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Preparation and Characterization of *N*-Halamine-based Antimicrobial Fillers

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Abstract

The purpose of this study was to demonstrate that the surface of CaCO₃ fillers could be coated with an *N*-halamine based fatty acid to make the filler surface organophilic and accomplish antibacterial activity simultaneously, rendering the resulting polymer-filler composites antimicrobial. Thus, a new bi-functional compound, 4, 4'-Dimethyl hydantoin-undecanoic acid (DMH-UA), was synthesized by treating the potassium salt of dimethyl hydantoin (DMH) with 11-bromoundecanoic acid (BUA). Upon chlorination treatment with diluted bleach, DMH-UA was transformed into 3-chloro-4, 4'-dimethyl hydantoin-undecanoic acid (Cl-DMH-UA). Alternatively, DMH-UA could be coated onto the surface of CaCO₃ to obtain the corresponding calcium salt, 4, 4'-dimethyl hydantoin-undecanoic acid-calcium carbonate (DMH-UA-CaCO₃). In the presence of diluted chlorine bleach, the coated DMH-UA on the surface of CaCO₃ was transformed into Cl-DMH-UA, leading to the formation of Cl-DMH-UA-CaCO₃. The reactions were characterized with FT-IR, NMR, UV, DSC and SEM analyses. Both Cl-DMH-UA and Cl-DMH-UA-CaCO₃ were used as antimicrobial additives for cellulose acetate (CA). The antimicrobial efficacy of the resulting samples was evaluated against both *Escherichia coli* (Gram-negative bacteria) and *Staphylococcus aureus* (Gram-positive bacteria). It was found that with the same additive content, CA samples with Cl-DMH-UA-CaCO₃ and Cl-DMH-UA had very similar antimicrobial and biofilm-controlling activity, but the former released less active chlorine into the surrounding environment than the latter.

Keywords

N-Halamine; mineral filler; antimicrobial additive; biofilm; cellulose acetate

INTRODUCTION

Microbial contamination of polymeric materials is a global concern and remains one of the most serious challenges in hospital equipment, medical devices, water purification systems, hygienic applications, and food packaging and food storage.^{1–6} Studies have indicated that certain microorganisms can stay alive for more than 90 days on various polymer surfaces, making the contaminated surfaces important sources for cross-contamination and cross-infection.^{3,4} Therefore, there is a clear need to develop antimicrobial polymers to reduce the risk of microbial contamination. To date, a number of antimicrobial polymers have been reported, and some studies have achieved very promising results.^{7–11}

The research interest in this lab is *N*-halamine-based antimicrobial polymers. An *N*-halamine is a compound containing one or more nitrogen-halogen covalent bonds that are

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formed by the halogenation of imide, amide, or amine groups.¹² The antimicrobial activity of *N*-halamines is attributable to halogen exchange reactions between *N*-halamines and microorganisms, leading to death of the microorganisms.¹² Unlike inorganic halogens, organic *N*-halamines are more stable, less corrosive, and have much less tendency to generate disinfection byproduct, making them attractive candidates as food and water disinfectants.¹²

Considerable efforts have been devoted to incorporate *N*-halamine moieties into polymeric materials to achieve antimicrobial effects. Worley and coworkers reported the first polymeric *N*-halamine, poly-1, 3-dichloro-5-methyl-5-(4'-vinylphenyl) hydantoin, which was synthesized by the functional modification of polystyrene.¹³ This covalent binding approach was successfully adopted later by a number of other researchers.^{14–18} An alternative approach is to use *N*-halamine compounds as antimicrobial additives for polymers.^{19–25} This approach is particularly suitable for the antimicrobial treatment of chemically inert polymers, in which direct covalent binding reactions cannot be easily performed in large-quantity real applications. We first reported a class of hindered amine-based *N*-halamine additives,¹⁹ and then developed amide-based and melamine-based *N*-halamine additives for antimicrobial treatments of polymers.²³ The simplicity and applicability are attractive features of the additive approach. Nevertheless, like any other polymer additives, migration and leaching of the *N*-halamine additives could be a concern for long-term application, although in our prior studies, leaching was not observed within one year of storage evaluation.²⁴

