**Regular Article**

**Biological Synergy and Antimicrobial Mechanism of Hydroxypropyltrimethyl Ammonium Chloride Chitosan with Benzalkonium Chloride**

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Preservatives in eye drops have always been the focus of people’s attention. Benzalkonium chloride (BAC) is one of the most frequently used bacteriostatic agents in eye drops, which has broad-spectrum and efficient bactericidal ability. However, the inappropriate dosage of BAC may lead to high cytotoxicity. Therefore, adding low-toxic hydroxypropyltrimethyl ammonium chloride chitosan (HACC) can not only achieve antimicrobial effect, but also have the advantages of moisturizing and biocompatibility. In this paper, the minimum inhibitory concentrations (MICs) of HACC and BAC were evaluated against *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Diphtheroid bacillus* and *Candida albicans*. Based on the MIC of each antimicrobial agent, an antimicrobial assay was performed to investigate the antimicrobial ability of disinfectant solution. Besides, cytotoxicity had also been assessed. When the HACC/BAC solution at weight ratio of 150/1 showed a highest antimicrobial efficiency and the cell proliferation rates were the highest in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Furthermore, the cell leakage was examined by UV absorption, indicating the great synergistic antimicrobial effect between HACC and BAC. What is more, the results of micromorphology research suggested that as the result of repulsive force between the two molecules, the average particle size of HACC would decrease. Finally, the impedance experiment showed that with the addition of BAC, current density would increase significantly, suggesting that more positive charge group was exposed to aqueous solution, leading the the increase of antimicrobial ability. Based on these results, HACC–BAC combination solution might be a promising novel antimicrobial group for biomedical applications.

**Key words** benzalkonium chloride; cytotoxicity; antimicrobial property; hydroxypropyltrimethyl ammonium chloride chitosan; electrochemistry

**Introduction**

Eye drops are the most common treatments for eye diseases, while the drug solutions in eye drops bottles can get contaminated during use due to contact of the tip with hands, eyelids, lashes or tears while instilling the drops. Gram-positive bacteria, Gram-negative bacteria and fungus, such as *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Corynebacterium diphtheriae*, and *Candida albicans*, are the main reasons for infection. Therefore, preservatives are needed in eye drops and other eye drug solutions. Whereas the large dosage of preservatives can be affected by their insecurity. Generally, increasing the concentration of preservatives can attain potent antimicrobial effect, but leading to cytotoxicity. Therefore, to explore a disinfection formulation with striking antimicrobial capability and nontoxicity is of cardinal significance.

Natural macromolecules are becoming widely used in clinics as antimicrobial agents due to their broad-spectrum bactericidal activities and high biological safety. Chitosan (CS), as the only alkaline polysaccharide in the nature, has attracted significant scientific attentions during the last two decades, which has several extraordinary advantages, including biocompatibility, biodegradability, and low cytotoxicity. However, the poor water solubility limits its application. Recently, the macromolecules of N-alkylammonium have been a promising candidate for antimicrobial materials. Janas et al. synthesized a series of new N-alkylammonium bromides and found that most active N-alkylammonium bromides of conjugated linoleic acid (CLA) were attached at the nitrogen of desosamine toward *Streptococcus pneumoniae* strains and *S. pyogenes*. Lin’s group synthesized fibrous membranes containing N-halamine quaternary ammonium salt (HQAS) as antimicrobial agent and manifested prominent antimicrobial efficacy. Another evidences proved that the PEGylated random copolymer of butyl methacrylate and quaternary ammonium showed the best antimicrobial activities against *E. coli* and *S. aureus* without hemolysis. Therefore, the combination of amino groups with chitosan seemed to be an effective antimicrobial material. Hydroxypropyltrimethyl ammonium chloride chitosan (HACC), which can be produced through the reaction of chitosan with glycidyltrimethylammonium chloride, is a polycationic compound, characterized by good water solubility, flocculation, moisture absorption, and antimicrobial properties with low cytotoxicity to cells. Therefore, HACC has been applied in textile and industrial water treatments, paper handling and the medical fields, especially in biological medicine.

