Durability of Amide *N*-Chloramine Biocides to Ethylene Oxide Sterilization

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The objective of this work is to study the stability of three novel topical antimicrobial dressings consisting of amide *N*-chloramine structures against ethylene oxide sterilization. Cotton gauze samples bonded with one of three amide *N*-chloramine structures were subjected to standard ethylene oxide (EtO) sterilization. The amounts of amide *N*-chloramine structures before and after the sterilization were quantified to indicate the stabilities of these amide *N*-chloramine structures to the sterilization. The samples after sterilization were challenged with a clinical isolate of healthcare-associated multidrug-resistant *Escherichia coli*. *N*-Chloramine structure converted from polymethacrylamide (dressing 2) had the highest durability (89.7% retained active chlorine) toward EtO sterilization; that from hydantoin (dressing 3; 86.3% retained active chlorine) followed; and poly(*N*-chloroacrylamide) (dressing 1) had the lowest (64.0% retained active chlorine). After EtO sterilization, all the samples still reduced *E. coli* presence at 5 minutes of contact, with dressing 2 retaining a log 6 reduction. The three tested amide *N*-chloramine structures could all survive EtO sterilization while retaining percentages of active chlorine ranging from 64.0 to 89.7%. Dressing 2 showed the best durability, whereas dressing 1 had the poorest durability. With the remaining amounts of amide *N*-chloramine structures after EtO sterilization, all the dressings could still reduce *E. coli* numbers within 5 minutes of contact, and dressing 2 resulted in a log 6 reduction in colony count. (J Burn Care Res 2012;33:e201–e206)

Infection is a big issue in wound care. Burn wound infections is a main reason for the failure of burn treatment and is associated with mortality. During the period 1994–2003, 10,229 Canadian children aged 0 to 19 years were admitted to hospitals due to burn-related injuries and 4.8% of them died.[1]

Treatment of bacterial infection in burns is a worldwide challenge. Antibacterial dressings can be developed to minimize colonization reducing the bacterial burden in burn wounds and preventing infection. Use of antimicrobial dressings has changed the face of modern burn care; however, consideration must be given to the fact that the majority of our wound care options are based on silver: silver nitrate, silver sulfadiazine, nanocrystalline silver, and silver-impregnated dressings to name a few. A recent international survey found that the most commonly used topical antimicrobials in burn care are based on silver.[2] However, emerging resistance associated with silver-based wound dressings is a growing concern. It has been found that *Escherichia coli* K-12 and O157:H7 are able to develop resistance to silver.[3] With the proliferation and broad use of silver-based products, many of which have little to no proven antibacterial activity,[4] the potential to see emergency of clinically relevant strains of silver-resistant bacteria is concerning. We are driven by the need to develop an antimicrobial dressing effective in those strains of bacteria that are known to be able to develop silver resistance before that resistance becomes an entrenched clinical problem.

A novel alternative to silver-based products is to use *N*-halamine (specifically *N*-chloramine) impregnated dressings. *N*-Chloramine refers to a group of compounds bearing N–Cl bond. The general formulas for organic *N*-chloramine are R_1R_2NCl and R_1NCl_2 (R_1 and R_2 are organic groups). If either R_1 or R_2 is a carbonyl group, it is called an amide *N*-chloramine. Chloramine chemicals are powerful biocidal agents and are

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also safe for the human body. As early as in 1916, inorganic *N*-chloramines were used in water disinfection as an alternative to chlorine. Worley, Bodor and their coworkers found that some cyclic organic *N*-chloramines have excellent potential to be used as water disinfectants.[5–8] Sun pioneered covalently bonding *N*-chloramine precurors-hydantoin derivatives onto various polymeric substrates.[9–12] Recently, acyclic *N*-chloramine structures have also been successfully bonded onto cotton and poly(ethylene terephthalate) fabrics for effective antibacterial functions.[13–15] Powerful and regenerable biocidal activity can be imparted onto those substrates after exposing them to chlorine bleach. Previous work has demonstrated the effectiveness of these products on multiple microorganisms including *E. coli* K-12, ATCC 15597, ATCC 29214, *Staphylococcus aureus* ATCC 6538, 14154, *Candida tropicalis* 62690, and *E. coli* bacteriophage MS2 15597-B1 virus.[14,16] Previously, we bonded polyacrylamide-based *N*-chloramine onto poly(ethylene terephthalate) fabric and challenged it with different multidrug-resistant bacteria including methicillin-resistant *S. aureus* and *Pseudomonas aeruginosa* (unpublished data). At 15 minutes of contact, there was log 6 reduction of bacteria.

