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Preparation and evaluation of a kind of bacterial cellulose dry films with antibacterial properties

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ABSTRACT

A kind of antibacterial bacterial cellulose (BC) dry film was developed and characterized as a potential functional wound dressing for acute traumas treatment. To achieve this, a freeze-dried BC film was immersed in a benzalkonium chloride solution, which belongs to cationic surfactant type antimicrobial agent, followed by another freeze-drying step. Some physical and antimicrobial properties of the prepared BC films were investigated and the results showed that the drug-loading capacity of the BC dry film was about 0.116 mg/cm^2 when soaked in 0.102% benzalkonium chloride solution. High water absorbing capacity, an important quality for wound dressings was also achieved with a swelling ratio of 26.2 in deionized water and of 37.3 in saline solution. With respect to the antimicrobial effect, a stable and prolonged antimicrobial activity for at least 24 h was obtained especially against *Staphylococcus aureus* and *Bacillus subtilis*, which were general Gram-positive bacteria that found on the contaminated wound.

1. Introduction

Bacterial cellulose (BC) is a kind of natural cellulose synthesized in abundance by *Acetobacter xylinum.* Although chemically identical to plant cellulose, BC is characterized by a unique fibrillar nanostructure which determines its distinguished physical and mechanical properties such as high porosity, high tensile strength, high water binding capacity and good biocompatibility (Hong & Qiu, 2008; Iguchi, Yamanaka, & Budhiono, 2000). Therefore, bacterial cellulose has been used widely as high quality audio membranes (Nishi et al., 1990), electronic paper (Shah & Brown, 2005), membranes of fuel cells (Evans, O'Neill, Malyvanh, Lee, & Woodward, 2003), and biomedical materials (Alvarez, Patel, Booker, & Markowitz, 2004; Czaja, Krystynowicz, Bielecki, & Brown, 2006; Czaja et al., 2007; Klemm et al., 2006; Mayal, Mayal, Dias Garcia Mayall, Rocha, & Narques, 1990).

As a kind of natural biomedical material, BC membrane for wound healing has attracted more and more attentions. BC is considered as an ideal wound dressing meeting the requirements of modern wound dressing materials used for chronic traumas (Czaja et al., 2006). A commercial product Biofill, which actually was a partially dried BC membrane, was investigated for wound healing of burns and chronic ulcers. The studies demonstrated that Biofill had a more effective performance than other wound dressing materials in accelerating the healing process, pain relief and so on (Fontana et al., 1990). Another BC wound dressing called XCell was used to heal chronic venous ulcers, and once again, the BC dressing showed satisfactory effect in treating these chronic skin abnormalities (Rovee, 2003).

However, BC itself does not have antibacterial property which is crucial to prevent wound infection during the wound healing. In order to obtain antibacterial activity in BC wound dressing, some researches have been carried out. The Xylos company developed an improved bacterial cellulose wound dressing product "XCell Antimicrobial Wound Dressing", which has been successfully marketed in the US since 2003 (Frankel, Serafica, & Damien, 2004). Maneerung et al. impregnated silver nanoparticles into bacterial cellulose and the freeze-dried BC film exhibited strong antimicrobial activity against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) (Maneerung, Tokura, & Rujiravanit, 2008). In 2009, Cai et al. developed bacterial cellulose-chitosan blends by immersing wet BC pellicle in chitosan solution followed by freeze-drying. The study demonstrated that the mechanical and antibacterial properties of BC-chitosan pellicle were suitable for potential biomedical applications such as tissue engineering scaffold and would dressing material (Cai, Jin, & Kim, 2009).

