Carbohydrate Polymers 84 (2011) 1365–1370

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Carboxymethyl chitosan prevents formation of broad-spectrum biofilm

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ARTICLE INFO

Article history:
Received 24 November 2010
Received in revised form 21 January 2011
Accepted 21 January 2011
Available online 3 February 2011

Keywords:
Carboxymethyl chitosan
Biofilm
Flocculation

ABSTRACT

In this study, we determined the effect of carboxymethyl chitosan (CM-chitosan) on biofilm formation and proposed a mechanism. Gram-positive and Gram-negative bacterial biofilm formation on microtiter plates was prevented 74.6% and 81.6% by CM-chitosan, respectively. Biofilm formation was also severely prevented in dynamic conditions. CM-chitosan inhibits the adhesion of bacteria with an efficiency of >90%. It prevents Gram-positive bacterial biofilm formation at efficiencies of 63.1% and Gram-negative bacterial biofilm formation at efficiencies of 70.6% when CM-chitosan is added at 1 h after biofilm initiation. The prevention of initial bacterial adherence and cell–cell interaction was ascribed to flocculation. The flocculation of Gram-positive and Gram-negative bacteria was 16.7% and 24.6% in the presence of CM-chitosan, respectively. CM-chitosan may neutralize bacterial surface charge and bridge the bacterial aggregates. CM-chitosan decreased 12.9% and 12.8% of the cell surface charge of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively. CM-chitosan may serve as an antibiofilm agent.

1. Introduction

A biofilm is described as a structured community of bacteria enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Wang et al., 2009). Such a mode of growth can be considered as a lifestyle different from the planktonic one. Biofilm forms in two steps: bacteria adhere to the surface of an exotic substratum first and then accumulate there to form complex film architecture. Microbial colonization and biofilm formation on medical devices significantly increase the risk of infection (Chaiban, Hanna, Dvorak, & Raad, 2005; Eiff, Jansen, Kohnen, & Becker, 2005; Stoodley, Costerton, & Stoodley, 2004). Bacteria within biofilms were found to be 1000-fold less susceptible to diverse antibiotics than planktonic ones (Houdt & Michiels, 2005; Stobie et al., 2008; Swidsinski, Loening-Baucke, Bengmark, Scholze, & Doerffel, 2008).

Direct application of antibiotics is not acceptable in clinic; such a practice promotes the formation of antibiotic resistance (Hetrick, Shin, Paul, & Schoenfisch, 2009). In addition, tight biofilms are difficult to be removed by diverse agents including detergents and proteases, and physical treatments including heating, sonication and vortexing (Fine, Furgang, Kaplan, Charlesworth, & Figurski, 1999). Biofilms are capable of releasing either single cells or small cell clusters, forming new biofilms (Kaplan & Fine, 2002). Antiseptic agents should kill surface bacteria; however, they may not be able to inhibit their resurgence (MacDonald, Santa, & Brozel, 2000).

The antibacterial activity of chitosan is due to the presence of amine functions. Chitosan is promising for diverse pharmaceutical applications, e.g. tapes for wound dressing, tooth paste and artificial tears (Felt, Carrel, Baehni, Buri, & Gurny, 2000; Kim et al., 1999). Recently, the effect of chitosan on preventing biofilm formation has been reported (Carlson, Taffs, Davison, & Stewart, 2008; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Ladet, David, & Domard, 2008; Rinaudo, 2006). Antibiofilm has been proposed to underline such effect. It is easy to modify chitosan with quaternization, hydrophobization, phosphorylation or attachment of polyethyleneoxide to obtain diverse derivatives (Amaral, Granja, & Barbosa, 2005; Ercelen et al., 2006; Sagnella & Mai-Ngam, 2005), some of which have achieved encouraging results (Jayakumar, Prabaharan, Reis, & Mano, 2005; Jayakumar, Nagahama, Furuike, & Tamura, 2007; Park, Saravanakumar, Kim, & Kwon, 2010; Prabaharan & Gong, 2008; Shi, Neoh, Kang, & Wang, 2006). Of these derivates, carboxymethyl chitosan (CM-chitosan) has been widely used in fields such as in drug delivery (Liu, Chen, & Park, 2005; Tan & Liu, 2009) and wound dressing (Muzzarelli, 2009; Wongpanit et al., 2005); it showed excellent biocompatibility, biodegradability, biological activity and low toxicity (Hiroki, Tran, Nagasawa, Yagi, & Tamada, 2009; Jayakumar & Tamura, 2006; Jayakumar, Nwe, Tokura, Tamura, 2007; Muzzarelli, 1988).