In addition to being effective antimicrobials, *N*-chloramine products do not result in damage to healthy tissue. The safety and tolerability of these products has been reported by Ye et al[17]; dimethyloldimethyl hydantoin-treated cotton fabrics (active chlorine: ca. 1100 ppm) did not generate any erythema or edema on the bare skin of 8-week-old New Zealand male rabbits after 4-hour skin contact.

However, *N*-chloramine structures are known to be not very stable on heating or exposure to ultraviolet,[18] implying that they might not survive autoclave or ultraviolet sterilization. Given that ethylene oxide (EtO) sterilization is a routine means of preparing surgical equipment, it may serve as an alternative sterilization method for *N*-chloramine-impregnated dressings. Although previously *N*-chloramines were impregnated on polyester dressings, standard surgical dressing in our institution is cotton gauze. This short communication reports the findings about the durability of three amide *N*-chloramine structures to EtO when bonded to cotton gauze. We hypothesized that amide *N*-chloramines would retain chlorine quantity and antibacterial activity after a standard EtO sterilization procedure.

METHODS

Materials

Wound dressing cotton gauzes were procured from our hospital supplies. Acrylamide, methacrylamide, potassium persulfate, hydantoin, and some other reagents were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Alkynyl monomer *N*-(2-methylbut-3-yn-2-yl)acrylamide (MBAA) was prepared according to a known procedure.[19]

Synthesis of 3-(3-Azidopropyl)-5,5-Dimethylimidazolidine-2,4-Dione

A new hydantoin derivative 3-(3-azidopropyl)-5,5-dimethylimidazolidine-2,4-dione (ADID) was synthesized in our lab.[20] To the 5,5-dimethylhydantoin (3.2 g, 25 mmol) solution in Me_2CO (120 ml) was added anhydrous K_2CO_3 (10 g, 75 mmol). The resulting suspension was heated to gentle reflux for 0.5 hours and then 1,3-dibromopropane (2.8 ml, 27 mmol) was added in one portion. The mixture was continued to reflux overnight and the hot reaction mixture was filtered off by passing through a thin layer of silica gel. The filtrate was evaporated to give a white solid which was further purified by column chromatography (EtOAc/hexanes = 1/3) to afford 3-(3-bromopropyl)-5,5-dimethylimidazolidine-2,4-dione (BDID) as white solid (5.1 g, 82%).

To dimethylformamide (20 ml) solution of BDID (2.4 g, 9.8 mmol), sodium azide (0.96 g, 14.7 mmol) was added. The resulting suspension was heated to 80°C and continuously stirred overnight. The cooled mixture was concentrated and the residue was partitioned between ethyl acetate and H_2O. The organic layer was concentrated again to give the crude compound which was further purified by column chromatography (EtOAc/hexanes = 1/3) to afford ADID as white solid (1.8 g, 90%).

The product was also confirmed using mass spectroscopy: theoretical mass of $[M + Na^+]$ 234.0961, found 234.0934.

Bonding and Clicking of *N*-Halamine Precursors to Cotton Gauze

Three monomers (acrylamide, methacrylamide, and MBAA) were graft polymerized onto the cotton gauze. The gauze samples were dried and stored in a desiccator (<20% relative humidity) for over 24 hours to reach constant weights. Percentage bonding of the polymers was calculated from the following equation:

$$\text{Bonding } \% = (W_2 - W_1)/W_1 \times 100 \qquad (1)$$

where W_1 and W_2 are the weights of the original and bonded fabrics, respectively.

