6. The ^{1}H NMR spectrum of the CD exhibits the double of doublet protons at δ 5.8 and 5.7 ppm due to the C2–OH and C3–OH protons, respectively. The doublet protons at δ 4.8 ppm were assigned to H1 proton of the CD, whereas the triplet protons at δ 4.5 ppm were assigned to the C6–OH protons of the CD. The multiplet protons at δ 3.7–3.2 ppm were assigned to the H2–H6 protons of the CD. The ^{1}H NMR spectrum of the CD-TS exhibits the new peaks, double of doublet proton signals, at δ 7.8–7.4 ppm due to the aromatic protons of the tosyl group. It was noted that the intensity of the double of doublet protons at δ 5.7 ppm was reduced. This was due to substitution of tosyl group at the C3–OH position of the CD. However, the intensity of the C2–OH did not change. Moreover, the new peak,

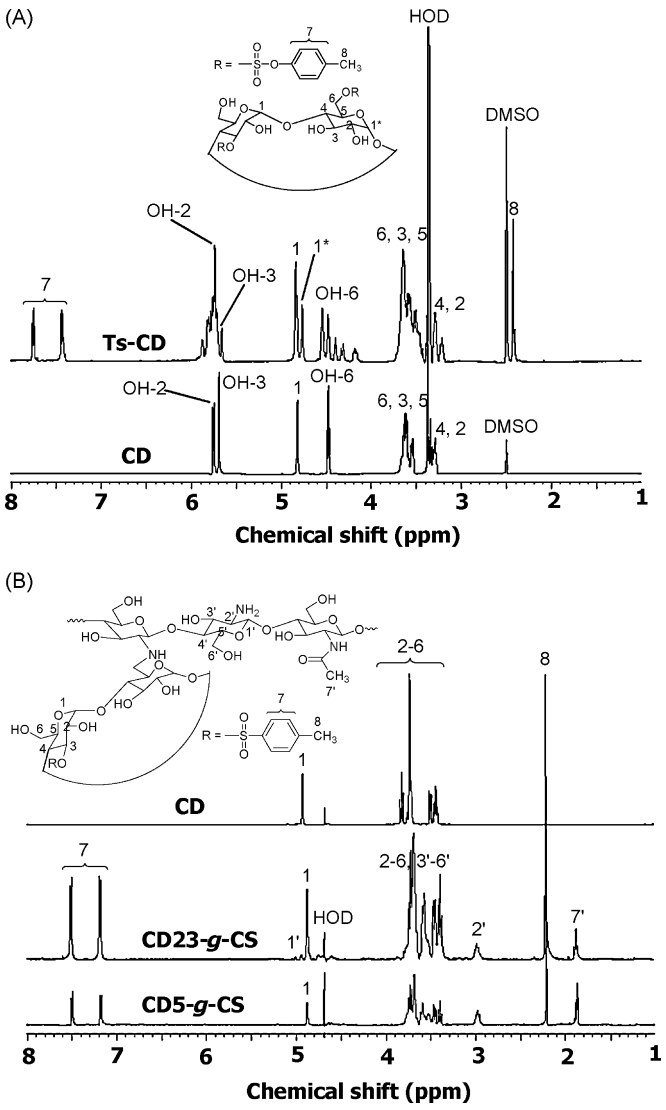


Fig. 2. ^{1}H NMR spectra of *O*-p-toluenesulfonyl-β-cyclodextrin (Ts-CD) and β-cyclodextrin (CD) in DMSO-d_6 (a), and β-cyclodextrin (CD), cyclodextrin grafted with chitosan 5% (CD5-*g*-CS) and cyclodextrin grafted with chitosan 23% (CD23-*g*-CS) in CD_3COOD/D_2O (b).

double of doublet proton signal, at δ around 4.8 ppm was assigned to H1* proton of the substituted CD. The multiplet proton signals at δ 4.6–4.2 ppm were assigned to the C6–OH group which substituted by tosyl group. The multiplet proton signals at δ 3.7–3.5 ppm and 3.4–3.2 ppm were assigned to H6, H3, H5 and H4, H2 of the CD, respectively. The singlet proton signal at δ 2.2 ppm was assigned to the methyl protons at para-position of aromatic moiety. The result revealed that a success of the tosylation of a set of hydroxyl groups of the CD could be verified by ^{1}H NMR spectroscopy, particularly in DMSO-d_6 as a solvent. Since the protons of the CD are clearly separated, therefore, it is easily to interpretation. In this study, the tosylation of the CD was occurred at C6–OH group and C3–OH group. When considering the reactivity of the hydroxyl groups of the CD, it can be ranked as follow C6–OH > C2–OH > C3–OH. However, the tosylation of the CD at C3–OH group under an alkali solution can be described via the mechanism which proposed by Onozuka et al. (Onozuka, Kojima, Hattori, & Tada, 1980). They proposed that the TsCl and the CD can form complex with ratio 1:1, and the methyl group of the TsCl, which is the hydrophobic part of the molecule, can locate near the C3–OH group of the CD. This was confirmed by using the scale molecular model (CPK model).