On the other hand, mineral fillers (such as silica, silicates, and precipitated CaCO₃) are often mixed with polymeric composite materials to facilitate processing and/or reduce costs.^{26,27} Calcium carbonate is one of the most widely used mineral fillers in the plastic, rubber, and paint industries due primarily to its wide availability and low cost.^{28,29} In order to aid dispersion of the inorganic filler with organic polymers and to promote interfacial adhesion between the polymer matrix and the filler, fatty acids with long alkyl chains (e.g., C₁₀–C₂₀) are often used to treat CaCO₃ and render the filler surfaces organophilic.^{30–32}

In this study, we therefore synthesized a bi-functional compound, 4, 4-dimethyl hydantoin-undecanoic acid (DMH-UA). It was hypothesized that the carboxylic acid group of DMH-UA could react with CaCO₃ to bring the long fatty acid alkyl chain onto the filler surfaces. Upon chlorination with diluted bleach, the amide bond of the coated DMH-UA could be transformed into *N*-halamines, producing 3-chloro-4, 4-dimethyl hydantoin-undecanoic acid (Cl-DMH-UA, an amide *N*-halamine) to render the treated CaCO₃ filler (Cl-DMH-UA-CaCO₃) antimicrobial. To our knowledge, such a strategy has never been reported. The current study provided a new potential route to alter the filler surface organophilic and accomplish antimicrobial activity simultaneously.

Both Cl-DMH-UA and Cl-DMH-UA-CaCO₃ were used as antimicrobial additives for cellulose acetate (CA). Because of its wide availability, low cost, and excellent film- and fiber-forming capability, CA is one of the extensively used polymers for the fabrication of biomedical devices, membranes, films, fibers, etc.,^{33,34} in which antimicrobial activities of the polymers are often desired. The antimicrobial efficacy and biofilm-controlling effects of the resulting CA samples were evaluated against both *Escherichia coli* (Gram-negative bacteria) and *Staphylococcus aureus* (Gram-positive bacteria) to fully evaluate the feasibility of the new approach.

EXPERIMENTAL SECTION

Materials

Potassium hydroxide, 5, 5-dimethylhydantoin, and potassium iodide were purchased from Acros Organics (Morris Plains, NJ). 11-bromoundecanoic acid (BUA), CA and Triton X-100 (TX-100) were obtained from Sigma-Aldrich (Milwaukee, WI). Calcium carbonate was purchased from Alfa Aesar (Ward Hill, MA). Clorox® regular bleach was obtained from a local store (titration confirmed that the bleach contained 6.0% NaClO). Other reagents were analytical grade and used as received. *Escherichia coli* (*E. coli*; ATCC 15597) and *Staphylococcus aureus* (*S. aureus*; ATCC 6538) were obtained from American Type Culture Collection (ATCC).

Instruments

FT-IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer. ¹H NMR study was carried out on a JEOL ECS-400 MHz NMR spectrometer at ambient temperature in deuterated chloroform (CDCl₃). UV spectra were taken using a Beckman Coulter DU 520 UV/VIS spectrophotometer. SEM images were obtained on a Quanta 450/FEI instrument. Thermal properties of the samples were characterized with DSC-Q200 (TA Instruments, DE) under N₂ atmosphere at a heating rate of 10 °C/min.

Synthesis of DMH-UA

Briefly, 0.025 mole of DMH was dissolved in 30 mL CH₃OH containing 0.03 mole of KOH. The mixture was heated at 60 °C for 1 h.²¹ The resulting potassium salt of DMH was dried in a vacuum oven at 60 °C for three days. The anhydrous salt was then dispersed in 100 mL N, N-dimethyl formamide (DMF) at 95 °C for 10 min with constant stirring. Afterwards, 0.025 mole of BUA was added to the mixture and the reaction was continued for 5 h at 95 °C under constant stirring. At the end of the reaction, the formed KBr was filtered, DMF was evaporated, and the crude product was recrystallized from ethanol. DMH-UA was obtained as white powders (melting point: 67.9 °C; yield: 60 %).

Synthesis of Cl-DMH-UA

A drop (~30 mg) of TX-100 (non-ionic surfactant) was added to 50 mL of 10 % of chlorine bleach (Clorox) and stirred for 5 min. To this, 1.0 g of DMH-UA was added and the mixture was vigorously stirred for 2 h at ambient temperature. The product was filtered, and washed thoroughly with distilled water to remove residual chlorines. The products were filtered and dried in a vacuum oven to constant weights. Cl-DMH-UA was obtained as white powders with a melting point of 74.8 °C at a yield of 81.4 %.