For easy understanding, the resulting modified cotton gauze samples were named as dressing 1 PAM-*g*-Cotton (cotton bonded with polyacrylamide), dressing 2 PMAM-*g*-Cotton (cotton bonded with polymethacrylamide), and PMBAA-*g*-Cotton (cotton bonded with poly(*N*-(2-methylbut-3-yn-2-yl)acrylamide)), respectively. PMBAA-*g*-Cotton underwent an additional Cu(I)-catalyzed azide-alkyne cycloaddition reaction (usually termed "click" reaction[21]) to bond with the cyclic *N*-chloramine precursor molecule ADID to yield dressing 3 (ADID-*g*-Cotton). "Click" reaction is an extraordinarily powerful strategy for the preparation of functional polymers because of its reaction specificity, quantitative yields, and high reactivity. Percentage of clicked ADID was calculated from the following equation:

$$\text{Clicking \%} = (W_2 - W_1)/W_1 \times 100 \qquad (2)$$

where W_1 and W_2 are the weights of the original and "clicked" fabrics, respectively.

Chlorination

The amide-bonded samples were converted to *N*-chloramine structures in a simple chlorination process: immersing the samples in a diluted chlorine bleach solution (300 ppm available chlorine for dressings 1 and 2 and 1500 ppm for dressing 3) at room temperature for 30 minutes. The liquid to fabric (liquor) ratio was 30:1 (w/w). The fabrics were then rinsed with copious amount of distilled water and air-dried for 24 hours. Then active chlorine contents on the samples were quantified with an iodometric titration.[13,14] The active chlorine concentration of the modified cotton gauze samples was then calculated from the following equation:

$$\text{Active chlorine concentration } [Cl^+] \text{ (ppm)} = 35.45 \times (V_1 - V_2) \times N \times 1000/(2 \times W) \qquad (3)$$

where V_1 and V_2 are the volumes (ml) of the iodine solution consumed in titrations of blank sodium thiosulfate solution and that with cotton gauze sample in, respectively, N is the normality of iodine solution, and W is the weight of the samples in grams.

EtO Sterilization

After chlorination, the samples were stored in the fume hood overnight to let them dry. Afterward, the samples were wrapped in plastic sterilization pouches and sterilized using EtO using our standard protocol for the operating room. EtO sterilization was carried out in SteriVac with a 2-hour cycle at 55°C followed by 12 hours and 8 minutes of aeration to remove residual EtO.

Antibacterial Assessment

Antibacterial properties of the bonded samples were examined according to a modified American Association of Textile Chemist and Colorists (AATCC) test method 100 against a clinical isolate of healthcare-associated multidrug-resistant *E. coli* (obtained from the Canadian ward surveillance study assessing antimicrobial resistance in Canadian hospitals, www.canr.ca). Given our desire to develop an alternative to silver-containing dressing, we chose this particular strain of bacteria as it allowed us to test our product against a bacterial species that has specifically been shown to develop silver resistance in the laboratory. Also this strain had the added advantage of having known multidrug resistance. The cotton gauze samples were cut into two small pieces (4.8 cm in diameter), and one piece contained four layers of overlapping cotton gauze made of open weaved cotton yarns. A 500 μl of an aqueous suspension containing 10^5 to 10^6 colony forming units (CFU)/ml *E. coli* was placed onto the surface of one piece of cotton gauze and covered with another piece. The cotton gauze set was then slightly pressed with a tweezer. After 5 minutes of contact, the inoculated samples were placed into 50 ml of 0.03% sodium thiosulfate aqueous solution to neutralize any active chlorine. The mixture was then vigorously shaken for 2 minutes. An aliquot of the solution was removed from the mixture and then serially diluted, and 100 μl of each dilution was placed onto a nutrient agar plate. The same procedure was also applied to the bleached unbonded cotton gauze samples as control. Viable bacterial colonies on the agar plates were counted after incubation at 37°C for 24 hours. All tests were performed in duplicate. Bacterial reduction is reported according to the following equation:

$$\text{Percentage reduction of bacteria (\%)} = (A - B)/A \times 100 \qquad (4)$$

$$\text{Log reduction} = \text{Log}(A/B) \text{ if } B > 0; = \text{Log}(A) \text{ if } B = 0 \qquad (5)$$

where A is the number of bacteria retrieved from control cotton gauze (CFU/ml) and B is the number of bacteria retrieved from treated cotton gauze samples (CFU/ml).