The aim of this study is to assess the effect of CM-chitosan on the formation of biofilms.

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doi:10.1016/j.carbpol.2011.01.036

2. Materials and methods

2.1. Bacterial strains, media and reagents

The bacterial strains used in this study were *Staphylococcus aureus* RN6390 and 15981, *Staphylococcus epidermidis* QY301, *Pseudomonas aeruginosa* PAO1 and FRD1, *Escherichia coli* K12 and *Chromobacterium violaceum* 12472. The strains were cultured in either Tryptic Soy Broth (TSB, Sigma) or Luria-Bertani (LB, Sigma) medium at 37 °C. For flow cell biofilm experiments, *S. aureus* RN6390 harboring pSB2019 and *P. aeruginosa* PAO1 harboring pMRP9-1 were incubated with an appropriate antibiotic (pSB2019: chloromycetin 15 μg/ml, pMRP9-1: carbenicillin 200 μg/ml).

CM-chitosan (deacetylation degree = 90% and viscosity = 80 MPa s) was obtained from Oddfoni Chemical Co. (Nanjing, China). The O-carboxymethylation degree, further calculated based on ^{1}H NMR spectrum, was 89%.

2.2. Biofilm assay

Biofilm formation in the wells of 96-well microtiter plates was conducted as described previously (Kaplan, Ragunath, Velliyagounder, Fine, & Ramasubbu, 2004). In brief, bacteria from an overnight culture were diluted to 1×10^6 colony-forming units (CFU)/ml with media containing CM-chitosan at different concentrations (range 0–2.5 mg/ml). The medium for *S. aureus* RN6390 was TSB supplemented with 0.5% (w/v) glucose and that for *P. aeruginosa* PAO1 was LB. Each well (Costar, Cambridge, MA, USA) was filled with 100 μl aliquots of the diluted culture. The plates were incubated at 37 °C for 24 h without shaking, and washed three times with 0.9% (w/v) NaCl. Biofilms were stained with 150 μl of 0.1% (w/v) crystal violet solution for 30 min, washed and incubated in 200 μl of 30% (v/v) acetic acid for 15 min to extract the crystal violet retained by the cells. The extract was used to determine the amount of biofilm by measuring its A_{590} with a microtiter plate reader (DynaTech, Chantilly, VA).

At least six replicates were conducted for each sample, and each experiment was performed at least in triplicate.

2.3. Biofilm formation in flow cells

Dynamic biofilm formation was performed in "once-through" flow cells (4.5 mm × 2 mm × 35 mm). Each flow cell was injected with 0.8 ml of the diluted culture of test strain, and incubated at room temperature for 40 min without flow. Flow was started at a constant rate of 0.5 ml/min. The images were obtained using confocal laser scanning microscope (CLSM). All assays were performed at least in triplicate.

2.4. Antibacterial activity assay

The antibacterial activity of CM-chitosan was tested using the method developed for MIC determination. In brief, cell suspension was inoculated into the bottles in which CM-chitosan was mixed at different final concentrations. The inoculum was incubated at 37 °C in a shaking incubator (150 rpm) for 24 h. After vortexing thoroughly, the bacteria were spread on TSB or LB agar plates in order to test the growth rate and cell viability. All the tests were carried out in triplicate.

2.5. Bacterial adhesion assay

Initial adhesion was performed as described previously (Fournier & Hooper, 2000). In brief, *S. aureus* RN6390 and *P. aeruginosa* PAO1 were cultured as described above to a density of 10^6 CFU/ml. One hundred microliters of culture was added to each well of a 96-well microplate and incubated at 37 °C for 1 h. The 96-well microplate was rinsed gently five times with 100 μl of 0.9% (w/v) NaCl. The adhered cells were stained with crystal violet as described above. All experiments were done in triplicate.