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reported that HACC with high molecular weight had high biocidal activity on the Gram-positive bacteria. Furthermore, it was shown that chitosan derivatives were nontoxic to COS-7 and MCF-7 cell lines, and the molecular weight of chitosans did not affect the cell viability while increasing the degree of trimethylation would increase the toxicity of the oligomer.\(^{12}\) Among them, the cell line COS-7 is a derivative of the simian kidney cell line curriculum vitae-1 (CVI) transformed with a mutant of simian virus \(^{40}\) and MCF-7 is breast cancer cell line which routinely used for drug screening study.\(^{14}\) Besides, there are still researches demonstrated chitosan derivatives have the promoting role in ocular delivery and conjunctival congestion like eye irritation, corneal edema.\(^{15}\) To sum up, HACC might be a promising polymeric antimicrobial agent with low cytotoxicity which available to ophthalmology.

Benzalkonium chloride (BAC) is a type of cationic surfactant and a mixture of alkyl benzyl dimethyl ammonium chlorides\(^{10}\) which shows strong effect on Gram-positive bacteria, but has less effect on \(P.\) aeruginosa, acid fast bacilli and bacterial spores. In addition, BAC has no absorption in the spectrum, but it has ultraviolet absorption with some surfactants (sodium dodecylbenzene sulfonate or dodecylmethylbenzyl ammonium chloride and so on), so as to obtain a rapid and sensitive detection method.\(^{16}\) Due to its broad-spectrum antimicrobial properties and convenient and fast detection methods, BAC is widespread applied in fabric softeners, personal hygiene and cosmetic products, as well as ophthalmic solutions and medications that use the nasal route of delivery.\(^{18,19}\) There have been a number of longitudinal studies involving BAC, which indicate that BAC is the most frequently used preservative in ophthalmic solutions today.\(^{20,21}\) However, with the application of BAC, there has been found that unsuitable concentrations could lead to serious adverse reactions, including burning or stinging sensations, dry eye and tearing.\(^{4}\) There is evidence that BAC has caused corneal and conjunctival toxicity, including cell loss, disruption of tight junctions, apoptosis and preapoptosis, cytoskeleton changes, and inflammatory reactions.\(^{22}\) Furthermore, it is possible that a relationship exists between chronic exposure to BAC and glaucoma. Besides, the hypothesis that BAC causes/worsens glaucoma have been tested experimentally in an animal model that closely reflects human physiology.\(^{23}\) Baudouin’s group argued that the incidence of these symptoms decreased significantly \((p<0.0001)\) by switching to a preservative-free formulation or by reducing the amount of preservative-containing treatment.\(^{24}\) Based on these data, the dosage of benzalkonium chloride should be balanced.

HACC and BAC can not meet the requirements of antimicrobial and non-toxic at the same time. Considering HACC has high biological safety and BAC has the broad-spectrum antimicrobial properties. Besides, although both of them are cationic polymers, the difference of molecular weight causes different antimicrobial mode. Therefore, combining HACC with BAC is a promising system with high antimicrobial energetically effect that can be applied in ophthalmic solutions and medications.

On the basis of the above statements, in this paper, we investigated the cytotoxicity and antimicrobial capability of HACC–BAC system in phosphate buffer solution (PBS). The antimicrobial capability of tested solutions was evaluated by spread plate method and the cytotoxicity was estimated by 3, [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assay. The Ultraviolet absorption experiment, Optical microscopy and Electrochemical experiment were applied to research the potential reasons regarding the HACC and BAC synergistic effect.

**Experimental**

**Material** HACC was obtained from Nanjing Kerui Technical Co., Ltd. (China) with a molecular weight of 10kDa and deacetylation degree of 81.5%. BAC was purchased from Shanghai Aoyuan Reagent Co., Ltd. (China). *E. coli* ATCC 25922, *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231 were all from Beijing huayueyang Biotechnology Co., Ltd. (China). *S. epidermidis* ATCC 12228 and *Diphtheroid bacillus* ATCC 10701 were obtained from Shandong top Bioengineering Co., Ltd. (China). Luria–Bertani (LB) broth was purchased from Haibo biology Co., Ltd. (China); Trypticase Soy Broth (TSB) was from Hefei Bomei Biotechnology Co., Ltd. (China) and Liquid Sabourand Medium was from Beijing solab Technology Co., Ltd. (China). Besides, Mueller–Hinton Agar and Sterile Defidrinated Sheep Blood used for *Diphtheroid bacillus* came from Shanghai Tongwei Biotechnology Co., Ltd. (China). The 96-well Culture Plates was obtained from Nan tong Feiyu Biotechnology Co., Ltd. (China).

