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Antimicrobial Activity of Nanoemulsion in Combination with Cetylpyridinium Chloride in Multidrug-Resistant *Acinetobacter baumannii*

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Acinetobacter baumannii has emerged as a serious problematic pathogen due to the ever-increasing presence of antibiotic resistance, demonstrating a need for novel, broad-spectrum antimicrobial therapeutic options. Antimicrobial nanoemulsions are emulsified mixtures of detergent, oil, and water (droplet size, 100 to 800 nm) which have broad antimicrobial activity against bacteria, enveloped viruses, and fungi. Here, we screened the antimicrobial activities of five nanoemulsion preparations against four *Acinetobacter baumannii* isolates to identify the most suitable preparation for further evaluation. Among them, N5, which contains 10% (vol/vol) Triton X-100, 25% (vol/vol) soybean oil, and 1% (wt/vol) cetylpyridinium chloride (CPC), showed the best efficacy against *A. baumannii* in both its planktonic and biofilm forms and was selected for further study. Our data demonstrate that, while the killing of planktonic forms of *A. baumannii* was due to the 1% CPC component of our nanoemulsions, the breakdown of biofilms was achieved via the emulsified oil and detergent fractions. Furthermore, we documented the effect of ethanol and NaCl in combination with N5 on planktonic *A. baumannii*. In killing curves of N5 combined with other agents (ethanol or NaCl), a synergistic effect of a ≥2-log decrease in CFU/ml was observed. The antibiofilm activity of N5 was confirmed via a cell proliferation test and scanning electron microscopy. The effects of exposure to severe environmental conditions, which simulates the field conditions in Iraq and Afghanistan, were evaluated, and this exposure did not affect the overall antimicrobial activity of N5. These studies lay a solid foundation for the utilization of nanoemulsions against the antibiotic-resistant forms of *A. baumannii*.

Over the past 15 years, *Acinetobacter baumannii* (an aerobic, Gram-negative coccobacillus) has become an emerging problematic pathogen with a wide array of antibiotic resistance (1), representing a serious threat not only to civilian hospital patients (2) but also to military service members wounded in Iraq and Afghanistan (3). Despite many approaches to find available treatment options (4), *A. baumannii*'s low permeability of the outer membrane (5), its ability to acquire genetic elements efficiently (6), and its ability to establish biofilms (7) have made treatment options limited.

Antimicrobial nanoemulsions are emulsified mixtures of detergent, oil, and water (particle size, 100 to 800 nm) which have been shown to have broad antimicrobial activity against bacteria, enveloped viruses, and fungi (8) at concentrations that are nontoxic in animals. When nanoemulsions function by fusing with lipid bilayers of cell membranes, the energy stored in the oil-and-detergent emulsion is released and destabilizes the lipid membrane of the bacteria; hence their antimicrobial activity (9, 10). The antimicrobial activity of nanoemulsions is nonspecific, unlike that of antibiotics, thus allowing broad-spectrum activity while limiting the capacity for the generation of resistance. These features make nanoemulsion a suitable candidate for both wound treatment (10) and surface decontamination.

Cetylpyridinium chloride (CPC) is a quaternary ammonium salt which has been utilized as an antimicrobial and disinfectant in many commercially available mouthwashes, toothpastes, lozenges, throat sprays, breath sprays, and nasal sprays. Quaternary ammonium compounds are active against bacteria through multiple mechanisms (11), with activity being maintained when the compound is incorporated into nanoemulsion formulations.

In this study, we investigated the antibacterial activity of various forms of nanoemulsions containing CPC by testing their MICs and minimal bactericidal concentrations (MBCs) on several strains of *A. baumannii*. We analyzed the kinetics of killing of the planktonic form of *A. baumannii* with and without the combination of ethanol and NaCl to determine any synergistic effects. Both ethanol and hypertonic solution of NaCl have membrane-destabilizing effects (12, 13), and their membrane-destabilizing effects have never been examined in conjunction with the membrane-destabilizing effect of nanoemulsion. The effectiveness of our lead nanoemulsion (N5) in dispersing *A. baumannii* biofilms was observed via scanning electron microscopy and quantitated by testing viability of remaining bacteria on biofilms. The stability of N5 under "field-like" environmental conditions was evaluated, including thermal shock (TS), high temperature/high relative humidity (HH), high temperature/low relative humidity (HL), and low temperature/low relative humidity (LL), based on current field guidelines.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. baumannii* strains (ATCC BAA-1605, ATCC 17961, ATCC 19606, and ATCC 19003) were procured from the ATCC (Manassas, VA). ATCC BAA-1605 is a multidrug-resistant strain isolated from the sputum of military personnel returning from Afghanistan in 2006 ([14]). ATCC 17961, ATCC 19606, and ATCC 19003 were isolated from blood, urine, and cerebrospinal fluid of patients, respectively. Nutrient broth (NB) (BD, Franklin Lakes, NJ) was used for bacterial culture. Bacterial stocks have been maintained at −80°C in NB with 30% (vol/vol) glycerol. Aliquots of stock culture were inoculated into fresh NB and cultured at 37°C overnight. Bacterial cultures with an optical density (OD) of 0.2 to 0.4 at 490 nm were used for all studies. Nutrient agar (NA) (BD, Franklin Lakes, NJ) was used for enumerating CFU, by standard assay procedures. Autoclaved Ringer's solution (7.2 g NaCl, 0.17 g CaCl$_2$, and 0.37 g KCl in 1 liter of distilled water) adjusted to pH 7.3 to 7.4 was used for the dilution of bacteria.