Up to now, the BC wound dressings were mostly investigated and applied for chronic wound treatment, and to our knowledge there were very little researches had been made on the wound

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healing of acute traumas which happened more frequently in people's daily life. Considering the convenience and portability in the practical applications, the dry form of BC film was preferred to the wet one. Hence, in this paper, a kind of BC dry film with antimicrobial properties was developed by using benzalkonium chloride as the antimicrobial agent. Benzalkonium chloride is a sort of antimicrobial cationic surfactant, which was widely used in commercial wound dressings and had a strong role against Gram-positive bacteria (Elder, 1989). Some properties of BC dry film including swelling ratio, slow release of benzalkonium chloride and antimicrobial effect were investigated in order to achieve good performances in acute traumas treatment for the future.

2. Materials and methods

2.1. Bacterial strains

A. xylinum (strain 1.1812), *E. coli* (strain 1.1100), *S. aureus* (strain 1.128) and *Bacillus subtilis* (strain 1.1630) were purchased from Institute of Microbiology Chinese Academy of Science and maintained on solid agar medium at 4 °C.

2.2. Culture media

Both the seed medium and the fermentation medium used for bacterial cellulose production contained 2.5% (w/v) mannitol, 0.3% (w/v) tryptone, 0.5% (w/v) yeast extract. Prior to autoclaving at 121 °C the pH value of the medium was adjusted to 5.0.

2.3. Culture conditions

Pre-inoculum for all experiments was prepared by transferring a single *A. xylinum* colony grown on agar seed medium into 100 mL of liquid seed medium. After 24 h of agitated cultivation at 30 °C, 6 mL of cell suspension was introduced into a 250-mL Erlenmeyer flask containing 100 mL of fermentation medium, and then incubated statically at 30 °C for 7 days.

2.4. Purification of bacterial cellulose

The harvested BC films were washed with distilled water to remove some medium components and then boiled in 1.0% NaOH solution for 2 h to eliminate attached cells and other impurity. After that, the BC films were further purified to remove other residues by distilled water washing until the pH of the washing liquid was neutral. Finally, the bacterial cellulose films were cut into a disc shape with a diameter of 15 mm.

2.5. Preparation of antimicrobial dry films

The disc-shaped bacterial cellulose films were freeze-dried for 24 h and then one piece of the dry BC film was immersed in 25 mL of the benzalkonium chloride solution for 24 h. After that the BC film was removed from the benzalkonium chloride solution and immersed briefly in distilled water (for 10 s) and wiped slightly once with filter paper to remove non-absorbed benzalkonium chloride. Each film was treated under the same way. Finally, the resulting BC films were freeze-dried again for 24 h to obtain the antimicrobial dry films.

2.6. Analysis of drug-loading capacity of BC films

The concentration of benzalkonium chloride solution was determined spectrophotometrically by measuring the absorbance value at 263 nm. In a typical experiment, one piece of freeze-dried BC film was weighted up and immersed in 25 mL of the benzalkonium chloride solution for 24 h under static condition. Then the cellulose piece was taken from the solution and treated with filter paper for the removal of extra free water on the BC film. Subsequently, the soaked film was weighed directly. The amount of the absorbed benzalkonium chloride in BC film was determined indirectly by comparing the difference between the amount of benzalkonium chloride before and after the film was soaked. Six pieces of films were applied to calculate drug-loading capacity of BC films for each feed drug concentration. The drug-loading capacity of BC film (mg/cm^2) was calculated as the amount of the absorbed benzalkonium chloride per square centimeter of BC dry film.

2.7. Analysis of swelling ratio of BC dry films

In a typical experiment, one piece of the antimicrobial BC dry film was weighted up and then immersed in a 25 mL of deionized water or 0.9% saline solution for 24 h at room temperature. Then the cellulose piece was removed from the deionized water or the saline solution, treated with filter paper for extra water removal and weighted up again. The swelling rate of BC dry film was calculated as follows: swelling ratio = $(W_{s,s} - W_i)/W_i$, where W_i was the initial weight of dry sample and $W_{s,s}$ was the weight of sample in swollen state.

