Development of a Benzalkonium Chloride Based Antibacterial Paper for Health and Food Applications

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Abstract: Pathogenic bacteria and other microorganisms pose a potent threat to humans by causing various infectious diseases. To control the spread of infection, different antibacterial products have been developed. However, most of them are known to be associated with health hazards, environmental pollution, complex fabrication, and/or higher cost. To address these issues, in this study, a low cost, biodegradable and human skin compatible antibacterial paper has been developed. A quaternary ammonium compound, benzalkonium chloride (BKC) has been used for paper surface treatment. The concentration of aqueous solution of BKC coated on paper was varied from 0.1 wt% to 0.2 wt%. No external binder was required for coating BKC onto paper. The efficacy of the coated paper was investigated against \textit{Staphylococcus aureus} ATCC 6538 and \textit{Escherichia coli} ATCC 8739 bacterial strains. This antibacterial paper is highly effective against both strains with the concentrations of BKC being within the allowable limit for cytotoxic effects. The optimum concentration of BKC coated on paper can be considered as 0.15 wt%, as nearly 100% inhibition was achieved with it against both strains. The developed antibacterial paper is suitable for being used in the industry for disinfection and food packaging purposes, and also by the public for hand sanitization.

Keywords: benzalkonium chloride; antibacterial paper; infectious diseases; hand sanitizer; food packaging

1. Introduction

Illnesses due to pathogenic bacteria are very common throughout the world. \textit{Staphylococcus aureus}, \textit{Escherichia coli}, \textit{Salmonella typhi}, \textit{Campylobacter jejuni}, \textit{Vibrio cholera}, etc. are some of the most common pathogenic bacteria, which can cause airborne, waterborne, and foodborne diseases. Diseases caused by these pathogens include bacteremia, mycobacterium tuberculosis, typhoid, diarrhea, cholera, pneumonia, plague, and various pulmonary and skin infections. The World Health Organization (WHO) estimates approximately 1 in 10 people fall ill each year due to consumption of contaminated food [1]. The death toll due to foodborne illness is estimated to be about 420,000 per year. Foodborne diseases have a huge financial cost as well. In the United States this cost is estimated to be about $8 billion per year [2]. About 2.2 million deaths and loss of nearly 12 billion US dollars per year occurs worldwide due to waterborne diseases as reported by WHO [3,4]. Around 3.2% of the global death is due to unsafe and unhygienic use of water [5]. The need for effective and user-friendly antibacterial devices which can be used as a preventive measure is evident. A paper based application could be ideal for this purpose, since paper is low-cost, abundant, biodegradable, and can be easily fabricated and functionalized [6–10]. Antibacterial papers possess a wide range of applications for healthcare and food industry. Sanitizing hands could be one of the potential applications of antibacterial papers. Antibacterial papers can also be used as a food packaging material and in the food production processes [11]. Previous studies demonstrated that antibacterial papers can be developed using graphene and carbon nanotubes [12,13], ZnO nanoparticles [14], and Ag nanoparticles [15]. In this study,
alkyldimethylbenzylammonium chloride, also known as benzalkonium chloride (BKC), was used as the antibacterial agent to develop effective and low-cost antibacterial papers.

The antibacterial property of BKC is well studied [16–18]. However, effectiveness of BKC on paper surface is less studied and less understood. BKC’s mechanism of antibacterial action involves altering the cell membrane permeability. The exact mechanism varies with the concentration of BKC solution [19]. Ionic and hydrophobic interaction between the positively charged BKC head and negatively charged bacterial cell membrane leads to loss of coherence in membrane. The bacterial cell loses its physical and ionic stability, resulting in the release of cytoplasmic materials and cellular lysis [20–22]. In addition, BKC is also effective in inactivating many viruses such as herpes simplex virus-1 [23], human immunodeficiency virus-1 [24], respiratory syncytial virus [25], and Measles morbillivirus [26].