Nanoemulsion preparation. Five oil-in-water nanoemulsions were prepared as previously reported by Baker et al. ([15]). Briefly, nanoemulsion 1 (N1) contained 2% (vol/vol) Triton X-100, 2% (vol/vol) tributyl phosphate, and 16% (vol/vol) soybean oil; nanoemulsion 2 (N2) contained 3% (vol/vol) Tween 60, 3% (vol/vol) soy sterol, 30% (vol/vol) soybean oil, and 0.35% (wt/vol) CPC; nanoemulsion 3 (N3) contained 15% (vol/vol) Tween 80, 3% (vol/vol) ethyl oleate, and 6% (vol/vol) octanol; nanoemulsion 4 (N4) contained 8% (vol/vol) Triton X-100, 8% (vol/vol) tributyl phosphate, 64% (vol/vol) soybean oil, and 50 μmol/liter EDTA; nanoemulsion 5 (N5) contained 10% (vol/vol) Triton X-100, 25% (vol/vol) soybean oil, and 1% (wt/vol) CPC. The solutions were emulsified using a Microfluidizer processor (M-110L; Microfluidics, Newton, MA). Two passes of emulsification at 20,000 lb/in^2 were conducted at room temperature. The droplet size distribution analysis was carried out using a dynamic light scattering method (90Plus particle size analyzer; Brookhaven Instruments Co., Worcestershire, United Kingdom).

Determination of MIC and MBC. To evaluate the antimicrobial activities of nanoemulsions against *A. baumannii*, the MIC and MBC were determined. Nanoemulsions were serially diluted with sterile NB in a 96-well plate, and each well, containing 180 μl of diluted nanoemulsion in NB, was inoculated with 20 μl of standardized bacterial culture with an OD of 0.2 to 0.4 at 490 nm. The cell density in the wells was 2 × 10^7/ml. Plates were incubated at 37°C overnight, and the MIC was determined as the highest dilution showing no bacterial growth. To determine the MBC, 100 μl of culture broth from wells containing no growth was plated onto NA and incubated at 37°C overnight. Because of the low number of surviving cells, no additional dilution was needed. The highest dilution that resulted in 99.9% reduction in the bacterial cell number was recorded as the MBC. The negative control was sterile deionized water in place of nanoemulsion, and 0.1% sodium hypochlorite was used as the positive control. Due to the different compositions of nanoemulsions, the MIC and MBC were expressed as the dilution of nanoemulsions.

Kinetics of killing. Overnight bacterial cultures (200 μl) were added to 1.8 ml of NB with 1:500, 1:1,000, 1:1,333, 1:2,000, or 1:2,677 dilutions of N5. At 1, 15, 30, and 60 min after mixing of bacterial culture and diluted N5, aliquots of samples were collected and diluted accordingly in Ringer's solution at room temperature for viability testing. Measured volumes (100 μl) were spread onto NA using a disposable spreader and incubated at 37°C overnight for colony formation. Overnight colonies were counted, and average counts were determined from at least three independent experiments for each set of conditions.

Effect of ethanol and NaCl. Overnight bacterial cultures (200 μl) were added to 1.8 ml of NB with 0, 10%, 20%, 30%, 50%, or 70% of ethanol mixed with 1:1,333, 1:2,000, 1:4,000, or 1:8,000 dilutions of N5. The cell density in the wells was 2 × 10^7/ml. At 60 min postmixing, the viability of cells was measured by CFU/ml determination. Similarly, overnight bacterial cultures (200 μl) were added to 1.8 ml of NB with 0, 2, 3, or 4 M NaCl mixed with 1:1,333, 1:2,000, 1:4,000, or 1:8,000 dilutions of N5. At 60 min postmixing, the viability of cells was measured by CFU determination.

Log killing (LK) was calculated as log$_{10}$(CFU/ml of untreated control sample) − log$_{10}$(CFU/ml of treated sample).

Effect of nanoemulsion on biofilms of *A. baumannii*. Overnight bacterial cultures were inoculated into 100 μl of fresh NB in flat-bottom 96-well cell culture plates (Costar 3599; Corning Inc. Corning, NY) and incubated for 72 h at 37°C. Every 24 h, the medium was removed and an equal volume of fresh medium was added. The negative control was an equal volume of NB without bacterial inoculation, and NB with 0.1% sodium hypochlorite was used as the positive killing control. After removal of the supernatant media, the biofilms were treated with 200 μl of N5 (1:400, 1:1,000, and 1:2,000 dilutions) for 1 h at 37°C. After that, the mixture of NB and N5 was removed, and the wells were gently washed twice with sterilized Ringer's solution. The quantification of remaining viability in biofilms was conducted using alamarBlue cell proliferation assay kit (AbDserotec, Kidlington, United Kingdom) as described in the manufacturer's manual. NB (120 μl) with 10% alamarBlue was added to the well. The reduction of alamarBlue in response to the chemical reduction of growth medium, which is the result of bacterial growth, was measured by OD at 570 nm and 630 nm after 30 min incubation at 37°C.