Bacterial adhesion and bacterial viability was quantified with microscopy immediately after the adhesion event. For each surface analyzed, 20 pictures were taken; covering the entire surface and the results represented the means of 20 different microscopic fields. Each image was then analyzed for determining the number of adhering cells.

2.6. Cell–cell interaction analysis

Cell–cell interaction was determined as described previously (Valle et al., 2006) with minor modifications. After inoculation, CM-chitosan was added at different times to different concentrations ranging from 0 to 2.500 mg/ml. After being incubated at 37 °C for an additional 24 h, biofilms were rinsed, stained with crystal violet and quantified as described above.

2.7. Flocculation assay

The flocculation assay was performed as described previously (Bratskaya, Avramenko, Schwarz, & Philippova, 2006) with some modifications. In brief, CM-chitosan solution was added to 10 ml of bacterial suspension with stirring on a Vortex mixer (2000 r/min, 5 s). The mixture was shaken for 10 min on a rotary shaker at 200 r/min and allowed to stand for 30 min. Then, OD_{600} of supernatant was measured. OD_{600} of bacteria treated at the same stirring condition but not added CM-chitosan was measured simultaneously. The flocculation was expressed as the relative decrease of turbidity to a reference without CM-chitosan and calculated as $(1 - OD_{sample}/OD_{control}) \times 100\%$. Experiments were performed at room temperature. All samples were run in triplicate and data represent mean values.

2.8. Cell surface charge assay

The cytochrome *c* binding assay was used to estimate the bacterial surface charge, which was carried out as previously described (Peschel et al., 1999). The bacteria were pelleted by centrifugation, rinsed and resuspended in morpholinepropanesulfonic acid (MOPS) buffer (20 mM; pH 7.0) to a final OD_{578} of 7.0. After being incubated with 0.5 mg/ml cytochrome *c* for 10 min, the cells were removed by centrifugation at 12,000 rpm for 5 min. The amount of cytochrome *c* in supernatant was determined with a photometer at 530 nm. The percentage of cytochrome *c* bound to the cells to the total was calculated as the following: $(1 - OD_{sample}/OD_{control}) \times 100\%$.

3. Results and discussion

3.1. CM-chitosan prevents biofilm formation in microtiter plate

Biofilm formation by all tested strains was significantly reduced in the presence of CM-chitosan (Table 1). With the increase of CM-chitosan concentration, a progressive reduction of biofilms was observed. CM-chitosan caused a 74.6% reduction of Gram-positive bacterial biofilm at 2.500 mg/ml and 81.6% reduction of Gram-negative bacterial biofilm at 0.156 mg/ml. CM-chitosan is active in preventing biofilm formation. CM-chitosan is more effective in preventing Gram-negative bacterial biofilm formation than in preventing the formation of Gram-positive bacterial biofilm, depending on the different physical and chemical properties of these bacteria.

Table 1
Prevention effect of CM-chitosan on (A) Gram-positive or (B) Gram-negative bacterial biofilm formations in 96-well microtiter plates. The amount of biofilm was quantified by measuring its A_{590}.

(A)	Biofilm biomass (OD_{590})			
	0.000 mg/ml	0.156 mg/ml	0.625 mg/ml	2.500 mg/ml
S. aureus 15981	2.35 ± 0.05	1.68 ± 0.06	1.30 ± 0.05	0.74 ± 0.03
S. aureus RN6390	2.80 ± 0.04	2.25 ± 0.04	1.42 ± 0.04	0.71 ± 0.03
S. epidermidis QY301	2.76 ± 0.04	2.55 ± 0.04	2.16 ± 0.01	1.09 ± 0.04
(B)	Biofilm biomass (OD_{590})			
	0.000 mg/ml	0.010 mg/ml	0.039 mg/ml	0.156 mg/ml
P. aeruginosa PAO1	1.25 ± 0.04	0.41 ± 0.04	0.25 ± 0.02	0.23 ± 0.02
P. aeruginosa FRD1	0.87 ± 0.02	0.55 ± 0.01	0.42 ± 0.02	0.29 ± 0.05
E. coli K12	0.41 ± 0.03	0.28 ± 0.03	0.22 ± 0.01	0.14 ± 0.01
C. violaceum 12472	0.37 ± 0.03	0.24 ± 0.03	0.21 ± 0.03	0.15 ± 0.01