**Test Solutions** HACC, BAC and phosphoric acid buffer solution (PBS, Solarbio, China) were dissolved in water to form HACC–BAC–PBS disinfection solution at BAC final concentration of 1 mg/L. The weight ratios of HACC and BAC were 1/1, 10/1, 30/1, 50/1, 100/1, and 150/1. The HACC–PBS solution was prepared with 1 mg/L HACC and PBS, and the BAC–PBS solution was prepared with 1 mg/L BAC and PBS. Here, the concentration of PBS was 5 mg/mL, and the water used was ultra-pure water.

**Determination of Minimum Inhibitory Concentration** Minimum inhibitory concentration (MIC) is the lowest concentration of a chemical which prevents visible growth of a bacterium.\(^{25,26}\) In this paper, the MICs of HACC and BAC were determined by a two-fold dilution method.\(^{27}\)*E. coli*, *S. aureus*, *S. epidermidis*, *Diphtheroid bacillus*, and *C. albicans* were cultural by sub-culturing in broth at 37°C under shaking conditions overnight. Different strains require different culture medium. LB broth was used for *E. coli*, Trypticase Soy Broth was for *S. aureus* and *S. epidermidis*, Mueller–Hinton Agar and Sterile Defidrinated Sheep Blood (5%) was for *Diphtheroid bacillus* and Liquid Sabourand Medium was used for *C. albicans*. After incubation, the bacterial suspension was double diluted for several times and added into 96-well plates. The absorbance value at 600 nm was detected by Microplate Reader (Thermo Fisher, U.S.A.). The concentration of the suspension was determined by comparing with the standard curve of bacterial suspension. Ultimately, the concentration was regulated to approx. \(10^6\) colony forming unit (cfu)/mL. Then the 0.1 mL bacterial suspensions were combined with 0.1 mL samples in the 96-well plates. The final concentration of HACC were 30, 24, 20, 16, 12, 8, and 4 mg/mL, respectively. The final BAC concentrations were 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.25 mg/L, respectively. The 96-well plates incubated at 37°C for 24–48h. Each sample was assayed for three times. The positive controls were prepared by combining bacterial suspension with broth, and the negative controls were prepared by mixing the samples with broth.\(^{28}\)
Antimicrobial Assays  Spread plate method was used to evaluated the antimicrobial activity of different test solutions.29) The 0.1mL bacterial suspensions (approx. 10⁶ cfu/mL) were added into 10mL tested solutions and mixed thoroughly. After incubation for 6h at ambient temperature, 0.5mL tested solutions were blended with 5mL neutralizing broth for 10min where the neutralizing broth was frequently used to neutralize preservatives and disinfectants so that the accurate antimicrobial activities could be determined. Eventually, 100μL of each sample was spread over an agar plate. The plates were incubated for 24–48h at 37°C and the number of growing bacterial colonies were counted.30) The PBS was equivalently treated as the positive control and each solution was conducted in triplicate to ensure the accuracy. The results of antimicrobial efficiency was calculated as the formula (1):

\[ LR = \frac{\log_{10}(I) - \log_{10}(F)}{F} \]  

Where \( I \) was the number of bacteria colonies of the control group (PBS) and \( F \) was the number of bacteria colonies after exposure to the tested solutions.