Scanning electron microscopy on the biofilm treated with N5. Acrylic slides were submerged in bacterial culture for 72 h with medium changes every 12 h. After 72 h of incubation, acrylic slides were gently washed twice with sterilized distilled water. Biofilms on acrylic slides were incubated with NB containing N5 alone, N5 without 1% CPC, or 1% CPC alone for 1 h and then gently washed twice with sterilized distilled water. Biofilms were fixed on acrylic slides by the method of Araujo et al. ([16]), and the fixed biofilms were dehydrated in a graded series of cold ethanol-water mixtures (50%, 70%, 80%, 90%, 95%, and 100% of ethanol) for 10 min each. An additional 10 min dehydration with 100% ethanol was done twice. With gentle rocking, biofilms on acrylic slide were treated with 50% ethanol–50% hexamethyldisilazane (HMDS) (Electron Microscopy Sciences, Hatfield, PA) for 5 min and 100% HMDS for 10 min. After dehydration, biofilms were air dried under the hood. The samples were coated using a Hummer 6.2 sputter coater (Anatech USA, Hayward, CA) with a gold-palladium (50%-50%) target. After processing, samples were observed with a Sigma VP40 field emission scanning electron microscope (Carl Zeiss, Inc., Germany) in high vacuum mode at 2 kV ([17]).

Effect of environmental exposure on the stability of N5. The stability of N5 (droplet size, MIC, and MBC) under four environmental conditions, including thermal shock, high humidity/high temperature, high humidity/low temperature, and low humidity/low temperature, which simulate field storage conditions, were tested based on the *Department of Defense Test Method Standard for Environmental Engineering Considerations and Laboratory Tests* ([18]), according to Bienek et al. ([19]). Briefly, for thermal shock conditions, N5 was placed in the environmental test chamber (model EWPH205-CCA; ESPEC North America, Hudsonville, MI) for 2 h at 23°C and 35% relative humidity, and then the temperature was lowered to −40°C over a 1.5-h period with the humidity kept at ~50%. This condition was maintained for 4 h. The environmental conditions were then changed to 65°C and 35% relative humidity over a 2-h period and maintained for 4 h. Temperature and relative humidity were adjusted to 23°C and 35% over a 1-h period, and then a new cycle resumed. A total of 5 cycles were conducted continuously. For high humidity/high temperature condition, the temperature and relative humidity of the chamber were adjusted to 65°C and 75% over a 1-h period. N5 was exposed to this condition for 96 h. At the end of exposure to the high-humidity/high-temperature condition, the environmental condition of the chamber was shifted to 23°C and 50% relative humidity over a 1-h period. After an additional 2 h of incubation, N5 was retrieved from the chamber. For the high-humidity/low-temperature condition, the chamber was primed to 65°C and ~15% relative humidity over a 1-h period. N5 was exposed to this condition for 96 h. At the end of exposure to the high-humidity/low-temperature condition, the environmental conditions were restored to 23°C and 50% relative humidity over a 1-h period. After 2 h of incubation, N5 was retrieved from the chamber. For the low-humidity/low-tempera-
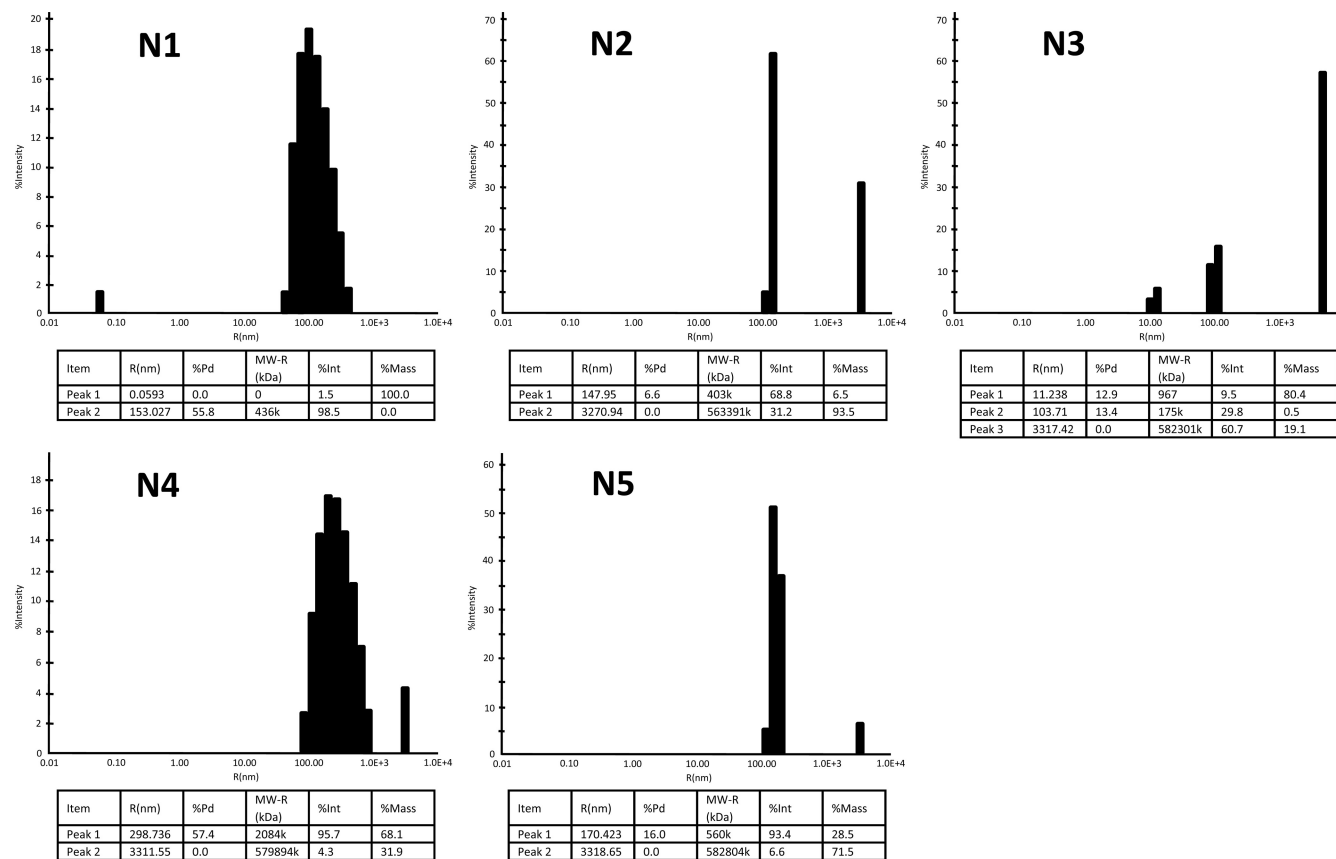
Item	R(nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	0.0593	0.0	0	1.5	100.0
Peak 2	153.027	55.8	436k	98.5	0.0