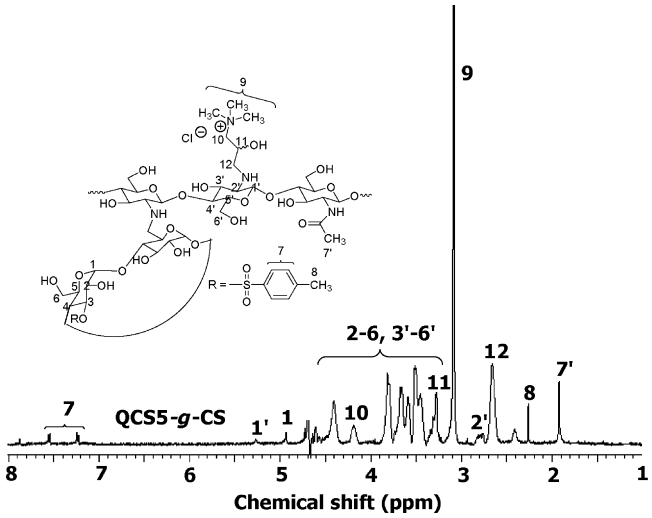


Fig. 3. ^{1}H NMR spectrum of quaternized cyclodextrin grafted with chitosan 5% (QCS5-*g*-CS) in D_2O.

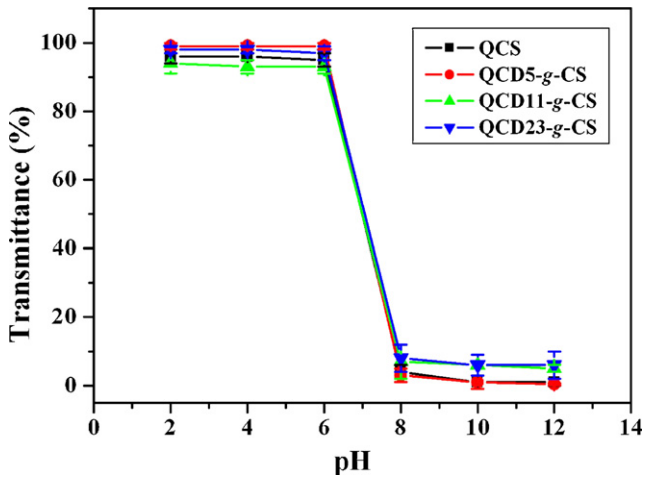


Fig. 4. The pH dependence of water solubility of quaternized chitosan (QCS), quaternized cyclodextrin grafted with chitosan 5% (QCD5-*g*-CS), quaternized cyclodextrin grafted with chitosan 11% (QCD11-*g*-CS) and quaternized cyclodextrin grafted with chitosan 23% (QCD23-*g*-CS) at 5 mg/mL.

The result revealed that the distance between S atom in the TsCl and the C3–O atom in the complex formed was a little shorter than the distance between S atom and the C2–O atom.

Fig. 2b shows the ^{1}H NMR spectrum of the CD, CD23-*g*-CS CD5-*g*-CS in D_2O/CD_3COOD. The ^{1}H NMR spectrum of the CD exhibits the doublet proton signals at δ 4.9 ppm and the multiplet proton signals at δ 3.9–3.4 ppm, which were due to the H1 proton and H2–H6 protons, respectively. The ^{1}H NMR spectrum of the CD in DMSO-d_6 and D_2O/CD_3COOD was different due to the difference of a polarity of the solvent. The ^{1}H NMR spectra of the CD23-*g*-CS and CD5-*g*-CS showed the double of doublet proton signals at δ 7.4–7.0 ppm due to the aromatic protons. Moreover, the multiplet proton signals at δ 5.0–3.0 ppm were due to the H2–H6 protons of CD and H3–H6 of CS, while two singlet signals at δ 2.9 and 2.1 ppm were due to the H2 proton of the GlcN and the methyl protons at para-position of aromatic group, respectively. Fig. 3 shows the ^{1}H NMR spectrum of the QCD5-*g*-CS. The double of doublet proton signals at δ 7.4–7.0 ppm were corresponded to the aromatic protons. The proton signal at 5.3 ppm was assigned to the H1′ proton of the GlcN, whereas the proton signal at δ 4.9 ppm was assigned to the H1 proton of CD. In addition, the multiplet proton signals at 5.0–3.0 ppm were corresponded to the protons of CD, and the proton signals at 3.1 and 2.7 ppm were assigned to the quaternary ammonium proton and methylene proton, respectively.