2.8. Release of benzalkonium chloride in BC dry films

The release behavior of benzalkonium chloride in one piece of BC dry film was carried out in a sealed beaker containing 25 mL of deionized water. The beaker was incubated statically at 37 °C for 24 h and samples drawn from the systems every 2 h were analyzed spectrophotometrically by measuring the absorbance at 263 nm. Triplicate experiments were carried out.

2.9. Analysis of antibacterial activity

The antimicrobial activities of the benzalkonium chloride-containing BC dry films were investigated by two methods against *E. coli* as a Gram-negative model bacterium and *S. aureus* and *B. subtilis* as Gram-positive model bacteria (Alagiachidambaram, Pavankumar, Vasanthamallika, & Sankaran, 2009).

2.9.1. Disc diffusion method

This method was performed in Luria–Bertani (LB) medium solid agar Petri dish. The BC dry films immersed in benzalkonium chloride solutions with different concentrations (0.026%, 0.051%, 0.077%, 0.102%, and 0.128%, w/w) were sterilized by autoclaving at 121 °C for 20 min and placed on *E. coli*-, *S. aureus*- and *B. subtilis*-cultured agar plates, respectively. The agar plates were then incubated at 37 °C for 24 h and the antimicrobial activity of BC film was determined by measuring the difference of the semi-diameter between inhibition zone and BC film.

2.9.2. Growth curve method

Single colony of *E. coli*, *S. aureus* and *B. subtilis* grown on agar culture medium were transferred into 100 mL of liquid seed medium separately. After 12 h of agitated cultivation at 37 °C, 5 mL of cell suspension from the seed culture was introduced into a 250-mL Erlenmeyer flask containing 100 mL of fermentation medium, and then a piece of antimicrobial BC dry film was put in the Erlenmeyer flask. The culture was kept at 37 °C for 24 h under agitation. Samples drawn from the systems every 2 h were analyzed spectrophotometrically by measuring the absorbance at 600 nm. The growth of the three model bacteria with pure BC films was also determined as control. Triplicate experiments for each bacterium were carried out.

2.10. Growth curve of E. coli in the cultures containing benzalkonium chloride

Single colony of *E. coli* grown on agar culture medium was transferred into 100 mL of liquid seed culture medium. After 12 h of agitated cultivation at 37 °C, 5 mL of cell suspension from the seed culture was inoculated into a 250-mL Erlenmeyer flask containing 100 mL of fermentation medium. Benzalkonium chloride stock solution was added in the Erlenmeyer flasks to the final concentration of 0.003%, 0.006%, 0.013%, 0.026%, 0.051%, 0.077% and 0.102%, respectively. The cultures were agitated at 37 °C for 9 h. Samples drawn from the cultures after 9 h were analyzed spectrophotometrically by measuring the absorbance at 600 nm. The growth of *E. coli* in the culture without benzalkonium chloride was also investigated as a negative control.

3. Results and discussion

3.1. Effect of antimicrobial agent concentration on antimicrobial activity

Five different concentrations of the benzalkonium chloride solution including 0.026%, 0.051%, 0.077%, 0.102% and 0.128% (w/w) were chosen to prepare the antimicrobial BC dry films. The concentration range investigated here was based on the common concentrations of benzalkonium chloride solution used in the treatment of wound, skin and mucous membrane as well as the sterilization of medical devices (Elder, 1989). The effects of concentration of the benzalkonium chloride solution on the antimicrobial activity were evaluated by the disc diffusion method and the results were shown in Fig. 1. It was found that all the BC films prepared under different concentrations of benzalkonium chloride solution exhibited antimicrobial activities against the three model bacteria, and the higher concentration of benzalkonium chloride solution brought about the higher antimicrobial activity. Additionally, based on the diameters of inhibition zones against the model bacteria, the best antimicrobial activity was displayed on *B. subtilis*, followed by *S. aureus* and *E. coli*.