Item	R(nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	147.95	6.6	403k	68.8	6.5
Peak 2	3270.94	0.0	563391k	31.2	93.5

Item	R(nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	11.238	12.9	967	9.5	80.4
Peak 2	103.71	13.4	175k	29.8	0.5
Peak 3	3317.42	0.0	582301k	60.7	19.1

Item	R(nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	298.736	57.4	2084k	95.7	68.1
Peak 2	3311.55	0.0	579894k	4.3	31.9

Item	R(nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	170.423	16.0	560k	93.4	28.5
Peak 2	3318.65	0.0	582804k	6.6	71.5

FIG 1 Distribution of droplet size of nanoemulsions. The five nanoemulsions with different detergents and oils were prepared by two rounds of emulsification at 20,000 lb/in^2 with a Microfluidizer. The size and mass distribution were analyzed using a dynamic light-scattering method.

ture condition, the environmental condition of the chamber was adjusted to −40°C and ~50% relative humidity over a 1-h period. N5 was exposed to this condition for 96 h. At the end of the exposure period, the temperature and relative humidity of the chamber were adjusted to 23°C and 50% over a 1-h period. After 2 h of incubation, N5 was retrieved from the chamber.

After the exposure to environmental conditions, the change in the droplet size distribution, expressed as effective diameter of the droplets, and their distribution width (Polydispersity) in treated N5 and control N5, which was stored at room temperature, were measured using a dynamic light-scattering method, and their MICs and MBCs were determined.

Statistical analysis. All experiments were conducted in triplicate, and each CFU/ml measurement was made from two separate plates. Their means and standard deviations were calculated from arithmetic CFU/ml. In case of log killing to examine the efficacy of N5 in conjunction with ethanol and NaCl, the arithmetic CFU/ml was converted to $\log_{10}$, and then statistical analysis was conducted. Student's t test was used to determine the level of significance ($P < 0.05$). Student's t test was conducted on the data set between 30% ethanol-treated samples and N5-plus-ethanol (1:4,000 and 30%) samples. The degree of freedom was 4; P values for strains ATCC BAA-1605 and ATCC 17961 were <0.05, and those of ATCC 19606 and ATCC 19003 were <0.01. The same method was used between 4 M NaCl-treated samples and N5-plus-NaCl (1:4,000 and 4 M) samples. The degree of freedom was 4, and P values for the 4 strains were <0.001.