Cell Culture  Mouse fibroblast cell (L929, Shanghai Yaji Biotechnology Co., Ltd., China) and Human Conical Epithelial Cells (HCEC, Wuhan punuosai Life Technology Co., Ltd., China) were selected to reflect the authenticity of cytotoxicity thoroughly according to GB 19192-2003 (Hygienic standard for contact lens care solution). L929 were cultured in 1640 medium (Sigma, U.S.A.) supplemented with 10% fetal bovine serum (Solarbio, China) and HCEC were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma, U.S.A.) containing 10% fetal bovine serum (FBS).31) Both of them were incubated in carbon dioxide incubator (Panasonic, Japan) with 5% CO₂. After reaching confluency, the cells were detached from the flask with trypsin–ethylene-diaminetetraacetic acid (EDTA) (Sigma-Aldrich, U.S.A.). Trypsin–EDTA contained 0.25% trypsin and 0.02% EDTA which could accelerate the detachment of cells from the culture dish and promote the cells to disperse into single cell. The cell suspensions were centrifuged at 1500rpm for 5min and then re-suspended in the growth medium for further studies.32)

Cytotoxicity Studies  The in vitro cytotoxicity of the tested solutions was evaluated with MTT (Sigma) assay according to the standard of ISO 10993-5 (Biological evaluation of medical devices–Part 5: Tests for in vitro cytotoxicity).33) After cultural, The cell counter (Thermo Fisher) was applied to determine the concentration of cells until the density is adjusted to approx. × 10⁵ cells and then planted the cells into 96-well plates, incubated for 24h at 37°C. Different test solutions with 1640 medium or DMEM were planted and incubated with the cells for another 24h. Next, 20μL MTT were added to the 96-well plates incubating for 6h. Finally, the medium was removed and dimethylsulfoxide (DMSO, Shanghai Yuanye Biotechnology Co., Ltd., China) was added to each well. The absorbance of each well was measured by Microplate Reader in the wavelength of 490nm.34) DMSO was used as positive control in each experiment.

Leakage of Cellular Metabolites  The integrity of the bacterial membrane was determined by UV absorption using a spectrophotometer (X-ma 3100, Human Co. Ltd., U.S.A.). E. coli and S. aureus treated with HACC, BAC, or HACC combined with BAC solutions were centrifuged at 12000 × g for 10min, respectively. The untreated samples were used as control. Measuring the absorbance of supernatants at 260 and 280nm (A260 and A280) to analyze the leakage of nucleic acid and protein, respectively.

The Optical Microscope Imaging  Optical microscope images of HACC-BAC-PBS solutions were obtained using Olympus BX35.

Electrochemical Experiment  Electrochemical measurements were carried out in a one-compartment with three-electrode electrochemical cell using an electrochemical workstation (CHI-650D, China). The working electrode was a Au electrode with the area of 0.795 mm² and a glassy carbon rod was served as the counter electrode. These experiments were conducted at room temperature (25 ± 2°C).

Prior to the test, the working electrode was polished by alumina powder with particle sizes of 1 and 0.05μm respectively. Electrodes were then cleaned with Milli-Q water for 5min by ultrasonic cleaner (Tianjin Hengtai Machinery Co., Ltd., China).

Statistical Analysis  All experiments were repeated three to six times. The significance of the deviations in the data for each sample was determined by Scheffe multiple comparison testing. In all cases, the results were considered statistically significant at a \( p < 0.05 \).35)

Results and Discussion  The MICs and Cytotoxicity of HACC and BAC  Antimicrobial activities of HACC and BAC were determined by MIC method with E. coli, S. aureus, S. epidermidis, Diphtheroid bacillus, and C. albicans. The results were shown in Table 1. It was seen that compared with bacteria, HACC exhibited better antimicrobial effect against C. albicans with the value of 8mg/mL. Correspondingly, BAC was competent to inhibit the reproductive of bacteria, and showed poor inhibitory effect to C. albicans. Moreover, of all the kinds of bacteria, BAC showed the worst bacteriostatic effect against S. aureus. In general, the MICs of HACC and BAC against these five strains were relatively low. Meanwhile, the cell proliferation rates of HACC and BAC were shown in Fig. 1. As can be seen, with the increase of concentration, the cell productions of L929 and HCEC were decreased. What is more, the L929 and HCEC showed the similar tendency to HACC and BAC. Compared to HACC, BAC exhibited stronger cytotoxicity, until the concentration were up to 0.25mg/L for L929 and up to 1mg/L for HCEC, the cell proliferation rates could reach.

| Microbial strains | E. coli | S. aureus | S. epidermidis | Diphtheroid bacillus | C. albicans |
|-------------------|---------|-----------|---------------|---------------------|------------|
| HACC (mg/mL)      | 12      | 12        | 10            | 12                  | 8          |
| BAC (mg/L)        | 4       | 8         | 4             | 4                   | 16         |

All results are shown as mean± standard deviation (S.D.) (n = 3),
nearly 90%. It is noteworthy that when the concentration of HACC was up to 8 mg/mL, there was still no toxicity. However, these concentrations could not hinder the growth of microorganisms. Thus, single HACC or BAC cannot be effectively antimicrobial when maintaining biological non-toxic.