The relationship between the feed drug concentrations and the drug-uploading capacities of BC films was demonstrated in Table 1. As the five concentrations of benzalkonium chloride were rather low, the drug-uploading capacities of BC films increased almost linearly with the rise of feed drug concentrations. Moreover, the comparisons of antibacterial activities between the benzalkonium chloride-containing BC dry film and the BC dry film without drug as control were also investigated. The BC dry film prepared in 0.102% of benzalkonium chloride solution was taken as a representative, and its drug-loading capacity of benzalkonium chloride was about 0.116 mg/cm^2. As seen in Fig. 2, no inhibition zone was observed with pure BC film against all the three model bacteria. It could be concluded that the antimicrobial activity was only attributed to benzalkonium chloride absorbed into bacterial cellulose film and not due to bacterial cellulose itself.

Table 1
Relationship between the feed drug concentrations and the drug-uploading capacities of BC films.

Drug concentrations	Absorbed benzalkonium chloride (mg)	Drug upload capacities (mg/cm^2)
0.026%	0.0512	0.0291
0.051%	0.1023	0.0579
0.077%	0.1535	0.0869
0.102%	0.2046	0.116
0.128%	0.2558	0.145

The purpose to show Fig. 2 was just to demonstrate qualitatively an antibacterial activity against one bacterium in a Petri dish. The sizes of wet disc-shaped bacterial cellulose films were same, but the shape of BC films would change slightly after freeze-drying. In order to quantificationally evaluate the antibacterial effects, three drug-loading films prepared at same benzalkonium chloride concentration were chosen to determine the antibacterial activity for each bacterium in the experiments. The difference of the semi-diameter between inhibition zone and BC film rather than the semi-diameter of inhibition zone was measured in order to reduce the disturbance from the film size change and the standard deviation from three films was included in Fig. 1.

3.2. Analysis of swelling ratio of bacterial cellulose dry films

The liquid absorbing capacity was important for the wound dressing during wound healing process for both the chronic and acute trauma. In this paper, the water absorption performance of the antimicrobial BC dry films reflected by swelling ratio was investigated in both deionized water and saline solution. The BC dry film prepared in 0.102% benzalkonium chloride solution was used as a model and five samples were used with the results exhibited in Table 2. Based on the data, good water-binding ability with a swelling ratio of 26.2 in deionized water and of 37.3 in saline solution was achieved by antimicrobial BC dry films; that is to say, the BC film prepared in our work could hold at least 26.2-fold water of its own weight within 24 h. The high water absorption ability of the antimicrobial BC dry film is crucial for wound dressing to

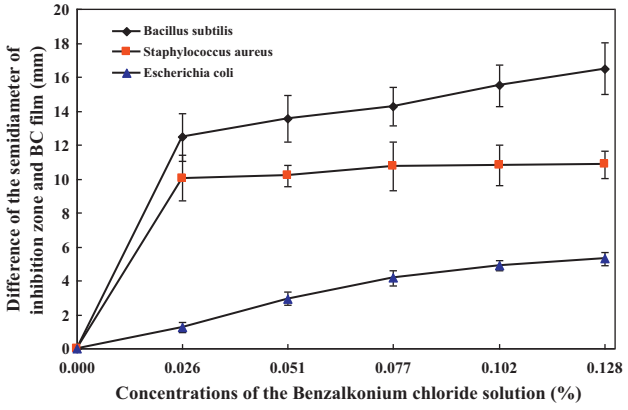

Fig. 1. Effect of concentrations of benzalkonium chloride solution on antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* on agar plates. Error bar represents the standard deviation for triplicate experiments.

Table 2
Swelling ratio of bacterial cellulose dry films in deionized water and saline solution.

Samples	Weight of dry film (g)	Weight of swollen film (g)	Swelling ratio
Deionized water			
1	0.0025	0.0697	26.88
2	0.0020	0.0510	24.50
3	0.0018	0.0481	25.72
4	0.0026	0.0718	26.62
5	0.0017	0.0481	27.29
Average	–	–	26.20 ± 0.99
Saline solution			
1	0.0017	0.0759	43.65
2	0.0023	0.0843	35.65
3	0.0024	0.0777	31.38
4	0.0020	0.0758	36.90
5	0.0019	0.0759	38.95
Average	–	–	37.30 ± 4.50

The BC film was prepared in a 0.102% benzalkonium chloride solution.