BKC has various commercial, domestic, and medicinal applications. It has been used as a preservative in various cosmetics, soaps, shampoos, and skin care products. Studies have reported the use of BKC as a hand sanitizer replacing alcohol [27]. Various disinfectants contain BKC at concentrations in the range of a few hundred ppm [28]. It is also used in the food industry to improve food production environment [29] by cleaning food processing lines and other surfaces [30].

Although BKC has wide-ranging applications, it has a potential to induce toxicity when used at a higher concentration. As such, different allowable limits have been set up for specific uses by the regulatory authorities. The acute toxicity of BKC after oral administration is reported to be approximately 344 mg/kg [31]. BKC is ionic, which prevents its absorption in the gastrointestinal tract or skin. When consumed orally, the vast majority of it is eliminated through feces, urine, and tissue residue, and approximately 10% is absorbed as per the assessment report on Directive 98/8/EC of the European Parliament [31]. Oral administration might cause some irritation of gut mucosa but no targeted organ is evidenced, nor any serious clinical sign reported. BKC can cause skin irritation in extreme rare cases only and is not regarded as a significant skin sensitizer [32]. Acute toxicity of BKC after dermal administration is reported to be 3.56 mL/kg (80% ethanol/water solution). There is a higher possibility of irritant dermatitis only if patients with infections such as leg ulcers, eczema, etc. are exposed to higher concentration of BKC [33]. Absorption of low concentrated BKC through healthy skin is insignificant, and if used within the recommended concentration range for exact uses, it has almost no adverse effect [34]. The American College of Toxicology (ACT) recommended maximum concentration of BKC solution that can be used safely in contact products is 0.1% and 0.13% for antiseptic wound cleaners [35,36].

In this study, an antibacterial paper has been developed by coating BKC solution onto paper surface. It can be a possible alternative to alcohol as it is much cheaper and requires less in volume. The antibacterial paper developed using BKC can be stored in dry conditions, hence it offers easier storage than wet tissue. It is also more easily fabricated than nanoparticle-based antibacterial papers, since nanoparticles may require chemical conjugation with cellulose fibers. On the other hand, BKC does not require any external binder to be coated onto paper cellulose. The antibacterial efficacy of the proposed antibacterial paper was evaluated against gram-positive (E. coli ATCC 8739) and a gram-negative (S. aureus ATCC 6538) bacteria. The experimental results of this study report that antibacterial papers coated with BKC at an acceptable concentration would substantially alter the growth kinetics of both gram-negative and gram-positive bacteria within a reasonably short exposure time.

2. Materials and Methods
2.1. Bacterial Strains, Culture Media and Agar Plate Preparation

S. aureus ATCC 6538 and E. coli ATCC 8739 on nutrient agar plates were collected from Renata Pharmaceuticals (Dhaka, Bangladesh) and stored at 4 °C.
2.2. Culture Media

Mueller-Hinton broth was collected from Lab M Ltd. (Lancashire, UK). The culture media was prepared by mixing 2.1 g of powdered broth in 1000 mL Milli-Q water. The broth solution was autoclaved at 121 °C and 15 psi pressure for 15 min. The pH of the broth solution was approximately 7.4.

2.3. Anti-Bacterial Paper Preparation

A 50% aqueous solution of BKC (Loba Chemie, Mumbai, India) was used as the antibacterial agent, which was then diluted to the desired concentrations of 0.1%, 0.15% and 0.2% via series dilution. Whatman no.1 filter paper was cut into 10 pieces of 1 cm² size by using a sterilized scissor. Each of the paper pieces were coated with 10 μL of previously prepared BKC solutions with the help of micropipette (Figure 1a).

Figure 1. Test methodology of benzalkonium chloride (BKC) coated antibacterial paper. (a) Coating small paper sample with BKC solution, (b) transferring bacterial culture from nutrient agar plate to nutrient broth solution, (c) immersion of coated paper into bacterial culture, (d) series dilution for transferring the bacterial culture from nutrient broth media to nutrient agar plates, (e) transferring the dilute bacteria culture in nutrient broth to agar plates for titer count.
2.4. Culture Conditions

Pre-inoculum was prepared by transferring a single colony of bacteria from agar plate to the nutrient broth in the test tube with the help of a sterilized inoculating loop. The inoculated broth solution was incubated at 37 °C (Figure 1b).