3.3. Solubility determination

Fig. 4 exhibits the pH dependence of the transmittance of the QCD-*g*-CS at 5 mg/mL compared to the QCS. It was found that the water solubility of QCD-*g*-CS and QCS was decreased with an increasing pH. At low pH, the QCD-*g*-CS and QCS showed excellent water solubility, since the free primary amino group can be protonated. On the other hand, the QCD-*g*-CS and QCS showed poor water solubility at high pH due to hydrophobicity of the CD moiety.

3.4. Molecular weight determination

The weight average molecular weight (M_w), number average molecular weight (M_n) and M_w/M_n of CS, QCS and QCD-*g*-CS were determined by gel permeation chromatography (GPC) presented in Table 2. The M_w of the native CS was found to be M_w 15 kDa, whereas the M_w of the QCS and QCD-*g*-CS were in the range of 74–97 kDa. The quaternized CS derivatives have M_w higher than the native CS due to the introduction of the quaternary ammonium and the CD moieties. However, it was noted that a relatively wide molecular weight distribution with a polydispersity index (PDI) of the quaternized CS derivatives was higher than that of the CS. This might be due to the branching of the quaternary ammonium and the CD moieties.

3.5. X-ray diffraction (XRD)

Fig. 5 shows the X-ray diffraction (XRD) patterns of CS and its quaternized derivatives. The XRD pattern of CS exhibited three characteristic peaks around $2\theta = 10.6^\circ$, 19.9° and 22.2° (Zhang & Neau, 2001). The reflection fall at $2\theta = 10.6^\circ$ assigned to the crystal form I and the strongest reflection appeared at $2\theta = 19.9^\circ$ corresponding to crystal form II. Compared with CS, the broad single XRD patterns of the QCS, QCD5-*g*-CS and QCD11-*g*-CS were observed. The decrease in crystallinity of QCS, QCD5-*g*-CS and QCD11-*g*-CS and QCD23-*g*-CS could be ascribed to the presence of quaternary ammonium moiety. This is possible since the steric hindrance of the quaternary ammonium group may obstruct the formation of inter- and extra-molecular hydrogen bonds of the CS backbone, leading to water-soluble derivatives. It is important to note that the QCD5-*g*-CS and QCD11-*g*-CS and QCD23-*g*-CS have lower crystallinity than that the QCS. This is due to the steric hindrance of the CD group. An increase in the CD moiety tended to decrease the crystallinity. However, the crystallinity of QCD23-*g*-CS is higher than QCD11-*g*-CS since the QCD23-*g*-CS depicted two small XRD peaks at 15° and 23°. It is possible that an increase in the CD moiety up to 23% resulted in a change in crystallinity of QCS backbone. This is attributed to the decreasing primary amino groups, which means less cationic charge density can be obtained on the CS backbone. In this study, it was confirmed that the QCS, QCD5-*g*-CS and QCD11-

Table 2
Weight average molecular weight (M_w), number average molecular weight (M_n) and M_w/M_n of chitosan (CS), quaternized chitosan (QCS), quaternized cyclodextrin grafted with chitosan 5% (QCD5-*g*-CS), quaternized cyclodextrin grafted with chitosan 11% (QCD11-*g*-CS) and quaternized cyclodextrin grafted with chitosan 23% (QCD23-*g*-CS).