3.2. Prevention of biofilm formation in flow cells

The tested strains, *S. aureus* RN6390 and *P. aeruginosa* PAO1, were tagged with green fluorescent proteins; therefore their observation is easy with a fluorescence microscopy. CM-chitosan prevented significantly the formation of biofilms (Fig. 1). When the concentration of CM-chitosan increased to 2.500 mg/ml (*S. aureus* RN6390) and 0.156 mg/ml (*P. aeruginosa* PAO1), biofilm formation in flow cells was not observable, demonstrating that CM-chitosan severely prevented bacterial biofilm formation in dynamic conditions.

CM-chitosan did not show both bactericidal and bacteriostatic activities to *S. aureus* and *P. aeruginosa* at concentrations mentioned in Table 2. The other bacteria showed similar results (data not

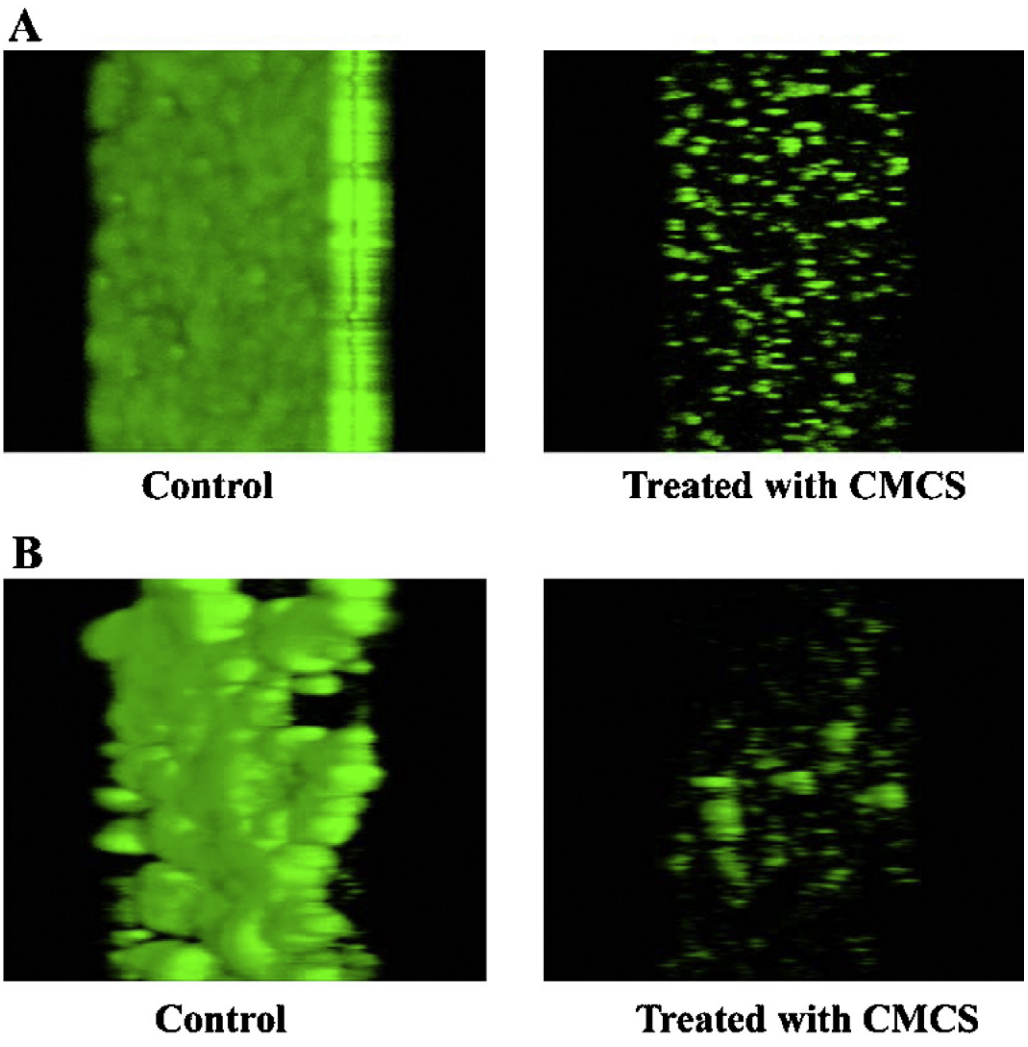

Fig. 1. CLSM images of biofilm formations in flow cell with media supplemented without (left) or with (right) CM-chitosan: (A) *S. aureus* RN6390, (B) *P. aeruginosa* PAO1.

Table 2
Cell viability of (A) *S. aureus* RN6390 or (B) *P. aeruginosa* PAO1 in medium with CM-chitosan.

Concentration (mg/ml)	Viable cells (CFU/ml)
(A)	
0.000	$(1.36 \pm 0.32) \times 10^8$
0.156	$(1.28 \pm 0.25) \times 10^8$
0.625	$(1.35 \pm 0.38) \times 10^8$
2.500	$(1.30 \pm 0.12) \times 10^8$
(B)	
0.000	$(1.38 \pm 0.31) \times 10^8$
0.010	$(1.33 \pm 0.40) \times 10^8$
0.039	$(1.36 \pm 0.35) \times 10^8$