2.5. Measuring the Optical Density of Bacterial Culture

After an incubation period of 6 h (for *S. aureus*) and 4 h (for *E. coli*), optical density (OD) of the bacterial culture in broth was measured using Hach DR 6000 spectrophotometer. The optical density was measured at 600 nm and an approximate bacterial concentration of the broth solution was obtained in colony formation units (CFU) per mL (OD<sub>600</sub> of 1.00 \(\approx\) 1.5 \(\times\) 10<sup>9</sup> CFU/mL for *S. aureus* and OD<sub>600</sub> of 1.09 \(\approx\) 3.0 \(\times\) 10<sup>8</sup> CFU/mL for *E. coli*) \([37,38]\). The optical density gave an idea about the initial bacterial load and the extent of dilution required before transferring to nutrient agar plates. The initial concentration of *S. aureus* culture was approximately 2.25 \(\times\) 10<sup>8</sup> CFU/mL, and 1.1 \(\times\) 10<sup>8</sup> CFU/mL for *E. coli*.

2.6. Preparation of Agar Plate

Mannitol Salt Agar (HiMedia Laboratories, Mumbai, India) was used to grow *S. aureus* bacteria in the laboratory. The agar solution was prepared by mixing 111.1 g of the agar powder for 1000 mL of Milli-Q water. MacConkey Agar (MERCK Life Science, Bengaluru, India) was used to grow *E. coli*. The liquid solutions were then sterilized in autoclave at 121 °C and 15 psi pressure for 15 min. The sterilized solutions were allowed to cool down to a temperature about 40–50 °C in the biosafety cabinet and transferred to petri dishes.

2.7. Analysis of Antibacterial Property

2.7.1. Immersion of Coated Paper into Bacterial Culture

The incubated bacterial culture in broth solution was divided into two equal parts into two test tubes. A total of 10 pieces of coated paper were suspended in one of the test tubes by using sterilized tweezer. The other part with non-coated clean paper samples served as the control medium. The duration of treatment was measured from the time of dipping of coated papers. Both tubes were then transferred to incubator and a temperature of 37 °C was maintained (Figure 1c).

2.7.2. Transfer of Bacterial Culture to Agar Plates

Time count started once coated paper samples were dipped in bacterial broth solution. After 30 min of incubation, both cultures (with and without immersed coated paper) were transferred to previously prepared agar plates. A volume of 100 \(\mu\)L of the solutions from both test tubes were transferred to agar plates after series dilution using vials. This step was repeated for exposure times of 90 min and 150 min as well. Then all agar plates were incubated at 37 °C overnight in the incubator for final titer count (Figure 1d,e).

2.7.3. Final Titer Count

Finally, after 24 h of incubation, agar plates were taken out of the incubator for titer count. Viable bacteria count in terms of CFU/mL was determined by gridding process manually. If N is the colony count from the culture exposed to coated paper and N<sub>0</sub> is the colony count from the control bacterial culture for a given duration, then, N/N<sub>0</sub> is the survival fraction and (1-N/N<sub>0</sub>) is the reduction in viability \([15]\).

2.8. Analysis of Swelling Ratio of Coated Paper

The swelling ratio analysis of coated paper was conducted following the reported procedure \([39]\). A weighted piece of 0.15% BKC coated paper was immersed into a 20 mL of deionized water for 24 h at room temperature. Then the paper sample was taken out from the water, treated with blotting paper to remove excess water, left to dry up and reach equilibrium, and weighted up again. The swelling rate of BKC dry film was calculated as
follows: swelling ratio = \( (W_f - W_0) / W_0 \), where \( W_0 \) was the initial weight of dry sample and \( W_f \) was the final weight of paper after immersion.

3. Results and Discussion

The experimental results demonstrated the effectiveness of the developed antibacterial paper against gram-positive and gram-negative bacterial strains. Figures 2 and 3 present experimental results of overnight incubated agar plates for \( S. aureus \) and \( E. coli \), respectively.