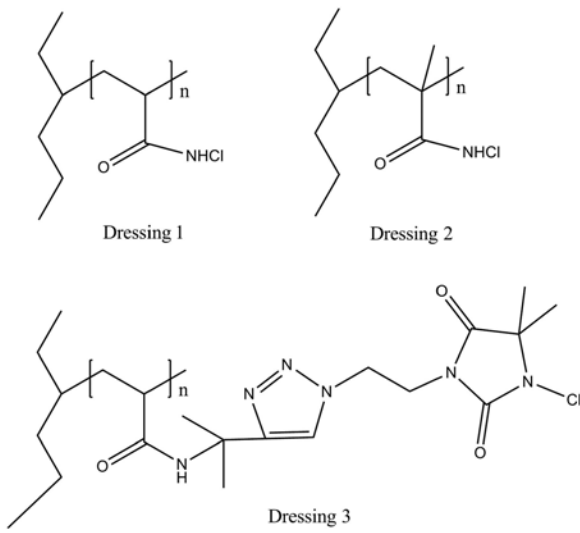

Figure 1. *N*-Chloramine-bonded cotton gauze (the alkyl chain represents cotton cellulose).

RESULTS

Three amide *N*-chloramines were bonded onto cotton gauze structures which are shown in Figure 1. The bonding percentages of all three amide *N*-chloramine precursor polymers are listed in Table 1. The bonding percentages were in the range of 0.85 to 3.13%. This is consistent with previously reported bonding percentages (0.7–2.5%).[13] The cyclic *N*-chloramine amine precursor hydantoin had been successfully bonded onto cotton in a one-step reaction.[10] However, formaldehyde was used in the modification process.[10] This study adopted a new and more environmental friendly method to introduce hydantoin onto cotton. First, PMBAA was bonded onto cotton to make a cotton gauze presenting alkynyl group. In the second step, azido hydantoin was "clicked" on PMBAA-cotton to yield ADID-*g*-Cotton (dressing 3).

Table 1. Bonding and clicking percentages of functional polymers on cotton gauze

Bonded Samples	Bonding %	Clicked Sample	Clicking %
PAM-*g*-Cotton (dressing 1)	1.22	—	
PMAM-*g*-Cotton (dressing 2)	0.85		
PMBAA-*g*-Cotton	3.13	ADID-*g*-Cotton* (dressing 3)	0.56

*ADID was clicked onto PMBAA-*g*-Cotton to yield ADID-*g*-Cotton.
PAM, polyacrylamide; *PMAM*, polymethacrylamide; *PMBAA*, poly(*N*-(2-methylbut-3-yn-2-yl)acrylamide).

As can be seen from Table 2, dressing 2 had the highest durability (89.7% retained active chlorine) toward EtO sterilization followed by dressing 3 (86.3% retained active chlorine). Even though dressing 1 had the highest relative drop of active chlorine (64.0% retained active chlorine), the amount of active chlorine remained was still higher than the other two dressings possibly due to better bonding percentage.

Table 3 presents the antibacterial results of cotton gauze samples bonded with the three *N*-chloramine structures. With the remaining amounts of amide *N*-chloramine structures after EtO sterilization, all the samples could still reduce *E. coli* numbers within 5 minutes of contact, and dressing 2 resulted in a log 6 reduction in colony count.

DISCUSSION

The chlorinated PMAM-g-Cotton (dressing 2) showed the least relative degradation with EtO sterilization, and although dressing 2 did not have the highest concentration of active chlorine before or after EtO sterilization, it did show the least relative reduction. It was reported that the side reaction happening in grafting PAM onto cotton could lead to the formation of branching polymer chain from the amide nitrogen.[22] Once amide is converted to *N*-chloramine, the hence introduced hydrogen alpha to amide nitrogen makes the structure less stable and can cause dehydrochlorination during heating. This explains why dressing 1 based on PAM is least stable against EtO sterilization.