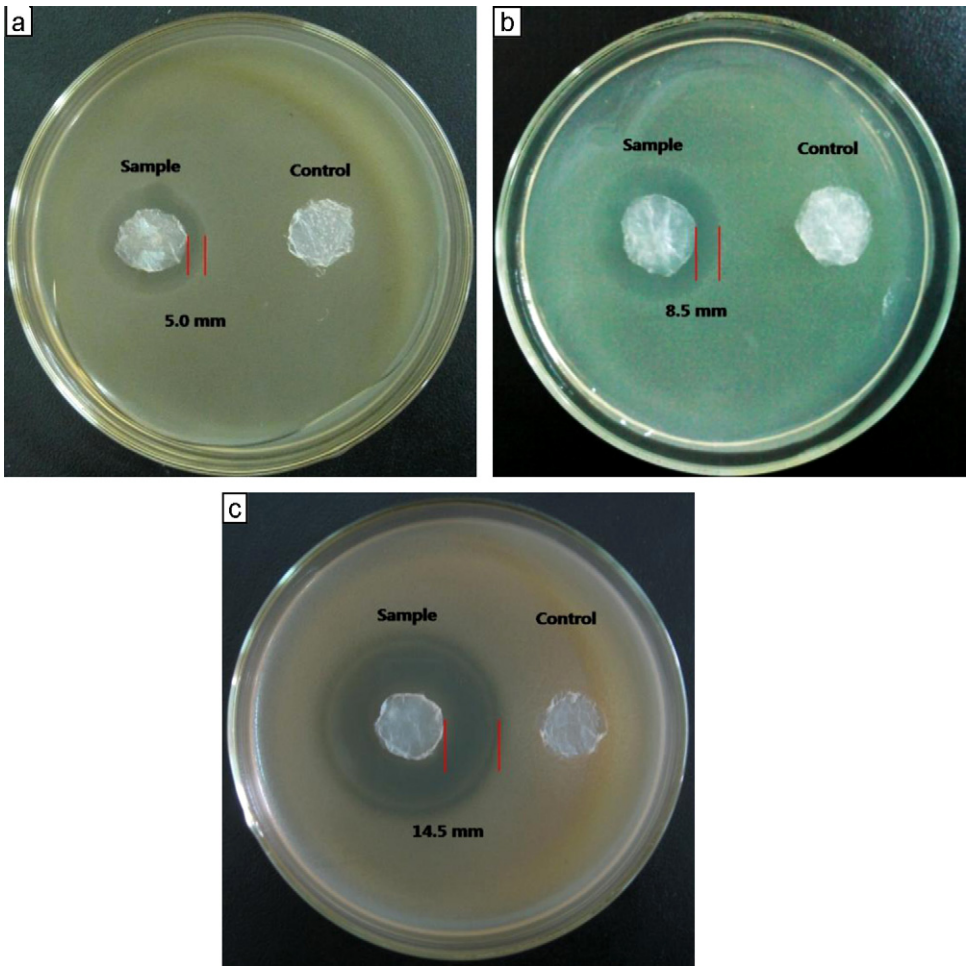

Fig. 2. Comparisons of antibacterial activity between benzalkonium chloride-containing BC dry films and BC without drug as control against (a) *Escherichia coli*, (b) *Staphylococcus aureus* and (c) *Bacillus subtilis*. The BC film was prepared in a 0.102% drug solution.

absorb blood and tissue fluid on acute traumas and would promote wound healing.