Samples	M_n (kDa)	M_w (kDa)	M_w/M_n
CS	7.78	15.73	2.02
QCS	20.62	97.45	4.75
QCD5-*g*-CS	19.33	75.67	3.91
QCD10-*g*-CS	22.63	81.24	3.58
QCD23-*g*-CS	16.61	74.56	4.48

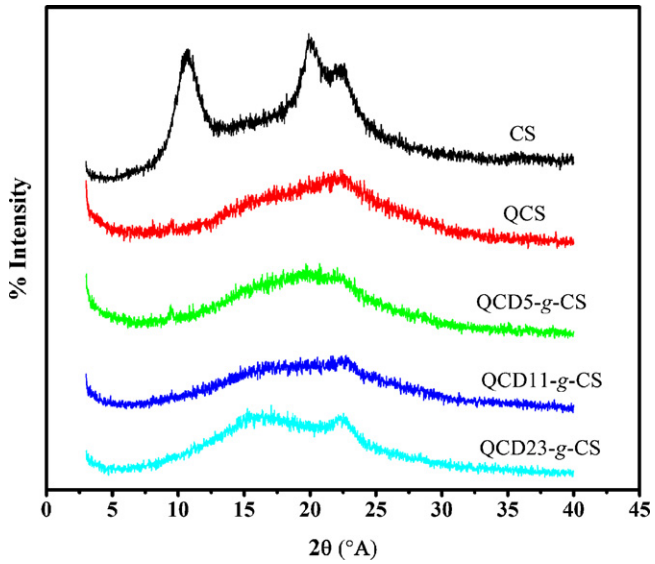


Fig. 5. X-ray diffraction patterns of chitosan (CS), quaternized chitosan (QCS), quaternized cyclodextrin grafted with chitosan 5% (QCD5-*g*-CS), quaternized cyclodextrin grafted with chitosan 11% (QCD11-*g*-CS) and quaternized cyclodextrin grafted with chitosan 23% (QCD23-*g*-CS).

g-CS and QCD23-*g*-CS were amorphous more than that of the CS. Moreover, the result revealed that the quaternary ammonium moiety was successfully introduced into the CS backbone.

3.6. Differential scanning calorimetry (DSC)

Thermal behavior of all materials was investigated by using DSC under nitrogen atmosphere which shown in Fig. 6. Native CS exhibited a broad endothermic peak at 85 °C and exothermic peak at 309 °C due to the loss of bound water and decomposition of the CS backbone, respectively (Kittur, Prashanth, Sankar, & Tharanathan, 2002; Mucha & Pawlak, 2005). The endothermic peak of the quaternized CS derivatives, QCS, QCD5-*g*-CS, QCD11-*g*-CS and QCD23-*g*-CS, were in the range of 70–120 °C with an on set at 26–92 °C (Table 3). Basically, polysaccharides have a strong

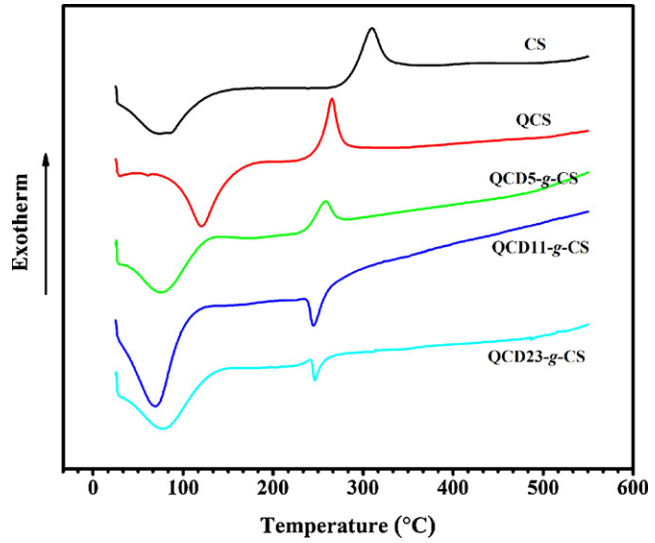


Fig. 6. DSC thermograms of chitosan (CS), quaternized chitosan (QCS), quaternized cyclodextrin grafted with chitosan 5% (QCD5-*g*-CS), quaternized cyclodextrin grafted with chitosan 11% (QCD11-*g*-CS) and quaternized cyclodextrin grafted with chitosan 23% (QCD23-*g*-CS).

Table 3
Peak temperature in DSC during the thermal degradation of chitosan (CS), quaternized chitosan (QCS), quaternized cyclodextrin grafted with chitosan 5% (QCD5-*g*-CS), quaternized cyclodextrin grafted with chitosan 11% (QCD11-*g*-CS) and quaternized cyclodextrin grafted with chitosan 23% (QCD23-*g*-CS).