Synthesis of DMH-UA-CaCO₃

A drop (around 30 mg) of TX-100 was added to 20 mL distilled water under constant stirring. To this, 0.2 g of DMH-UA was added and the mixture was stirred for 5 minutes. Afterwards, 1.0 g of CaCO₃ filler was added, and the mixtures were shaken at 40 rpm at ambient temperature for 2 h. The product was collected with filtration, washed with distilled water, filtered, and dried in a vacuum oven to constant weight.

Synthesis of Cl-DMH-UA-CaCO₃

A drop (around 30 mg) of TX-100 was added to 50 mL of 10 % of chlorine bleach (Clorox) and stirred for 5 minutes. To this, 1.0 g of DMH-UA-CaCO₃ was added and the mixture was stirred at ambient temperature for 1 h. After filtration, the product was washed with distilled water, filtered, and dried in a vacuum oven to constant weights.

Iodometric titration

The active chlorine content of Cl-DMH-UA and Cl-DMH-UACaCO₃ were determined by iodometric titration.^{21,23} About 0.05 g of the sample was taken into a flask containing 40 mL of CHCl₃ with 1.0 wt % acetic acid. 1.0 g of potassium iodide was added and the mixture was stirred for 30 min at ambient temperature under N₂ atmosphere. The released iodine was titrated with 0.01 N of Na₂S₂O₃. The percentage of active chlorine was calculated based on the following formula (1), where V_s and V₀ were the final and initial volumes (mL) of Na₂S₂O₃ consumed during the titration, W_s was the weight of the sample (mg), and C_{Na₂S₂O₃} was the concentration of the sodium thiosulfate used (0.01N). Each test was repeated three times and the average value was reported.

$$\%Cl = \frac{35.5}{2} \times \frac{(V_s - V_0)}{W_s} \times c_{Na_2S_2O_3} \times 100 \quad (1)$$

Preparation of polymer films containing Cl-DMH-UA and Cl-DMH-UA-CaCO₃

CA was used as the model polymer. In the preparation of polymer films, predetermined amounts of Cl-DMH-UA or Cl-DMH-UA-CaCO₃ were added to 5 % CA solution in CHCl₃ and mixture was stirred for 1 h at ambient temperature. The solution was placed onto glass slides in a fume hood for two days at ambient temperature to evaporate the solvent. The obtained films were further dried in vacuum at ambient temperature to achieve constant weights.

Antimicrobial activity of Cl-DMH-UA and Cl-DMH-UA-CaCO₃ in water

All the microbial studies were performed in a biosafety level-2 hood, and the guidelines provided by the U.S. Department of Health and Human services were followed to ensure lab safety.³⁵ *S. aureus* was grown in Tryptic soy broth at 37 °C overnight. The cells were harvested by centrifuge at 6000 rpm for 5 minutes, washed twice with phosphate buffer (PBS), and then re-suspended in PBS to concentrations of 10⁷–10⁸ colony forming units per milliliter (CFU/mL). A known amount of Cl-DMH-UA or Cl-DMH-UA-CaCO₃ was added to 10 mL sterilized water, vortexed for 60 s, and sonicated for 10 min to disperse the compounds. Afterwards, 100 µL of the above bacterial solution was added into the Cl-DMH-UA or Cl-DMH-UA-CaCO₃ suspension. The mixture was vortexed for 60 s and then shaken at 40 rpm. After a certain period of contact time, 100 µL of this suspension was added to 900 µL of 0.03 % sodium thiosulfate solution to quench the active chlorine. The resulting solution was vortexed for 10 s, serially diluted, and each dilution was placed onto nutrient agar plates. After overnight incubation at 37 °C, the CFUs on the plates were counted.

Antimicrobial activity of Cl-DMH-UA or Cl-DMH-UA-CaCO₃ in CA films as additives

E. coli and *S. aureus* were used as model microorganisms to challenge the antimicrobial activity of CA films containing Cl-DMH-UA or Cl-DMH-UA-CaCO₃. The bacteria were grown in their corresponding broth solutions (Luria-Bertani broth for *E. coli* and Tryptic soy broth for *S. aureus*) at 37 °C overnight, harvested, washed with PBS, and resuspended in PBS to 10⁷–10⁸ CFU/mL, as described above. 20 NL of the bacterial suspension was placed on the surface of a CA film containing Cl-DMH-UA or Cl-DMH-UA-CaCO₃ (2×2 cm²). The film was then covered with another identical film and 100 g of sterile weights were placed on the “sandwich” to ensure contact. After a certain period of contact, the films were transferred to 10 mL of 0.03 wt % sodium thiosulfate aqueous solution to quench the active chlorine without affecting bacterial growth.¹⁹ The mixture was vortexed for 60 s and sonicated for 3 min to transfer the bacteria into the solution. An aliquot of the resulting