3.3. Release of benzalkonium chloride molecules in BC dry films

For an antimicrobial material, the permanence of the antimicrobial activity is important in practical applications, which need a stable and prolonged release of antimicrobial agents. The release behavior of benzalkonium chloride molecules from the BC dry film, prepared in the benzalkonium chloride solution of 0.102% as a representative, was studied and the data were illustrated in Fig. 3. It shows that the benzalkonium chloride molecules were released gradually from the BC dry films within one day with about 66% release of benzalkonium chloride after 24 h. The unique fibrillar nanostructure and three-dimensional network of BC material might attribute to the slow release behavior of benzalkonium chloride in water.

Generally in the area of drug delivery, if drug embedded in nanoparticles or nanofibers, three-dimensional porous structure can facilitate the drug release process due to their larger surface areas. So nanoparticle drug carriers are required to facilitate intramural drug transport and uptake (Song et al., 2009). Nanofibers also have a faster release rate than microfibers do (Sill & von Recum, 2008). In our study the drug benzalkonium chloride was not embedded in fibers but entrapped in the network of bacterial cellulose hydrogels or adsorbed on nanofibers of BC. In hydrogel, the rate of drug release depends on the water content of the swollen hydrogel, as well as on its network parameters such as degree of crosslinking and mesh size (Sannino, Demitri, & Madaghiele, 2009). A SEM image regarding the fibrillar nanostructured and three-dimensional network in the BC film used in our study is shown in Fig. 4. The highly porous structure can easily permits loading of drugs into the gel matrix and subsequent drug release at a rate dependent on the diffusion coefficient of the small molecule or macromolecule through the gel network (Hoare & Kohane, 2008).

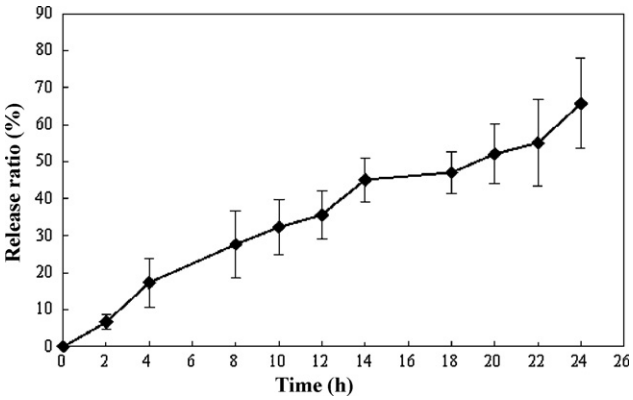

Fig. 3. Benzalkonium chloride molecules releasing behavior of the bacterial cellulose dry films. The BC film was prepared in a 0.102% drug solution. Error bar represents the standard deviation for triplicate experiments.

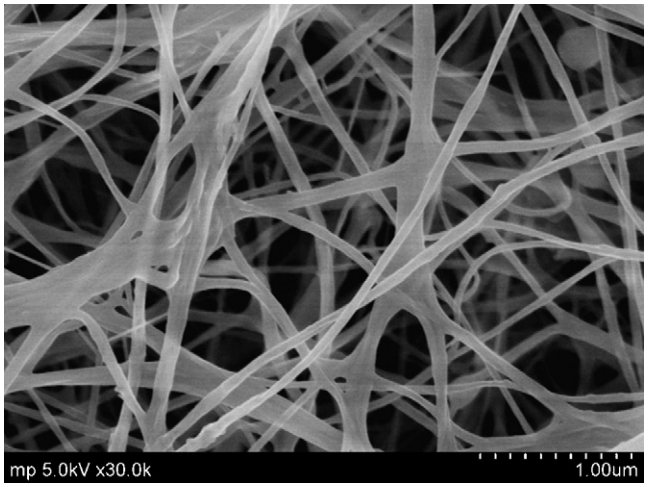

Fig. 4. SEM image of bacterial cellulose film to show the fibrillar nanostructured and three-dimensional network.