RESULTS

Size distribution of nanoemulsion particles. The five nanoemulsions prepared by Microfluidizer emulsification were evaluated based on the particle size distribution using the ZetaPlus particle sizing software of the 90Plus particle size analyzer. Nanoemulsion 1 (N1) contained particles with an average size of 153 nm, nanoemulsion 2 (N2) contained two particle sizes with averages of 148 nm and 3,271 nm, nanoemulsion 3 (N3) had three major particle size peaks at 11, 104 nm, and 3,317 nm, nanoemulsion 4 (N4) had two major peaks with averages of 299 nm and 3,312 nm, and nanoemulsion 5 (N5) had a major peak at 170 nm and a minor peak at 3,316 nm (Fig. 1). All nanoemulsion size evaluations were repeated after 6 months, with no significant change in particle size distribution (data not shown.)

Determination of MIC and MBC. The five nanoemulsion preparations (N1 to N5) were tested for antimicrobial activity on four strains of *A. baumannii* obtained from the ATCC (strains ATCC BAA-1605, ATCC 17961, ATCC 19606, and ATCC 19003). N1, N2, N3 and N4 did not have significant antibacterial activity as measured in either MIC and MBC studies (Table 1). N5 showed exceptional antibacterial activity against all four strains in both the MIC and MBC assays at high dilution ranges, 1:2,187 for MIC and 1:729 to 1:2,187 for MBC (Table 1). In the case of N5, the 1:729 and 1:2,187 dilutions were equivalent to 13.7 µg/ml and 4.6 µg/ml of cetylpyridinium chloride. Based on this data set, we selected only N5 for additional studies.

Kinetics of killing. The kinetics of antimicrobial activity of N5 was evaluated against *A. baumannii* strains ATCC BAA-1605, ATCC 17961, ATCC 19606, and ATCC 19003 at dilution ranges of 1:500 to 1:3,333. Representative data from the 1:1,333 dilution are

TABLE 1 Comparative MICs and MBCs of 5 nanoemulsions on *Acinetobacter baumannii* strains[a]

Test strain	MIC						MBC					
	N1	N2	N3	N4	N5	1% CPC	N1	N2	N3	N4	N5	1% CPC
BAA-1605	NE	1:81	1:81	NE	1:2,187	1:2,187	NE	1:27	NE	NE	1:729	1:729
17961	NE	1:81	1:81	NE	1:2,187	1:2,187	NE	1:27	1:27	NE	1:2,187	1:2,187
19606	NE	1:81	1:81	NE	1:2,187	1:2,187	NE	1:81	1:27	NE	1:2,187	1:2,187
19003	NE	1:243	1:81	NE	1:2,187	1:2,187	NE	1:81	1:27	NE	1:2,187	1:2,187

[a] Due to the different compositions of nanoemulsions, the MIC and MBC are expressed as the dilution of nanoemulsions. For N5, dilutions of 1:729 and 1:2,187 are equivalent to 13.7 μg/ml and 4.6 μg/ml of cetylpyridinium chloride. NE, no effect.

shown (Fig. 2). At a 1:1,333 dilution of N5, bacterial colony counts were reduced >2 logs for strains ATCC BAA-1605 and ATCC 19606 and ~3 logs for strains ATCC 17961 and ATCC 19003 at 15 min. By 30 min, all bacterial strains had been reduced by ≥2 logs. At dilutions of >1:1,333, the viability of all bacterial strains was reduced by ≥2 logs by 30 min (data not shown).

Effects of ethanol and NaCl on N5 antimicrobial activity. The additive effect of ethanol or NaCl with N5 in killing *A. baumannii* was evaluated. Increasing concentrations of ethanol ranging from 0% to 70% were added to dilutions of N5 (1:1,333, 1:2,000, 1:4,000, and 1:8,000). Strains ATCC BAA-1605 and ATCC 17961 showed less susceptibility to the addition of 30% ethanol than strain ATCC 19606 and ATCC 19003, with log reductions of 1.34 and 1.26, respectively, for ATCC BAA-1605 and ATCC 17961 compared to log reductions of 2.81 and 3.44, respectively, for strains ATCC 19606 and ATCC 19003 after 1 h of incubation. This additive effect was further evaluated at a 1:4,000 dilution of N5, in which the addition of 30% ethanol resulted in a ≥1- to 3-log increase in killing effect (CFU/ml) of N5 against all four bacterial strains tested (Fig. 3). The additive effects of NaCl on N5 were also evaluated. Strains ATCC BAA-1605 and ATCC 17961 showed less susceptibility to 4 M NaCl, with 0.55- and 0.70-log reductions, respectively, compared to the susceptibility of strains ATCC 19606 and ATCC 19003, which were reduced by 1.42 and 1.41 logs, respectively. Similarly to the effects of ethanol, NaCl also showed

FIG 2 Effect of N5 on the planktonic form of *A. baumannii*. The antimicrobial activity of N5 was evaluated against *A. baumannii* strains (□, ATCC BAA-1605; ▲, ATCC 17961; ○, ATCC 19606; ■, ATCC 19003). Overnight bacterial cultures (200 μl) were added to 1.8 ml of NB with ~1:500 to 1:2,677 dilutions of N5. After ~1 to 30 min incubation, CFU/ml of samples were measured by plating on NA with serial dilution. Kinetics of killing of N5 from the 1:1,333 dilution is shown.

significant enhancement of the killing effect of N5, with a ≥3- to 4-log additional decrease in CFU/ml of all four strains of *A. baumannii* (Fig. 4).