solution was serially diluted, and 100 μL of each dilution was placed onto nutrient agar plates. The plates were incubated and CFUs were counted, as described above. The same procedure was applied to pure CA films (without Cl-DMH-UA or Cl-DMH-UA- CaCO_3) to serve as controls. Each test was repeated three times and the average value was reported.

Biofilm-controlling function

In this study, CA films containing Cl-DMH-UA or Cl-DMH-UA- CaCO_3 ($1.0\text{ cm} \times 1.0\text{ cm}$) were immersed in 1 mL of PBS containing $10^7 - 10^8$ CFU/mL of *S. aureus*. The samples were gently shaken at 40 rpm for 30 min at 37°C to facilitate bacterial initial adhesion. Afterwards, the films were taken out of the bacteria solution and gently washed with PBS ($3 \times 10\text{ mL}$). Each film was immersed in a well containing 2 mL of tryptic soy broth and incubated at 37°C for 48 h for biofilm formation. After incubation, each film was gently rinsed with 0.1 M sodium cacodylate buffer (SCB) and the bacteria were fixed with 2.5 % glutaraldehyde in SCB at 4°C for 24 h. After gently washing with PBS, the films were dried through an alcohol gradient method,³⁶ sputter coated with gold and observed under scanning electron microscope (SEM). Pure CA films were tested under the same conditions to serve as controls.

Stability study

To investigate the stability of the chlorines in the *N*-halamine-based films, a series of CA films ($1 \times 1\text{ cm}^2$) containing 5 wt % of Cl-DMH-UA or Cl-DMH-UA- CaCO_3 were immersed individually in 10 mL of deionized water under constant shaking (40 rpm) at ambient temperature. At specified intervals (3h, 6h, 24h, 48h, 72h and 120 h), the films were taken out and the water was iodometrically titrated to determine the content of released chlorines following the NEMI (National Environmental Methods Index) 4500-Cl B. For each interval, triplicate samples were analyzed and the average value was reported.

RESULTS AND DISCUSSION

Synthesis and Characterization of DMH-UA and Cl-DMH-UA

As illustrated in scheme 1, DMH-UA was synthesized by the nucleophilic reaction of BUA with the potassium salt of DMH. In this study, BUA was selected as it could bring long fatty acid alkyl chains to DMH, and compared with other bromo fatty acids with even longer alkyl chains, BUA is readily available with low costs. Upon chlorination, the amide group of DMH-UA was transformed into Cl-DMH-UA.

FT-IR analysis was used to characterize the products. Fig. 1 showed the FT-IR spectra of DMH, DMH-UA and Cl-DMH-UA. In the spectrum of DMH, the broad peak centered around 3218 cm^{-1} could be attributed to N-H stretching vibrations, and the 1702 cm^{-1} and 1770 cm^{-1} band were caused by imide and amide groups.²¹ Upon alkylation, the C-H stretching vibrations of the alkyl chain were clearly observed at 2851 cm^{-1} and 2918 cm^{-1} in the spectrum of DMH-UA.²¹ The peaks at 3284 cm^{-1} and 3436 cm^{-1} were assigned to the N-H and O-H vibrations of DMH-UA. In addition, the carbonyl peaks shifted to 1727 cm^{-1} and 1732 cm^{-1} , and a new carbonyl band appeared at 1695 cm^{-1} , which must be caused by carboxylic group of the undecanoic acid moiety. After chlorination, the N-H band was transformed to N-Cl band. As a result, the band at 3284 cm^{-1} disappeared (N-H band) and only O-H vibration at 3434 cm^{-1} could be detected.