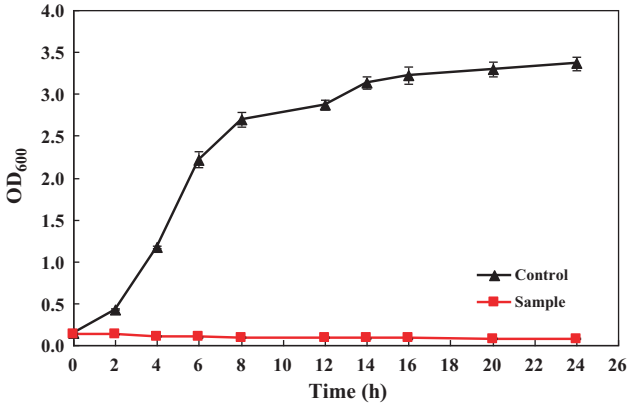

Fig. 5. Growth curves of *Bacillus subtilis* in the cultures with antimicrobial BC dry film or with the pure BC film.

3.4. Antibacterial effect studies using growth curves

The antimicrobial effects of the antimicrobial BC dry films were tested against *B. subtilis*, *S. aureus* and *E. coli* using the growth curves of tested strains. The growth curves of the model bacteria in the culture broth with the antimicrobial BC dry film or with pure BC as control were exhibited in Figs. 5–7. All the antimicrobial BC dry films investigated here were prepared in 0.102% benzalkonium chloride solutions. In Figs. 5 and 6, obvious increases in OD_{600} of *B. subtilis* and *S. aureus* were obtained within 24 h when

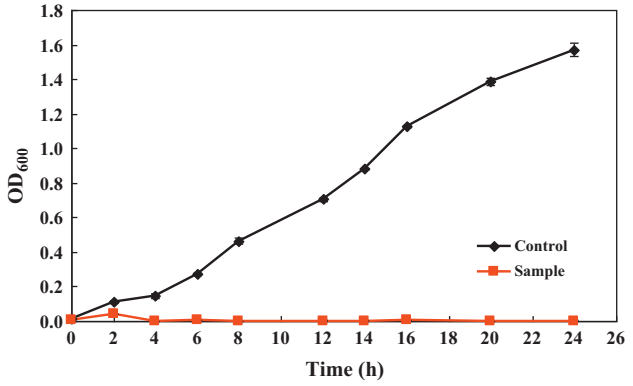

Fig. 6. Growth curves of *Staphylococcus aureus* in the cultures with antimicrobial BC dry film or with the pure BC film.

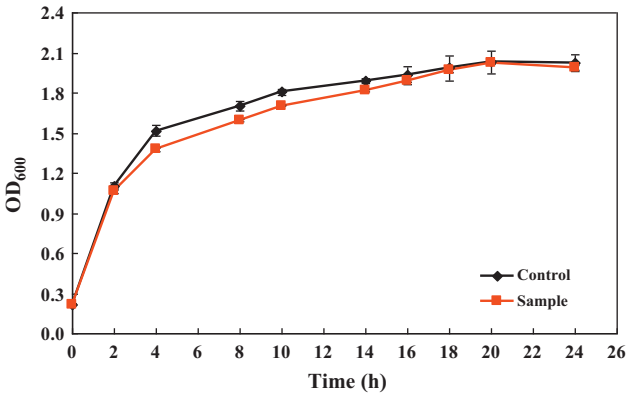

Fig. 7. Growth curves of *Escherichia coli* in the cultures with antimicrobial BC dry film or with the pure BC film.

the pure BC film was added as control. The OD_{600} of *B. subtilis* and *S. aureus* reached 3.363 and 1.572 at 24 h, separately. However, almost no OD_{600} changes happened in the cultures, where benzalkonium chloride-containing BC dry films were added. The lowest OD values for the samples from the cultures of *B. subtilis* and *S. aureus* were 0.077 and 0.003, respectively. This means that the prepared BC dry films possessed good antimicrobial activities against *B. subtilis* and *S. aureus*, and this result was in accordance with that exhibited in Fig. 1. However, for *E. coli*, almost no OD_{600} differences were observed between the cultures with an antimicrobial BC dry film and those with a pure BC film control in 24 h (Fig. 7), which means that the benzalkonium chloride-containing BC dry film exhibited inconspicuous influences on the growth of *E. coli*. The result was opposite to that shown in Figs. 1 and 2.