Biofilm studies. A key component of the lack of efficacy of standard antibiotics and antimicrobials against *A. baumannii* is the presence of biofilms, which serve to protect the bacteria and prevent penetration of antimicrobial agents (20). In order to evaluate the antimicrobial activity of N5 against *A. baumannii* biofilms, we measured the metabolic activities of biofilm integrity posttreatment. The effects of N5 on *A. baumannii* ATCC BAA-1605 in biofilms were measured utilizing an alamarBlue cell proliferation assay kit, which measures the reducing power of living cells. At a 1:1,000 dilution of N5, 80% of metabolic activity of *A. baumannii* was lost within 1 h (Fig. 5) of treatment, which increased to ~90% loss of metabolic activity when the concentration was increased to a 1:400 dilution (Fig. 5). We utilized scanning electron microscopy to investigate the effect on morphology of *A. baumannii* ATCC BAA-1605 (antibiotic-resistant strain) biofilms treated with N5, N5 without 1% CPC, or 1% CPC alone. After 72 h of incubation, *A. baumannii* generated robust biofilms with approximately 100% confluence (Fig. 6a). N5 without 1% CPC appeared to disrupt and disperse the bacterial biofilms, with a clear reduction in overall bacteria present (Fig. 6b). When 1% CPC alone was applied on the biofilm, it deformed the superficial bacteria, but the biofilm structures remained intact (Fig. 6c). Considering the intact biofilm structures after treatment and the low metabolic activities of the bacteria deep inside the biofilm, 1% CPC alone had limited antibacterial activities on the biofilm. N5, which contains 1% CPC, led to a complete disruption of the biofilm, leaving only a small amount of damaged bacteria present (Fig. 6d).

Evaluation of the effect of environmental exposure on stability of N5. *A. baumannii* remains a major problem in troops injured in Iraq and Afghanistan, where environmental conditions are drastically different from those in a laboratory environment. In order for a novel antimicrobial therapeutic to be effective, it must maintain its activity under these extreme conditions. Elevated temperature is known to modify the macroscopic phase separation behavior of nanoemulsions. In extreme environments, such as those in which many military units are stationed, the quality of many diagnostic products exposed to environmental conditions is in question. The effects of environmental factors like temperature and humidity on the stability of N5 were tested. Four sets of environmental exposure conditions were tested. After incubation in HH conditions for 96 h, the nanoemulsion particle size of N5 increased 2.5 times (494.1 nm) compared with the size under lab conditions (213.9 nm) (23°C and ambient relative humidity). Following incubation of N5 under HL conditions, the particle size
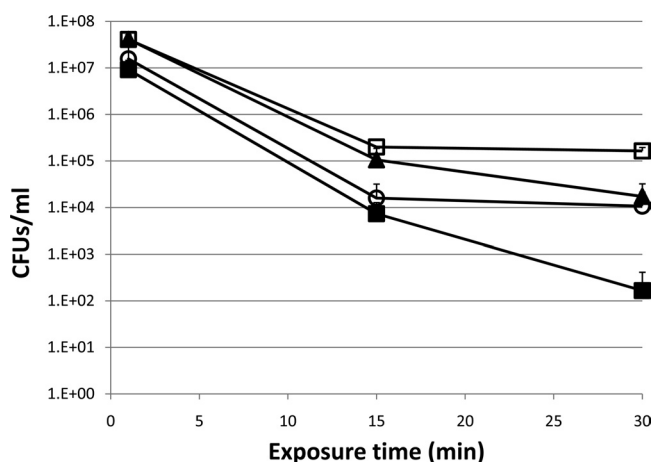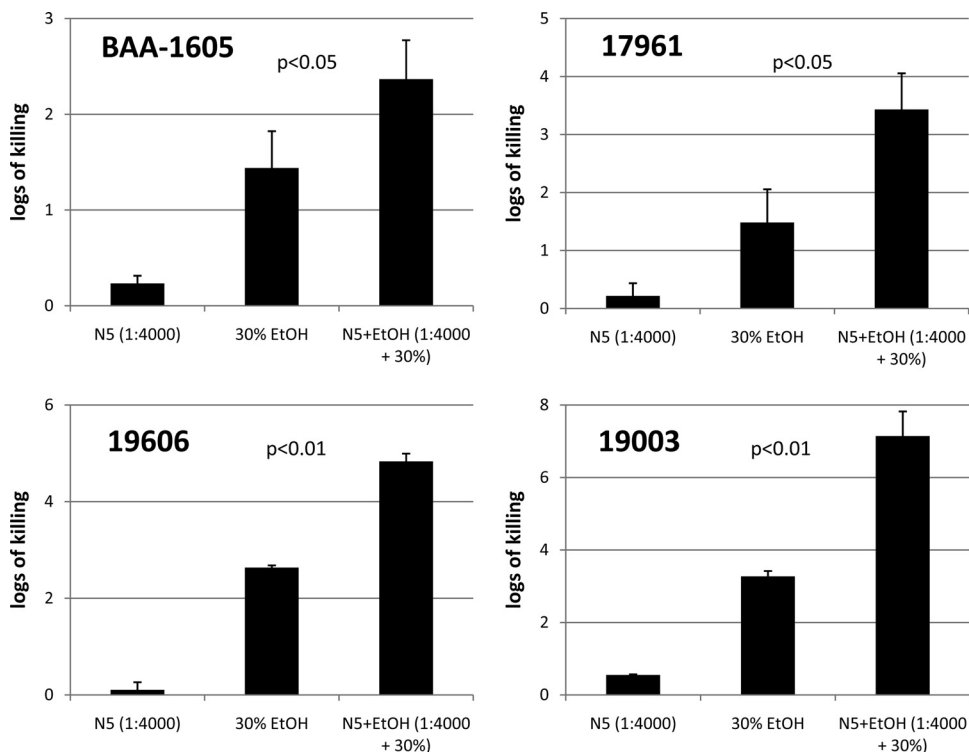
FIG 3 Additive effect of N5 combined with ethanol on *A. baumannii* viability. Bacterial cultures (2×10^7/ml) were incubated for 1 h in NB with the indicated concentrations of single agents (N5 and ethanol) or combinations, in which the addition of 30% ethanol resulted in a >1- to 3-log increase in killing effect (CFU/ml) of N5 against all four bacterial strains tested.