FT-IR results were further supported by ^1H NMR studies, as shown in Fig. 2. In the ^1H NMR spectrum of DMH, the methyl protons showed a signal at 1.47 ppm, and the imide proton and amide protons could be observed at 8.2 ppm and 5.8 ppm, respectively. After the reaction with 11-bromo undecanoic acid, new signals caused by the protons on the alkyl

chains appeared at 4.0 ppm (E), 2.3 ppm (A), 1.6 ppm (B,D), and 1.3 ppm (C). In the ^{13}C NMR spectrum of DMH (Fig. 3), the peaks at 156 ppm (C2) and 178 ppm (C4) were due to the carbonyl carbons. The signal at 60 ppm could be attributed to the quaternary carbon in the DMH ring and the signal near 25 ppm was attributed to methyl carbons. After reacting with 11-bromoundecanoic acid, a new signal at 174 ppm (F) was observed in the spectrum of DMH-UA, which was caused by the carboxylic carbon. The carbonyl carbon signals shifted to 158 ppm (C2) and 180 ppm (C4), and the peak at 60 ppm shifted to 64 ppm (C5). The new signals observed in the range of 34–24 ppm (A, B, C, D, E) were attributable to the methylene groups of the alkyl chains, confirming the structure of DMH-UA. Upon chlorination, the signal at 64 ppm (C5) shifted to 65 ppm in the spectrum of Cl-DMH-UA, which might be caused by the replacement of the N-H bond by N-Cl bond as the latter had stronger electron-withdrawing effect on C5 than the former. The N-H $\rightarrow$ N-Cl transformation was further supported by UV analysis. As shown in Fig. 4, the absorption centered at 240 nm in the spectrum of DMH-UA was caused by the hydantoin ring structures.^{37–40} After chlorination, in addition to the 240 nm peak, a broad band at 270–280 nm could be observed, and this was caused by the disruption of the N-Cl bond and/or the transition from a bonding to an antibonding orbital of the *N*-halamine structure.¹⁹

Thermal properties of the samples were examined by DSC, as shown in Fig. 5. According to the supplier, DMH melts at 175–180 °C. In the DSC curves, BUA showed a sharp melting point at 50.5 °C. Upon the alkylation of DMH with BUA, the melting point of DMH-UA appeared at 67.9 °C. These were reasonable findings since the alkylation reaction would reduce the hydrogen-bonding sites of the resulting compound, and the long alkyl chain of BUA would prevent the close pack of the molecules, both of which would reduce the melting point of DMH-UA. Upon chlorination, the N-H bond in DMH-UA was transformed into N-Cl bond, making the compound more polar, and the melting point of Cl-DMH-UA increased to 74.8 °C. Furthermore, an exothermal peak at 162.3 °C was observed, which could be caused by the thermal decomposition of the N-Cl bond in Cl-DMH-UA.

Characterization of Cl-DMH-UA- CaCO_3

Iodometric titration showed that under our experimental conditions, Cl-DMH-UA- CaCO_3 had 1.18 % of active chlorines, suggesting that the CaCO_3 filler was coated with 36.42 % of Cl-DMH-UA. Fig. 6 showed the FT-IR spectra of the original CaCO_3 and Cl-DMH-UA- CaCO_3 . In the spectrum of CaCO_3 , the peaks at 1420–1470 cm^{-1} , 874 cm^{-1} , and 713 cm^{-1} were attributed to C-O stretching and bending vibrations of the carbonate anion (CO_3^{2-}).⁴¹ After coating with Cl-DMH-UA, in addition to the characteristic bands of calcium carbonate, new bands appeared at 2851 and 2914 cm^{-1} as well as 1732 and 1795 cm^{-1} , which were attributable to C-H stretching vibrations and C=O stretching vibrations of the Cl-DMH-UA moieties, respectively.

In DSC studies (Fig. 7), the pristine CaCO_3 did not show any significant thermal changes at lower than 250 °C. In the DSC curve of Cl-DMH-UA- CaCO_3 , a melting peak at 75.7 °C and an exothermic peak at 164.2 °C were observed, confirming that Cl-DMH-UA was coated onto CaCO_3 (see the similarities between Fig. 5 and Fig. 7).

The surface morphology of CaCO_3 and Cl-DMH-UA- CaCO_3 was examined by SEM and both of these fillers exhibited scalenohedral calcite morphology (Fig. 8) with particle sizes in the range of 2–4 μm . No significant morphological change was observed upon coating Cl-DMH-UA onto CaCO_3 . Nevertheless, the coated CaCO_3 fillers became less agglomerate as compared to the uncoated CaCO_3 , which could be caused by the Cl-DMH-UA moieties on the filler surfaces that prevented closer contact. In previous studies, similar changes were observed when CaCO_3 was coated with other organic compounds/polymers.⁴²