0.156	$(1.33 \pm 0.31) \times 10^8$

shown). This analysis suggested that CM-chitosan did not interfere bacterial growth.

3.3. CM-chitosan impairs cell-surface initial interaction

After incubation, more than 90% cells of both *S. aureus* RN6390 or *P. aeruginosa* PAO1 were restrained from adhering to surfaces in 1 h. CM-chitosan may inhibit the adhesion of bacteria in preventing biofilm formation (Fig. 2).

Table 3
Prevention effect of CM-chitosan on biofilm formations of (A) *S. aureus* RN6390 or (B) *P. aeruginosa* PAO1 by addition of CM-chitosan at 1 or 6 h after biofilm initiation. The amount of biofilm was quantified by measuring its A_{590}.

Concentration (mg/ml)	Biofilm biomass (OD_{590})	
	1 h	6 h
(A)		
0.000	2.79 ± 0.04	2.76 ± 0.03
0.156	2.27 ± 0.03	2.65 ± 0.03
0.625	1.50 ± 0.02	2.14 ± 0.02
2.500	1.03 ± 0.05	1.80 ± 0.03
(B)		
0.000	0.85 ± 0.03	1.14 ± 0.04
0.010	0.51 ± 0.03	0.89 ± 0.04
0.039	0.33 ± 0.01	0.66 ± 0.05
0.156	0.25 ± 0.03	0.62 ± 0.02

3.4. CM-chitosan reduces cell–cell interaction

CM-chitosan was added to biofilms already developed at different stages. CM-chitosan blocked the further development of biofilms which have developed for either 1 or 6 h (Table 3), suggesting that CM-chitosan prevented biofilm formation by reducing intercellular adhesion.

CM-chitosan caused a reduction of bacterial adhesion. The process of biofilm formation was also interfered by CM-chitosan. The