The bottom three plates in Figures 2 and 3 refer to bacterial culture treated with coated paper and the top plate correspond to bacterial culture without any treatment. Bacterial cultures of \( S. aureus \) with immersed coated papers showed significantly less bacterial growth in comparison to the culture in control medium (Figure 2a,b). It can be inferred from the figure that BKC concentration as well as exposure time affects its bacterial growth inhibition. With an increase in time of exposure to BKC coated paper, bacterial viability reduced for a definite concentration. Coated papers with higher concentration of BKC solution were associated with higher colony inhibition of \( S. aureus \). Trends similar to that of were observed for the bacterial culture of \( E. coli \) when treated with BKC coated papers of different concentrations (Figure 3a,b). Increased exposure time and higher concentration were found to increase bacterial inhibition.

**Figure 2.** Bacterial cell culture (\( S. aureus \)) in control experiment and against (a) 0.1% BKC and (b) 0.15% BKC coated paper at different times of exposure.
**Figure 3.** Bacterial cell culture (*E. coli*) in control experiment and against (a) 0.1% BKC and (b) 0.15% BKC coated paper at different times of exposure.

3.1. Effect of Antibacterial Agent Concentration and Exposure Time

Results showed strong antibacterial activity of BKC coated papers against both *S. aureus* and *E. coli* (Table 1). Increased exposure time and higher concentration were found to increase bacterial inhibition. BKC treated paper’s affects the growth of both *S. aureus* and *E. coli* with respect to exposure time and BKC concentration. For a definite exposure time, increasing concentration led to higher bacterial inhibition. This is due to the fact that more antibacterial agents are exposed to the same amount of bacterial load, leading to greater reduction in viability. Increasing the exposure time for a definite concentration led to higher bacterial inhibition, and it was found that for 90 min exposure time, a satisfactory
level of inhibition of around 90% was observed within 90 min for BKC solutions of all three concentrations. After 150 min of exposure, nearly 100% inhibition was observed for all the cases except for 0.1% BKC coated paper when treated against *E. coli*. When the concentration of BKC solution was increased from 0.1% to 0.15%, there was a significant increase in growth inhibition. However, the effect of increase in concentration from 0.15% to 0.20% was negligible as seen from the data. Therefore, 0.15% can be regarded as the optimum concentration.

Table 1. The antibacterial activity assay of BKC coated paper against *S. aureus* and *E. coli* for all three concentrations.

| Duration of Treatment (min) | Reduction in Viability (%)(1-N/N₀) × 100% |
|-----------------------------|------------------------------------------|
|                             | 0.1% BKC coated papers                   |
| 30                          | 58.20                                    |
| 90                          | 87.76                                    |
| 150                         | 99.16                                    |
|                             | 0.15% BKC coated papers                  |
| 30                          | 80.00                                    |
| 90                          | 97.72                                    |
| 150                         | 98.99                                    |
|                             | 0.2% BKC coated papers                   |
| 30                          | 94.34                                    |
| 90                          | 97.80                                    |
| 150                         | 99.03                                    |
|                             | 0.1% BKC coated papers                   |
| 30                          | 74.67                                    |
| 90                          | 90.78                                    |
| 150                         | 91.85                                    |
|                             | 0.15% BKC coated papers                  |
| 30                          | 99.38                                    |
| 90                          | 99.31                                    |
| 150                         | 100.00                                   |
|                             | 0.2% BKC coated papers                   |
| 30                          | 99.55                                    |
| 90                          | 100.00                                   |
| 150                         | 100.00                                   |

The antibacterial activity of the coated paper could be explained by the fact that, when dipped into bacterial solution, BKC diffused to the solution from the paper and usual antibacterial mechanism took place. When exposed, BKC is found to induce oxidative stress and upregulation of fatty acid metabolism in bacteria under growth arresting and lethal concentration [40,41]. It disrupts the cell consistency and stability of the cell membrane both ionically and physically. The electrostatic interaction between positively charged BKC head and negatively charged bacterial membrane causes permeation of BKC side chains into intramembrane region resulting in the leakage of cytoplasmic contents and precipitation of cellular materials [21].