Antimicrobial activities of Cl-DMH-UA and Cl-DMH-UA-CaCO₃ in water

The antimicrobial activities of Cl-DMH-UA and Cl-DMH-UA-CaCO₃ against *S. aureus* in water were summarized in Table I (each test was repeated three times). With only 300 ppm of active chlorine, Cl-DMH-UA provided a total kill of 10⁷–10⁸ CFU/mL of *S. aureus* in 120 min. When the chlorine content was increased to 500 ppm, the minimum contact time for a total kill of the bacteria decreased to 60 min. On the other hand, Cl-DMH-UA-CaCO₃ showed a much lower antimicrobial potency against the same bacteria. With 500 ppm of active chlorine, Cl-DMH-UA-CaCO₃ only provided a 93 % and 99.5 % of reduction of *S. aureus* after 60 min and 120 min of contact, respectively. This lower antimicrobial activity of Cl-DMH-UA-CaCO₃ in water could be caused by contact, i.e., since Cl-DMH-UA was coated onto CaCO₃ surfaces, the *N*-halamine moieties had limited mobility to make full contact with the bacteria, leading to weak antimicrobial potency.

Antimicrobial activities of Cl-DMH-UA and Cl-DMH-UA-CaCO₃ in CA films as antimicrobial additives

The antimicrobial activities of Cl-DMH-UA and Cl-DMH-UA-CaCO₃ in CA films were summarized in Table II. It was interesting to note that in the tests against *S. aureus* (Gram-positive bacteria), the two systems demonstrated very similar antimicrobial efficacy, in contrast to the trend in the water-testing system (see Table I). This could still be explained by contact: in CA films, Cl-DMH-UA could form its own domains (“islands”) that were dispersed in the matrix of CA polymers (“sea”), a very similar morphology to the dispersion of Cl-DMH-UA-CaCO₃ (“islands”) in CA polymers (“sea”). In the antimicrobial tests, only *N*-halamines on the surfaces of the “islands” could make full contact with *S. aureus* for the chlorine exchange reactions,^{43–45} leading to similar antimicrobial potency of the Cl-DMH-UA and Cl-DMH-UA-CaCO₃ systems.

On the other hand, in the test against *E. coli* (Gram-negative bacteria), at 1% and 3% of additive contents, CA films with Cl-DMH-UA showed more potent antimicrobial effects than CA films with Cl-DMH-UA-CaCO₃ (Table II).

This difference could be attributed to the different structures of the bacteria. A major structural difference between Gram-negative bacteria and Gram-positive bacteria is that the cell wall of the former is overlaid with an outer membrane comprising lipopolysaccharide. This lipopolysaccharide layer offers a supplementary barrier limiting or preventing the penetration of antimicrobial agents into the cell.^{46–48} Therefore, a slight difference in the level of contact might result in a significant difference in antimicrobial potency against *E. coli* (Gram-negative bacteria). When the additive content was further increased to 5 %, the difference became unobvious, and the two systems demonstrated similar potency against *E. coli*.

Biofilm-controlling function

Biofilm-formation is a serious problem in a broad range of applications.⁴⁹ Since CA films containing Cl-DMH-UA and Cl-DMH-UA-CaCO₃ showed potent antimicrobial effects, it was highly likely that they could also prevent microbial adhesion/biofilm-formation. To evaluate this effect, CA films with or without the new additives were first exposed to *S. aureus* for 30 min, and then incubated in broth solution for 48 h to facilitate biofilm-formation.⁵⁰ As shown in Fig. 9A, on the control CA film surface, a large quantity of bacterial cells aggregated together and formed several layers, suggesting biofilm-formation. However, on CA film surfaces with 5 % of Cl-DMH-UA or Cl-DMH-UA-CaCO₃ (Fig. 9B and Fig. 9C), the microbial adhesion level was much lower, and no biofilms could be observed. The biofilm-controlling function of CA films containing Cl-DMH-UA or Cl-DMH-UA-CaCO₃ could be attributed to the antimicrobial activities of the *N*-halamines, i.e.,

when bacteria came into contact with the films, most of them were inactivated during and/or after adherence/colonization, resulting in cleaner surfaces. It might also be possible that when bacteria were approaching to the discs, they retreated when they sensed that the surface was inappropriate for adhesion.²³ More studies are needed to determine the action mode(s) of the samples for the further development of biofilm-controlling materials.