Interestingly, dressing 2 also was the only product that demonstrated a log 6 reduction in *E. coli* after only 5 minutes of contact, compared with silver-based wound dressings which generally need 2 hours to demonstrate significant kill of bacteria.[23,24] Kill of bacteria by *N*-chloramines occurs by two mechanisms. One is based on release of free chlorine and another on direct transfer of chlorine to biological receptors. Chlorine can be transferred from polar N–Cl bond to water, generating chlorine in the "+1" oxidation state as hypochlorous acid or hypochlorite anion. In the second mode of action, chlorine is directly transferred to biological receptors to form a thermodynamically more stable species. Worley et al[25] designed a model study to explore the antibacterial mechanism of one typical *N*-chloramine and concluded that the disinfecting action of 3-chloro-4,4-dimethyl-2-oxazolidinone against *S. aureus* actually was the result of the interaction of the whole *N*-chloramine molecule with the bacterium instead of the limited amount of dissociated free chlorine. So, the major biocidal mechanism for *N*-chloramine is believed to be through

Table 2. Impact of ethylene oxide sterilization on the *N*-chloramine structures

Dressings*	Active Chlorine Before Ethylene Oxide Sterilization (ppm)	Active Chlorine After Ethylene Oxide Sterilization (ppm)	Retained Active Chlorine (or retained *N*-halamine) (%)
Dressing 1	620 ± 6	397 ± 2	64.0
Dressing 2	263 ± 6	236 ± 6	89.7
Dressing 3	146 ± 3	126 ± 5	86.3

*They are chlorinated dressings. Dressing 1, PAM-*g*-Cotton; dressing 2, PMAM-*g*-Cotton; and dressing 3, ADID-*g*-Cotton.
PAM, polyacrylamide; *PMAM*, polymethacrylamide; *ADID*, 3-(3-azidopropyl)-5,5-dimethylimidazolidine-2,4-dione.

chlorine transfer. The methyl group on both PMAM and ADID is speculated to anchor onto the cell wall of bacteria so as to speed up the process of chlorine transfer (further study is currently underway to test this hypothesis). This explains that the better or comparable antibacterial efficacy resulted from dressings 2 and 3 than dressing 1 even though the former two have lower levels of active chlorine.

Because this experiment only looked at 5-minute kill effects, the other two dressings only resulted in around log 2 reductions. However, they still demonstrated a measurable active chlorine level that is sufficient to result in log 5-log 6 colony reductions at longer contact duration as demonstrated in previous studies.[13]

CONCLUSIONS

The three tested *N*-chloramine structures could all survive EtO sterilization with retained relative percentages of active chlorine ranging from 64.0 to 89.7%. Dressing 2 showed the best durability, whereas dressing 1 had the poorest durability. The levels of active chlorine on the dressings were still at levels known to demonstrate adequate bacterial kill.[13] One dressing (dressing 2) had in vitro effect sufficient to result in a log 6 reduction in multidrug-resistant *E. coli*. Thus, we have demonstrated that it is possible to engraft standard cotton dressings with *N*-chloramines, expose them to standard EtO sterilization procedures, and still retain sufficient biologically active chlorine to result in log 6 reduction in bacteria exposed to the dressing. Having demonstrated this essential feature of the dressings, we are pursuing in vitro studies assessing cytotoxicity profile of these dressings on dermal fibroblasts and keratinocytes, as well as animal studies to assess the in vivo effectiveness of the dressing on bacterial load, including standard burn wound bacteria (*S. aureus*, methicillin-resistant *S. aureus*, and *P. aeruginosa*)

Table 3. Antibacterial results of *N*-chloramine-grafted cotton gauze samples

Dressing*	Active Chlorine (ppm)	Reduction of *E. coli* at 5 min of Contact	
		Percentage Reduction	Log Reduction
Dressing 1	397 ± 2	96.6	1.46
Dressing 2	236 ± 6	100	6.0
Dressing 3	126 ± 5	98.0	1.7

*They are chlorinated dressings. Dressing 1, PAM-*g*-Cotton; dressing 2, PMAM-*g*-Cotton; and dressing 3, ADID-*g*-Cotton.
PAM, polyacrylamide; *PMAM*, polymethacrylamide; *ADID*, 3-(3-azidopropyl)-5,5-dimethylimidazolidine-2,4-dione.

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