Samples	Temperature (°C)	Onset (°C)	Peak (°C)	ΔH (J/g)
CS	25–150	27.08	85.17	335.27
	265–340	286.6	309.24	−196.96
QCS	65–180	92.7	120.67	357.42
	265–360	251.48	265.34	−173.02
QCD5-*g*-CS	25–150	29.14	88.99	428.92
	220–280	238.39	257.88	−84.21
QCD11-*g*-CS	25–120	27.63	70.03	531.94
	230–270	238.61	245.13	67.64
QCD23-*g*-CS	25–130	26.56	78.31	455.11
	235–255	242.98	246.44	25.34

affinity with water, so they can be disordered structures in order to be hydrated (Kacurakova, Belton, Hirsch, & Ebringerova, 1998; Phillips, Takigami, & Takigami, 1996). Therefore, the endotherm related to the evaporation of water is expected to reflect physical and molecular change during the introduction of the CD and quaternary ammonium moieties into the CS backbone. It can be observed in differences of peak area and position of the endothermic peak, indicating that these CS derivatives backbones differ in their water holding capacities, strengths of water and the CS derivatives backbone interactions. In Table 3, the CS has enthalpy (ΔH) as 335 J/g, whereas the QCS has ΔH as 357 J/g. It can be explained that introduction of the quaternary ammonium moieties into the CS backbone caused an increase in the water holding capacity. In the same way, the ΔH of the QCD5-*g*-CS and QCD11-*g*-CS was increased with an increasing DS. This result was consistent of Kittur et al. (2002), who suggested that the ΔH increased with an increase in *N*-deacetylation and carboxylation which indicated that a definite correlation exists between the water holding capacity and chemical and supramolecular structure of these polymers. On the other hand, the ΔH of the QCD23-*g*-CS was decreased when the DS was 23%. This was due to the reduction of the quaternary ammonium moiety into the CS backbone or increasing the hydrophobicity of the CD moiety. This result was confirmed by the X-ray diffraction pattern of the QCD23-*g*-CS. Due to the differences in chemical and structure characteristics, remarkable differences in the exothermic transitions in CS, QCS, QCD5-*g*-CS, QCD11-*g*-CS and QCD23-*g*-CS were observed. The exothermic peak at 309 °C was due to the decomposition of the CS backbone, while the exothermic peak of the QCS was shifted to lower temperature at 265 °C. The result revealed that an introduction of the quaternary ammonium moiety into the CS backbone attributed to a decrease in thermal stability as a consequence of decreased crystallinity. The QCD5-*g*-CS showed the exothermic peak at 257 °C, while the QCD11-*g*-CS and QCD23-*g*-CS showed the endothermic peaks at 245 °C and 246 °C, respectively. The exotherm and endotherm at lower temperatures can be attributed to decomposition of highly CD substituted regions in the CS backbone. Similar result has been observed in our research group. We found that the exothermic peak at 320 °C was due to the decomposition of the CD backbone (Nuchuchua et al., 2009). In our study, it can be concluded that the QCS derivatives have lower thermal stability than the native CS, particularly in the QCD-*g*-CS.

3.7. Antimicrobial activity

The antimicrobial activity of the QCD-*g*-CSs was evaluated by using a minimum inhibitory concentration (MIC) and a minimum bactericidal concentration (MBC) procedure against *S. mutans*, *S. oralis* and *C. albicans* at pH 7.2 in accordance to methods of the National Committee for Clinical Laboratory Standards (NCCLS) compared to the QCS (National Committee for Clinical Laboratory

Table 4
Antimicrobial activity of quaternized chitosan (QCS), quaternized cyclodextrin grafted with chitosan 5% (QCD5-*g*-CS), quaternized cyclodextrin grafted with chitosan 11% (QCD11-*g*-CS) and quaternized cyclodextrin grafted with chitosan 23% (QCD23-*g*-CS).

Samples	DS/DQ	MIC/MBC (mg/mL)		
		C. albicans	*S. mutans*	*S. oralis*
QCS	–	1.25/5	10/10	20/20
QCD5-*g*-CS	0.06	1.25/5	10/20	20/20
QCD11-*g*-CS	0.15	1.25/5	10/10	10/20
QCD23-*g*-CS	0.38	0.625/1.25	10/10	10/20