**Antimicrobial Properties and Cytotoxicity in Different HACC–BAC–PBS Solutions**

PBS is usually used in contact lens care solutions or eye drops, therefore we chose PBS as the buffer solution in this study. As be described before, considering the MICs and the cell cytotoxicity of single antimicrobial agent, HACC and BAC were unable to meet antimicrobial and biocompatible requirements. So it was essential necessary to combine these two compounds together. Based on the data of MICs, we selected HACC–BAC weight ratios of 1/1, 10/1, 30/1, 50/1, 100/1, and 150/1 to investigated. Antimicrobial activities and cytotoxicity of HACC–BAC–PBS system were showed in Figs. 2 and 3. Antimicrobial capacity of HACC–BAC–PBS solutions against *E. coli* was displayed in Fig. 2-(1). According to formula (1), LR was taken as the parameter of sterilization efficiency. The results showed a clear tendency with the weight ratios from 1/1 to 150/1, the log reductions increased, from 2.329-, 3.121-, 3.544-, 3.731-, 4.845- to 5.845-log, respectively. According to the standard, the log reduction should not be less than 3.0 logs (99.9%) for bacteria. Thus, the samples all could achieve the requirements of the national standard except the weight ratios of 1/1.

Moreover, Fig. 3A provided more details of the tested results. As the weight ratios increased, fewer bacterial colonies were observed on plates when incubated with test samples, and the weight ratios of 150/1 exhibited the best antimicrobial efficacy, there were almost no colonies after antimicrobial activity. Obviously, the HACC–BAC–PBS system had obvious antimicrobial advantage over single HACC–PBS solution or BAC–PBS solution with the same concentration.

The direct outcome was reported in Fig. 2-(2) about the antimicrobial efficacy against *S. aureus*. HACC–BAC–PBS solutions at weight ratios from 1/1 to 150/1 exhibited 2.583-, 2.711-, 2.743-, 2.756-, 3.255-, 5.556-log reductions, respectively. It was clear that the ratios of 1/1 to 50/1 failed to achieve >3-log reductions. In the contrary, the other samples showed superior antimicrobial performance against *S. aureus* and HACC/BAC weight ratio of 150/1 was the most competent to kill *S. aureus*. The lack of survival of *S. aureus* colonies in plate after incubated with 150/1 samples, which displayed in Fig. 3B, further confirmed its excellent antimicrobial properties. Single HACC–PBS and BAC–PBS solution with maximum concentration could not meet the standard, which log reduction against *S. aureus* was less than 3.

What is more, the *S. epidermidis* and *Diphtheroid bacillus* were chosen to exhibit the antimicrobial efficacy of HACC–BAC–PBS solutions, which were more closely related to diseases around the eyes, so that these data could preferably reflect the effectiveness of the experiment. Antimicrobial capacity of HACC–BAC–PBS solutions against *S. epidermidis* was shown in Fig. 2-(3). Similar to previous tendency, along with the weight ratio of HACC–BAC–PBS increased, from 1/1 to 150/1, the antimicrobial efficacy gradually improved, from 2.478- to 5.3-log reductions. When the weight ratio achieved
10/1, the log reductions exceed 3. When the weight ratio of HACC/BAC was up to 150/1, the antimicrobial efficiency was close to 100%, and the log reduction achieved the maximum. Figure 2-(4) showed the antimicrobial efficiency of HACC–BAC–PBS against Diphtheroid bacillus. There was a clear trend that with the weight ratio of HACC/BAC increased, the antimicrobial efficiency improved, from 3-, 3.29-, 3.69-, 3.901-, 4.29- to 5.1-log reductions, respectively. HACC/BAC weight ratio of 150/1 was the most competent to kill Diphtheroid bacillus. More details were exhibited in Figs. 3C and 3D.