The antibacterial activity was found to be slightly higher for *E. coli* than *S. aureus* for the first 90 min of incubation. This was due to differences in cell structure between gram-positive *S. aureus* and gram-negative *E. coli*. *S. aureus* has a thick cell wall made of 50% peptidoglycan by weight [42] and a single plasma membrane, whereas the gram-negative *E. coli* has a thin cell wall sandwiched between inner and outer membrane. The thick peptidoglycan cell wall gives *S. aureus* extra protection against the antibacterial agent. These differences were small, and it can be said that the coated papers are effective in inhibiting both *E. coli* and *S. aureus* as after 150 min of incubation nearly 100% inhibition was achieved.
The optimum concentration of BKC to develop antibacterial paper was found to be 0.15%; however, the effective concentration of BKC solution that comes in contact with the human skin through antibacterial paper would be much less due to the slow transfer rate of BKC from paper to human skin. As such, the issues related to toxicity will not be of any concern.

The BKC coated antibacterial paper was naturally air dried, hence, attained an equilibrium moisture content. Should the paper be used for hand sanitization, the equilibrium moisture of coated paper along with the moisture of skin or sweat will instigate the diffusion and activation of BKC on skin.

Different research groups have demonstrated nanoparticle (such as Ag and ZnO nanoparticles), and polymer (such as chitosan, ε-poly(L-lysine) and carboxymethyl cellulose) coated multilayered antibacterial papers which require sophisticated fabrication [13,14,43,44]. Some of the reported works also require long exposure time to achieve high inhibition [13,14,43]. Antibacterial paper reported in this article uses a quaternary ammonium salt (BKC) solution as the antibacterial agent, which is easy to manufacture, and provides better inhibition at short exposure time.

3.2. Analysis of Swelling Ratio of the Antibacterial Paper

The water binding capacity of the filter paper coated with BKC solution (0.15%) is shown as swelling ratio (Table 2).

Table 2. Swelling ratio of coated antibacterial paper in deionized water.

| Sample No. | Dry Paper Weight $W_0$ (g) | Swollen Paper Weight $W_f$ (g) | Swelling Ratio $(W_f - W_0)/W_0$ |
|------------|-----------------------------|-------------------------------|---------------------------------|
| 1          | 0.2484                      | 0.5988                        | 1.415                           |
| 2          | 0.2531                      | 0.6070                        | 1.398                           |
| 3          | 0.2542                      | 0.6133                        | 1.413                           |
| 4          | 0.2484                      | 0.5991                        | 1.412                           |
| 5          | 0.2542                      | 0.6026                        | 1.371                           |
| **Average**|                            |                               | **1.402 ± 0.0166**             |

Swelling ratio has been used to evaluate the swelling and wicking of the proposed bioactive papers. Swelling often changes the structural arrangement and dimensions of porous materials [45]. Swelling ratio is an indirect measure of the free volume between knots, and can be used to predict the wicking and wettability of various porous polymers [46–48]. A swelling ratio of 1.402 was observed for the coated filter paper used in our paper, whereas a very high swelling ratio of 26.20 was obtained for a wound dressing bacterial cellulose dry film [39]. Having a comparatively lower swelling ratio, the cross-link density is high in our paper and will have a relatively lower wicking and wettability. Higher wicking and wettability might dilute the coated agent which may result in quality degradation, and may cause losing active agent during packaging and storing. This lower value of swelling ratio is suited for the paper storing and packaging.

4. Conclusions

In this work, a low cost, reliable, biodegradable and biocompatible antibacterial paper based on a quaternary ammonium salt, benzalkonium chloride (BKC) has been developed. The paper has been found to resist and inhibit the growth of both gram-positive (S. aureus ATCC 6538) and gram-negative (E. coli ATCC 8739) bacterial strains. This has high potential for a wide range of industrial applications, and can be commercially produced for hand sanitizing and food packaging purposes. Further studies will be conducted for better understanding of the effect of BKC in paper structure and diffusivity from paper to human skin. BKC is effective against many viruses, therefore, it is highly likely that the BKC coated paper will also be effective as antiviral surface.
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