Stability studies

The stability of chlorines in Cl-DMH-UA and Cl-DMH-UA-CaCO₃ inside CA films was evaluated by immersing the sample films in deionized water. As shown in Fig. 10, after 120 h, CA films with 5% of Cl-DMH-UA and Cl-DMH-UA-CaCO₃ released 0.57 µg/mL and 0.49 µg/mL of active chlorines into the immersing water, respectively, which were much lower than the current EPA maximum residual disinfectant level of 4 ppm in drinking water^{51,52}, pointing to even greater potentials of the new materials.

CONCLUSIONS

In summary, we have developed an *N*-halamine precursor DMH-UA, which could be coated onto the surface of mineral filler CaCO₃. After chlorine bleach treatment, Cl-DMH-UA was formed on CaCO₃ surfaces, providing potent antimicrobial effects. In water-based tests, Cl-DMH-UA showed faster antimicrobial effects than the corresponding Cl-DMH-UA-CaCO₃, primarily because the former had higher mobility than the latter to make full contact with bacterial cells. However, when used as antimicrobial additives in CA films, Cl-DMH-UA and Cl-DMH-UA-CaCO₃ provided the resulting films with very similar antimicrobial potency, particularly at higher additive content. Further, CA films with Cl-DMH-UA-CaCO₃ released less active chlorines into the surrounding environment than the films with Cl-DMH-UA. Although more investigations are needed to further evaluate the long-term stability of the antimicrobial fillers inside CA and other polymeric materials, the findings from the current study suggested that it could be an effective strategy to use *N*-halamine-coated mineral fillers as antimicrobial additives of polymers to achieve potent antimicrobial and biofilm-controlling effects.

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Figure 1.
FT-IR spectra of (a) DMH, (b) DMH-UA, and (c) Cl-DMH-UA

Figure 2.
 ^1H NMR spectra of (a) DMH, (b) DMH-UA, and (c) Cl-DMH-UA

Figure 3.
 ^{13}C NMR spectra of (a) DMH, (b) DMH-UA, and (c) Cl-DMH-UA

Figure 4.
UV spectra of DMH-UA and Cl-DMH-UA

Figure 5.
DSC curves of (a) 11-bromo undecanoic acid, (b) DMH-UA, and (c) Cl-DMH-UA

Figure 6. FT-IR spectra of (a) Cl-DMH-UA, (b) unmodified CaCO₃, and (c) Cl-DMH-UA-CaCO₃.

Figure 7.
DSC curves (a) untreated CaCO_3 (b) Cl-DMH-UA and (c) Cl-DMH-UA- CaCO_3

Figure 8.
SEM images of (A) unmodified CaCO_3 , and (B) Cl-DMH-UA-CaCO_3

Figure 9. SEM images of biofilm-controlling function against *S. aureus* of (A) Pure CA film as control, (B) CA film containing 5 wt % of Cl-DMH-UA, and (C) CA film containing 5 wt % of Cl-DMH-UA-CaCO₃

Figure 10. Active chlorine level released into the immersing solution from CA films containing 5 wt % of CI-DMH-UA and 5 wt % of CI-DMH-UA-CaCO₃

Scheme 1.

Synthesis of calcium salt of 3-chloro-4,4-dimethyl hydantoin-undecanoate (Cl-DMH-UA-CaCO₃)

Table I

Influences of active chlorine contents in Cl-DMH-UA or Cl-DMH-UA-CaCO₃ on percentage reduction of 10⁷–10⁸ CFU/mL of *S. aureus* after 60 min and 120 min of contact *

Active chlorine content	Cl-DMH-UA		Cl-DMH-UA-CaCO ₃	
	60 min	120 min	60 min	120 min
100 ppm	99.5 %	99.5%	34%	66%
300 ppm	99.9 %	100 %	39%	94%
500 ppm	100%	100%	93%	99.5%

* Cl-DMH-UA and Cl-DMH-UA-CaCO₃ were dispersed in water and made contact with the bacterial suspension. See text for details.

Table II

Influences of Cl-DMH-UA or Cl-DMH-UA-CaCO₃ contents in CA films on percentage reduction of 10⁷–10⁸ CFU/mL of *S. aureus* and *E. coli* after 120 min of contact

Additive content in CA	Cl-DMH-UA		Cl-DMH-UA-CaCO ₃	
	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>
1 wt %	98 %	95 %	95 %	66 %
3 wt %	99 %	99.9 %	99.9 %	73 %
5 wt %	99.9 %	100 %	100 %	100 %