In addition, Fig. 2-(5) presented the data provided by the experiments on the antimicrobial activity against C. albicans. The data obtained were similar to other strains before. Along with the increase of HACC in the disinfection system, log reduction raised from 2.529, 2.921, 3.344, 3.631, 4.845 to 5.545. Similarly, the weight ratio of 150/1 exhibited the utmost antimicrobial efficiency against C. albicans, which exceeded the single HACC or BAC in PBS at the same concentration level. Figure 3E was consistent with these results.

Considering the inhibitory effect on the bacteria growth of HACC–BAC–PBS samples, we proceed to evaluate their cytotoxicity at the same concentration for the antimicrobial assays. The cytotoxicity assays were conducted by six replicates for the tested samples. As can be noticed in Fig. 4, the cell proliferation of HACC–BAC–PBS solutions with the weight ratios of 100/1 and 150/1 were 103.9 and 98.6% for L929 and 96.3 and 94.1% for HCEC, suggesting that solutions exhibited no cytotoxicity to L929 and HCEC after incubating with cells for 24h.

In the present study, high molecular weight HACC and broad spectrum of antimicrobial BAC are selected together as disinfectants in tested solutions, which have been used as biocidal agents, in medical applications and water treatment. At the same time, all of them have the ammonium group. There have been reported that its antimicrobial characteristics against some bacteria are caused by the electrostatic interaction between the NH$_{4}^{+}$ groups and the phosphoryl groups of phospholipid components of cell membranes, which could increase the permeability of bacterial membrane. However, the antimicrobial mechanism of HACC and BAC is also of great difference. High molecular weight HACC could also cause metabolic disorder, because the polymer membrane formed by HACC on the surface of bacteria can affect the absorption of nutrients and the excretion of metabolic waste, resulting the death of bacteria. Small molecular BAC has another distinct antimicrobial mechanisms. Benzalkonium chloride shows antimicrobial activity in aqueous solution by adding soluble membrane lipids and reacting with other lipids through surfactants, which destroy the integrity of cells. Besides, BAC may also enter cellular internal and then bind with nucleic acids or denature the protein to intervene microbial proliferation. Thus, the different antimicrobial mode of HACC and BAC can exert their respective antimicrobial advantages in aqueous solution. HACC is external bacteriostasis, while BAC kills microorganisms entering the cellular interior.

Fig. 2. The Antimicrobial Efficacy of Different Samples against (1) E. coli, (2) S. aureus (3) S. epidermidis, (4) Diphtheroid bacillus, and (5) C. albicans at Ambient Temperature for 6h

Test samples were HACC–PBS solution (0.15mg/mL HACC in 5mg/mL PBS), BAC–PBS solution (1mg/L BAC in 5mg/mL PBS), HACC–BAC–PBS disinfection solutions with 1mg/L BAC in 5mg/mL PBS at HACC/BAC weight ratios of 1/1, 10/1, 30/1, 50/1, 100/1, and 150/1, respectively. Results were mean ± S.D. (n = 3).

Fig. 3. The Surviving Colonies after Exposed to Tested Solutions

(A) E. coli, (B) S. aureus, (C) S. epidermidis, (D) Diphtheroid bacillus and (E) C. albicans after incubated with different solutions: (1) PBS; HACC–BAC–PBS solutions at different HACC/BAC weight ratios of (2) 50/1, (3) 150/1.
What is more, owing to the lack of objective conditions and theoretical knowledge, the other N-alkyl substituents of HACC against antimicrobial efficiency have not been made a profound study.