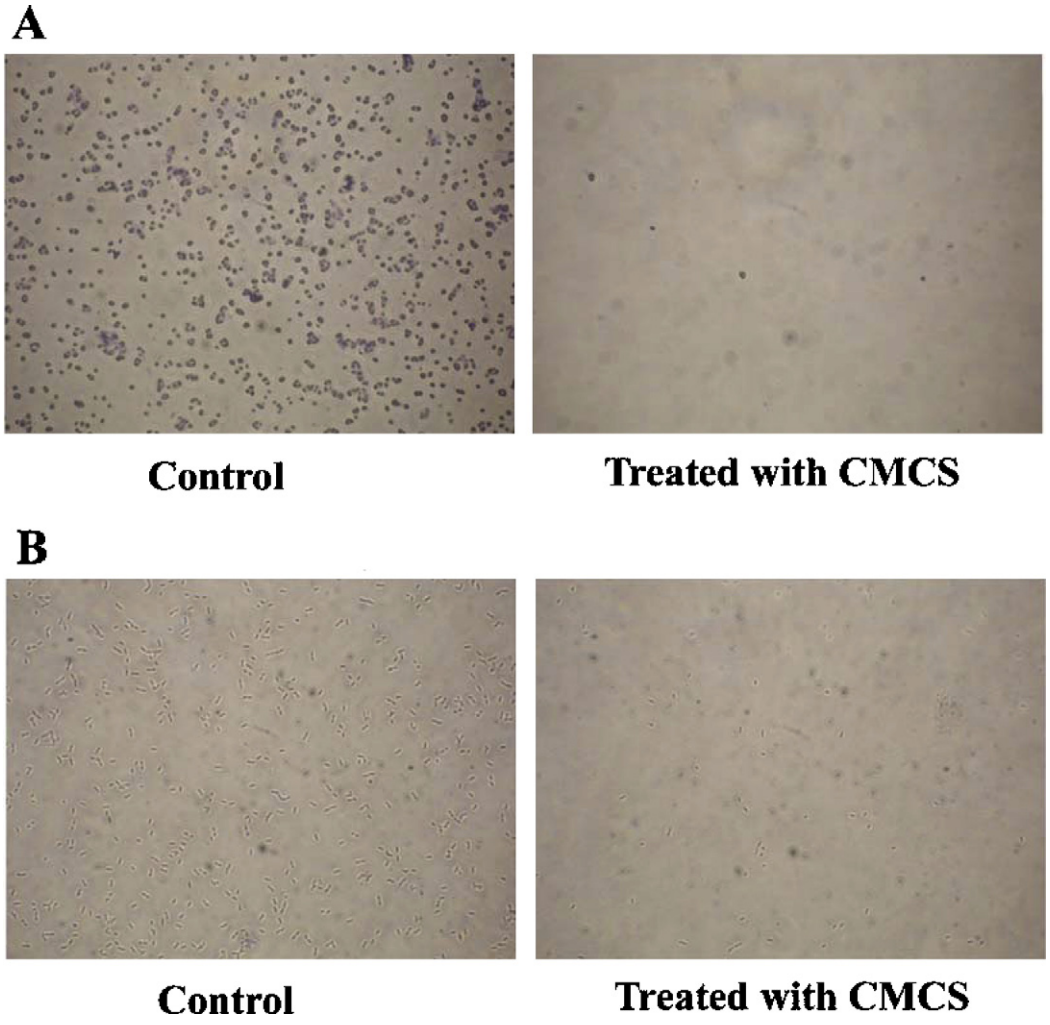

Fig. 2. Light microscopy images of the initial adhesion of (A) *S. aureus* RN6390 or (B) *P. aeruginosa* PAO1 attached on the surfaces without (left) or with (right) CM-chitosan in cultures (×600).

individual bacterial cell will succumb to relatively fast moving fluid and high wall shear stress; therefore being easily removed under dynamic flow conditions if initial bacterial adhesion and subsequent cellular contacting were prevented by CM-chitosan. CM-chitosan promises to be a new, non-antibiotic agent of preventing biofilm formation on medical devices.

3.5. Flocculation caused by CM-chitosan

Flocculation efficiency increased with the concentration of CM-chitosan. When the concentration of CM-chitosan was 2.500 mg/ml, the flocculation of *S. aureus* RN6390 became visible at the naked eye. Even at the lowest concentration, the flocculation of bacteria took place (Fig. 3). We proposed that biofilm formation was prevented by bacterial aggregation caused by inhibition of bacterial adherence and reduction of cell–cell interaction. The formation of N-carboxybutyl chitosan flocs on the microbial cell wall was documented by Muzzarelli et al. (1990).

When cultured in broth, bacteria form extremely tenacious biofilms on surfaces. However, we observed that cells tended to flocculate when CM-chitosan is added into medium. Flocculation of polymers should be a process in which bacteria adhere to each other due to the existence of flocculants on their surfaces. Such a process is closely related to bacterial adhesion to surfaces (Sabin, Stranda, Nordengen, & Østgaard, 2002). As bacteria aggregate, the increase in total particulate mass reduces the likelihood that attached aggregates remain adherent (Liljemark, Bloomquist, & Germaine, 1981). Bacterial aggregates are easier to be removed than individual bacterial cells (Liljemark et al., 1981; Magunuson, Ericson, & Pruitt, 1976). Our findings demonstrated that the inhibition of bacterial adherence and cell–cell interaction was indeed ascribed to the aggregation of bacteria.