increased ~2 times (441.6 nm). Exposure to TS and LL conditions had little effect on particle size; sizes were 291.2 nm and 279.7 nm, respectively. Upon visualization of nanoemulsion following environmental exposure studies, phase separation was observed in the TS-, HH-, and HL-treated samples; however, the antimicrobial activity of N5, as measured by MIC and MBC assays, was not affected (Table 2).

DISCUSSION

Recently, skin, soft tissue, and bloodstream infections caused by multidrug-resistant *A. baumannii* have increased among service members from battlefields as well as nosocomial patients in civilian hospitals (21). *A. baumannii* is resistant to almost all conventional antibiotics by a wide range of mechanisms and can survive for prolonged periods on the surfaces of instruments in hospital settings (1). These factors in addition to its ability to form biofilms and its special cell surface properties (pilus assembly and production of Bap surface adhesion protein) have made current medical approaches ineffective (22, 23). Several new approaches to development of antimicrobial therapeutics have been reported, including the generation of nitric acid-producing nanoparticles (24), photodynamic therapy (25), and gallium maltolate treatment (26). Although the experimental results of these approaches seem positive, the therapeutic efficacy and the potential secondary effects of these treatments need further investigation. This lack of effective and safe treatment options for *A. baumannii* has led to the continued development of novel antimicrobial agents.

Antimicrobial nanoemulsions are surfactant-containing oil-in-water emulsions (particle size, 100 to 800 nm) which are very

effective against many bacteria, enveloped virus, fungi, and spores (8, 27) at concentrations that are nonirritating to skin or mucous membranes of animals (28). The antimicrobial activity and mechanism of nanoemulsions are believed to function as a result of the ability of the nanoemulsions to fuse with the outer membranes of microorganisms, with the electrostatic interaction between the cationic charge of the nanoparticles and the anionic charge on the microorganisms (10) ultimately destabilizing the membrane's lipid bilayers and its cellular permeability, leading to disruption (29); hence the broad spectrum activity of these particles.

In this study, we compared the bacteriostatic and bactericidal characteristics of five nanoemulsion preparations against four *A. baumannii* strains isolated from sputum, blood, urine, and cerebrospinal fluid of patients. Our lead formulation, N5, which contains 10% (vol/vol) Triton X-100, 25% (vol/vol) soybean oil, and 1% (wt/vol) CPC, inhibited *A. baumannii*, with MICs and MBCs at dilutions of 1:2,187 and 1:729 to 1:2,187, respectively, depending on the strains, and reduced the number of CFU/ml over 10^2 to 10^6 times within 15 to 60 min at a 1:500 to 1:3,333 dilution.