**Cell Leakage Determination by UV Absorption** In order to get more information about the synergistic effect of HACC and BAC in antimicrobial activity, the determination of leakage of cellular metabolites had been implemented. The results were shown in Fig. 5. Obviously, the untreated bacteria (control) exhibited the lowest A260 and A280 values against both *E. coli* and *S. aureus*, suggesting that relatively lower amounts of extracellular nucleic acids and proteins than that of HACC or BAC treated bacteria. The figure also showed that compared with the single HACC and BAC solution, the A260 value significantly increased to 0.346 and 0.387, respectively after exposed to mixed solutions, indicating containing the maximum nucleic acid in the solution, and there was significant difference with other solutions. However, the A280 value increased relatively slowly. After exposed to the combined solution, the A280 value increased to 0.192 and 0.199, respectively, showing that no matter what it was a single solution or a mixed solution, the impact on protein leakage was less than that of nucleic acid. It was worthy noting that the significant difference between single solution and combined solution against A260, indicating a different pattern with respect to the bacterial inhibition. Sundfors et al. found that significant changes in cell membrane permeability and cell morphology resulting from the combined treatment of clove oil and EAP were evidenced by increasing in UV absorption of cell supernatants, increasing cell staining with propidium iodide, and changing in cell structure revealed by transmission electron microscopy. 44) Therefore, HACC and BAC have a great synergistic antimicrobial effect, which the combined solution synergistically damages the cell membrane and improves the permeability of cell membrane, resulting in greater leakage of nucleic acid and protein.

**Micromorphology of HACC–BAC Aggregates** The optical microscope of the above tested solution was carried out, which had been used to clarify the microscopic morphology of two molecules in the solution. The results were shown in Fig. 6. Figure 6a clearly showed particle morphology of HACC in aqueous solution. HACC molecules were crowed together loosely to take shape many small spheres of uniform size. Figure 6b gave the results of the images of HACC–BAC solution with the weight ratios of 150/1. Compared with the same concentration of single HACC solution, the average particle size of HACC in the combined solution was smaller. This phenomenon were understandable because there were the hydrophobic interaction between the benzene ring of HACC and the aliphatic chain of BAC, and the repulsive force between
the two molecules. As the result that BAC could accelerate the dispersion of HACC molecules.

**Impedance Spectroscopy of HACC and Combined Solution** The impedance method is based upon a measurement of the response of the electrochemical cell to a small-amplitude alternating potential. The response is often shown in the complex-impedance presentation. Typical Nyquist diagrams was shown in Fig. 7. The impedance spectroscopy of the figure was divided by two distinct regions: a semicircle and a slant line. The semicircle in higher frequency is with relation with charge transfer process. Semicircle diameter increases suggesting an decrease in exchange current density and a slower charge transfer. It was clear that the impedance of mixed solution was smaller than single solution, suggesting that more transfer charge was exposed in solution. The result was consistent with our previous speculation. This can be explained that hydrophobic regions of HACC and BAC could agglomerate inside by hydrophobic interaction between these two molecules and the hydrophilic group such as –NH₂, –OH, and NH₄⁺ were exposed to the surface of the aggregates in the solution, leading the higher current density.

In summary, the synergism of HACC/BAC solution can be explained from that hydrophobic interaction between two molecules accelerates the dispersion of HACC and leads more hydrophilic group such as quaternary ammonium group to expose to aqueous solution. Thus, more positive charges would to combine with the negative charge on bacterial membrane, resulting the bacterial membrane to be more permeable, so that BAC can enter the bacteria more quickly and combine with the bacterial nucleus to achieve the purpose of antimicrobial efficacy.

**Conclusion**

In summary, this article argued the HACC and BAC as the antimicrobial molecules in disinfectant solvents. Antimicrobial assays and cell cytotoxicity tests were testified by changing the weight ratio of two molecules. The results turned out that the antimicrobial efficiency of the weight ratio of 150/1 had an advantage over the single component, and exhibited the remarkable biological friendliness. In addition, the synergistic antimicrobial mechanism of HACC and BAC had been explored. The leakage of cellular metabolites suggested a different pattern with respect to the bacterial inhibition of combined solution. The microscopic morphology carried out by confocal laser scanning microscopy indicated that the existence of BAC could make HACC more decentralization in aqueous solution. The impedance spectroscopy showed that exchange current density increased by a wide margin with the addition of BAC. We speculated that due to the more exposed hydrophilic group, HACC combined with BAC made great cooperation in respect to the antibiosis. Therefore, considering its low toxicity and biocompatibility, HACC–BAC combined solution should have promising application in ophthalmological disinfection products such as eye drops or contact lens care solutions. We hope that the above work can play an auxiliary role in the research of ophthalmic disinfection products or other disinfection formula systems in the future.

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**Conflict of Interest** The authors declare no conflict of interest.
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