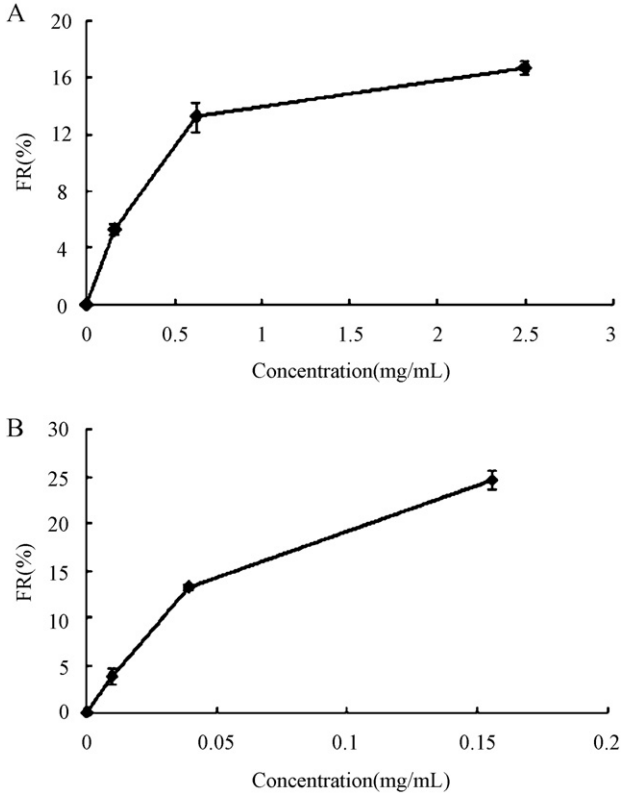

Fig. 3. Flocculation ratio of (A) *S. aureus* RN6390 or (B) *P. aeruginosa* PAO1.

Table 4
Surface charge of (A) *S. aureus* RN6390 or (B) *P. aeruginosa* PAO1 cultured with CM-chitosan.

Concentration (mg/ml)	Cell surface charge (%)
(A)	
0.000	67.18 ± 0.23
0.156	66.12 ± 0.41
0.625	65.90 ± 0.15
2.500	54.28 ± 0.38
(B)	
0.000	58.39 ± 0.13
0.010	57.39 ± 0.39
0.039	48.33 ± 0.40
0.156	45.58 ± 0.33

3.6. CM-chitosan affects cell surface charge

Generally, attractive electrostatic interaction between bacteria and chitosan is often implicated as important parameters in floc formation. To determine a possible relation between flocculation and bacterial surface charge, cytochrome *c* was used to estimate the relative surface charge of cells. The optical density of respective supernatant decreased with the increase of CM-chitosan concentration. The percentage of cytochrome c bound to bacterial pellet was lower (Table 4). Therefore, bacterial cell walls were less negatively charged because they were partially or completely neutralized by CM-chitosan. The flocculating effect of CM-chitosan can be enhanced by the bridging mechanism due to its high molecular weight. CM-chitosan is involved in, at least in part, the aggregation through charge neutralization and flocculation by bridging mechanism.

4. Conclusion

The biofilm-preventing activity of CM-chitosan promises it to be a simple and practical agent for biofilm control. However, bacterial surface is structurally complex and the function of CM-chitosan in biofilm-controlling deserves further studies. Although the mechanism is not fully understood, our findings demonstrated a potential application of CM-chitosan as a non-antibiotic biofilm-related infections preventing agent.

Acknowledgements

This work was supported by National High-tech RandD Program (2007AA091506 and 2007AA09Z418), National SandT Major Project (2009ZX09301-013), Program for Changjiang Scholars and Innovative Research Team in University (IRT0944) and Special Fund for Marine Scientific Research in the Public Interest (201005024).

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