The main reason for this phenomenon should be ascribed to poor antimicrobial capacity of the benzalkonium chloride against Gram-negative bacteria and drug dilution effect in liquid culture medium. Although in Figs. 1 and 2, the antibacterial BC film showed antibacterial ability against *E. coli* (a Gram-negative bacterium), it had the worst performance compared to the inhibiting effects on the Gram-positive bacteria (the differences of the semi-diameter between inhibition zone and BC film were 5.0, 8.5 and 14.5 mm against *E. coli*, *S. aureus* and *B. subtilis*, respectively, with the films prepared at 0.102% drug concentration). This is because benzalkonium chloride is a kind of cationic surfactant type antimicrobial agent and has a poor role against Gram-negative bacteria. The results obtained in liquid cultures also showed that the antibacterial ability against *E. coli* became much weaker or no antibacterial ability at all (Fig. 7).

The growth curve of *E. coli* in the cultures containing benzalkonium chloride of different concentrations was exhibited in Fig. 8.

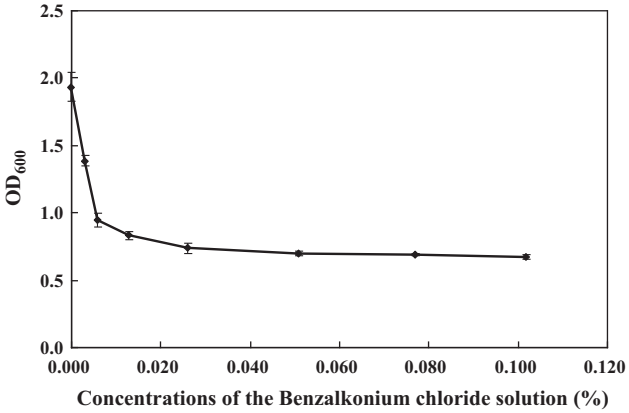

Fig. 8. Growth curve of *E. coli* in the cultures containing benzalkonium chloride of different concentrations.

It showed that the OD_{600} value of negative control without drug reached 1.93 and the cell density could also reach around 0.7 at benzalkonium chloride concentration of 0.102%. At the concentrations from 0.026% to 0.102%, no remarkable effect on the growth of *E. coli* was found. The lowest inhibition concentration was about 0.006% (Fig. 8). When the BC dry film (containing 0.2046 mg drug, Table 1) prepared in 0.102% of benzalkonium chloride solution was placed in liquid culture media, the concentration of benzalkonium chloride diffusing from the dry antibacterial film might be diluted theoretically to about 0.0002%, which could not inhibit *E. coli* any more (Fig. 8). Therefore the antibacterial ability of drug-loading dry films became worse or completely no antibacterial function in liquid cultures.

4. Conclusions

In the research, a kind of new functional dry BC film containing benzalkonium chloride as a potential antimicrobial wound dressing was developed. It possesses good portability, high water absorption capability and strong antibacterial properties especially resists both *S. aureus* and *B. subtilis* (Gram-positive bacteria). A stable and lasting release of the antimicrobial agent within at least 24 h was achieved by the prepared BC dry film, which could afford a nice permanence of the antimicrobial activity as wound dressing. The preparative procedure of the antimicrobial BC dry film was quite simple, and other antimicrobial agents like antibiotics, silver antibiotic agents and antimicrobial surfactants besides benzalkonium chloride could also be applied according to the introduced method. The antimicrobial BC dry film would be prospective to be developed as a commercial product for the acute traumas treatment in the future.

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