Standards, 2000, 2002). The QCS and QCD-*g*-CSs exhibited antimicrobial activity against *S. mutans*, *S. oralis* and *C. albicans* with MIC values ranging from 1.25 to 20 mg/mL and 0.625 to 5 mg/mL, whereas MBC values were in the range of 5 to 20 mg/mL and 1.25 to 20 mg/mL, respectively (Table 4). The QCS and QCD-*g*-CSs showed antimicrobial activity against *C. albicans* better than *S. mutans* and *S. oralis*. This might be due to the fundamental differences in microbial cell wall between fungi and bacteria. The fungal cell wall are composed of chitin and other polysaccharides such as β-1,3-glucan (zymosan) and mannose-containing glycoproteins, while the Gram-positive bacteria cell wall are composed of peptidoglycan, an acidic polymer, such as teichoic acid, lipo teichoic acid and teichuronic acid (Franklin & Snow, 1981). Therefore, it is possible that the quaternized CS containing CD moiety can be easily absorbed and penetrated onto the fungal cell surface leading to the leakage of proteinaceous and other intracellular constituents. Similar result has been observed by Sagoo et al (Sagoo, Board & Roller, 2002). They found that spoilage yeasts were more sensitive to the CS than Gram-positive and Gram-negative bacteria. In comparison to the QCS, antimicrobial activity of the QCD-*g*-CSs increased with an increasing DS/DQ ratio of 0.15 and 0.38 against *S. oralis* and *C. albicans*, respectively. It is noted that even though the DS/DQ ratio increased, however, the antimicrobial activity of the QCD-*g*-CSs did not increase against *S. mutans*. In fact, even though the DS/DQ ratio increased, but the DQ decreased with an increasing DS. Therefore, the positively charged density of the QCD-*g*-CSs still played an important role on the antibacterial activity. Our result indicated that an introduction of the CD moiety on the CS was more effective with *C. albicans* than *S. soralis*, but it did not affect much with *S. mutans*. Previously, it has been reported that the MICs of CS against *C. albicans* were in the range of 0.5 to >1.25 mg/mL (Tsai, Su, Chen, & Pan, 2002; Hongpattarakere & Riyaphan, 2008). Moreover, chitooligosaccharide (COS) showed antibacterial activity against *S. mutans* as reported by several research groups (Kim, Lee, Lee, & Park, 2003; Tarsi, Corbin, Pruzzo, & Muzzarelli, 2007). Recently, Ji et al. (2009) reported that *N*-[1-hydroxy-3-(trimethylammonium)propylchitosan chloride] (HTCC) showed the antimicrobial activity against *S. mutans* with MIC values 0.5 and 1 mg/mL in lactic acid and water, respectively, whereas the MIC of the CS in lactic acid was 2.5 mg/mL. Although many reports recorded on MIC and MBC values with the same microorganisms such as *C. albicans*, *S. mutans* and *S. oralis*, however, it could not be compared directly with our results because of the non-identical assay conditions and the different species of microorganism, in which the strains might vary considerably.

The antifungal mechanism of the CS involved cell wall morphogenesis with CS molecules interfering directly with fungal growth, similarly to the effects observed in bacteria cells (El Ghaouth, Arul, Grenier, & Asselin, 1992). Up to the present time, the mechanism of antimicrobial activity of the CS and their derivatives is still not clear. However, it is proposed that the positive charge density of the CS absorbed onto the negatively charged cell surface of bacteria leads to the leakage of proteinaceous and other intracellular constituents, causing cell death. Moreover, microscopic techniques such as transmission electron microscopy (TEM) (Chung & Chen, 2008), atomic force microscopy (AFM) (Eaton, Fernandes, Pereira, Pintado, & Malcata, 2008), scanning electron microscopy (SEM) (Kong et al., 2008) have been used to study and confirm the antimicrobial mechanism. The result revealed that antimicrobial activity of the QCS and QCD-*g*-CSs was dependent on the DS/DQ ratio and type of microorganism.

4. Conclusion

Novel water-soluble CD-*g*-CS was successfully synthesized by quaternizing the CD-*g*-CS with glycidyltrimethyl ammonium chloride under mild acidic condition. Introduction of the quaternary ammonium moiety onto the CD-*g*-CS enhanced the water solubility, while crystallinity and thermal stability decreased when compared to the CS. The QCS and QCD-*g*-CSs showed higher antimicrobial activity against *C. albicans* than *S. mutans* and *S. oralis*. It was due to the fundamental differences in microbial cell wall between fungi and bacteria. The antimicrobial activity of QCD-*g*-CSs increased with an increasing in DS/DQ ratio against all microorganisms except *S. mutans*. Introduction of the CD moiety into the CS was more effective with *C. albicans* than *S. oralis* and *S. mutans*. The result revealed that the DS/DQ ratio and type of microorganism played an important role on the antimicrobial activity.

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