One way in which antimicrobial activity of nanoemulsion can be enhanced is with the addition of a cationic halogen-containing compound such as CPC, which provides a positive surface charge on the particle of nanoemulsions and ultimately efficiently attracts the negatively charged bacterial surface (30). Additionally, CPC can function as a cosurfactant (15), thereby aiding in bacterial cell membrane destabilization, and has been shown to possess independent antimicrobial activity (31). In the case of the planktonic form of bacteria, our data demonstrate that the killing is due to the 1% CPC present in NS. However, the additional components of
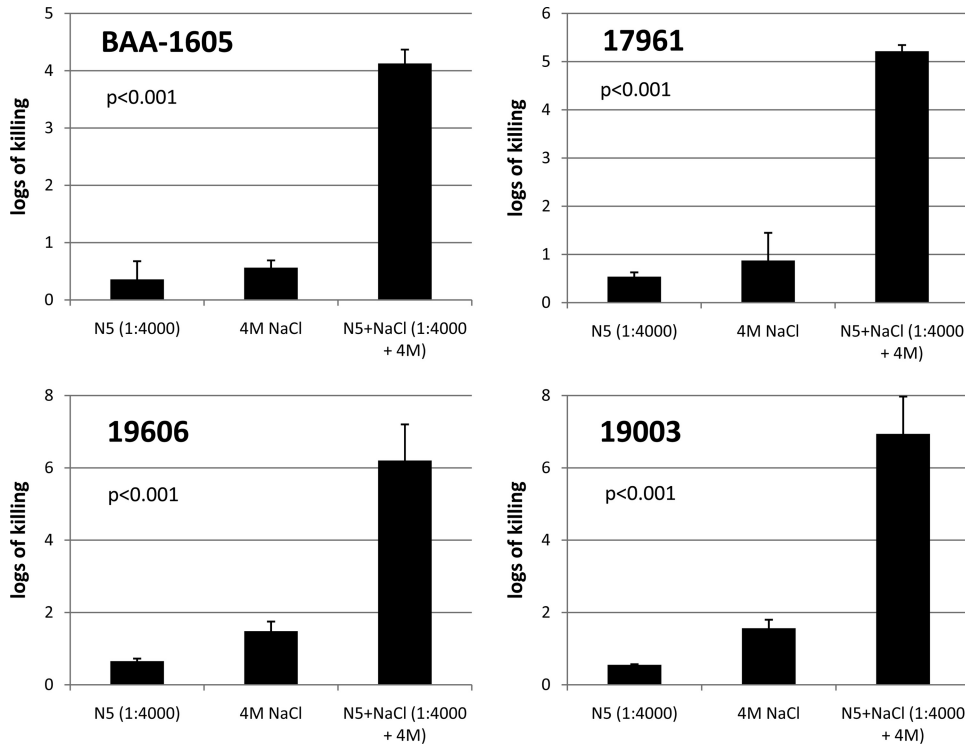
FIG 4 Additive effect of N5 combined with NaCl on *A. baumannii* viability. Bacterial cultures (2×10^7/ml) were incubated for 1 h in NB with the indicated concentrations of single agents (N5 and NaCl) or combinations, in which the addition of 4 M NaCl resulted in a >3- to 4-log increase in killing effect (CFU/ml) of N5 against all four bacterial strains tested.

N5 have a significant effect on disruption of the *A. baumannii* biofilm, thus enhancing killing over that achieved with 1% CPC alone.

Additionally, we evaluated the additive effects of ethanol and a hypertonic solution of NaCl on *A. baumannii* killing, both of which demonstrated enhanced killing versus N5 alone.

Bacteria within a biofilm show susceptibilities to antibiotics and to the host immune response different from those of their
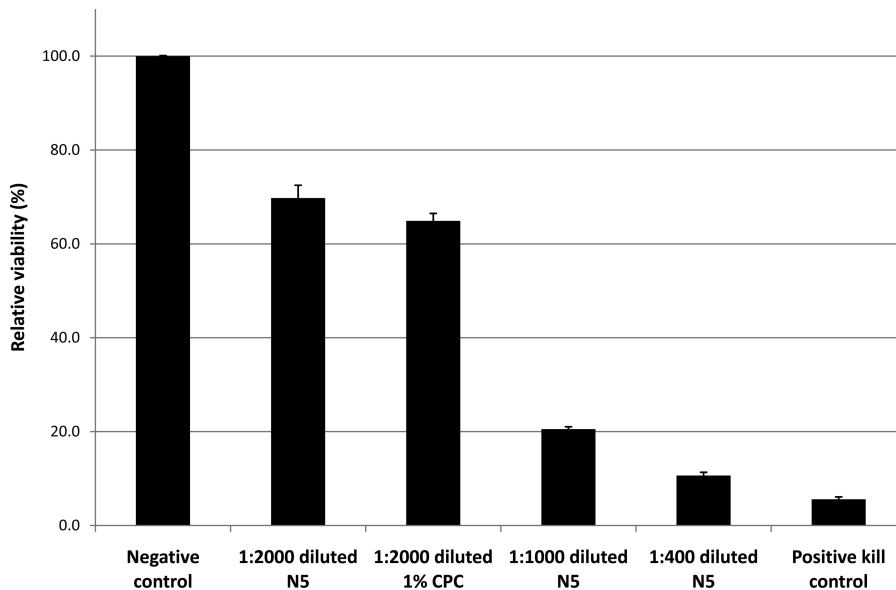

FIG 5 Effect of N5 on the metabolic activity of *A. baumannii* ATCC BAA-1605 biofilms. The biofilms formed in flat-bottom 96-well cell culture plates after 72 h of incubation at 37°C were incubated with 200 µl of N5 (1:400, 1:1,000, and 1:2,000 dilutions) and 1% CPC alone (1:2,000 dilution) for 1 h at 37°C, and the remaining metabolic activity in biofilms was measured using an alamarBlue cell proliferation assay kit. The negative control was an equal volume of NB without N5, and the positive killing control was an equal volume of NB with 0.1% sodium hypochlorite.
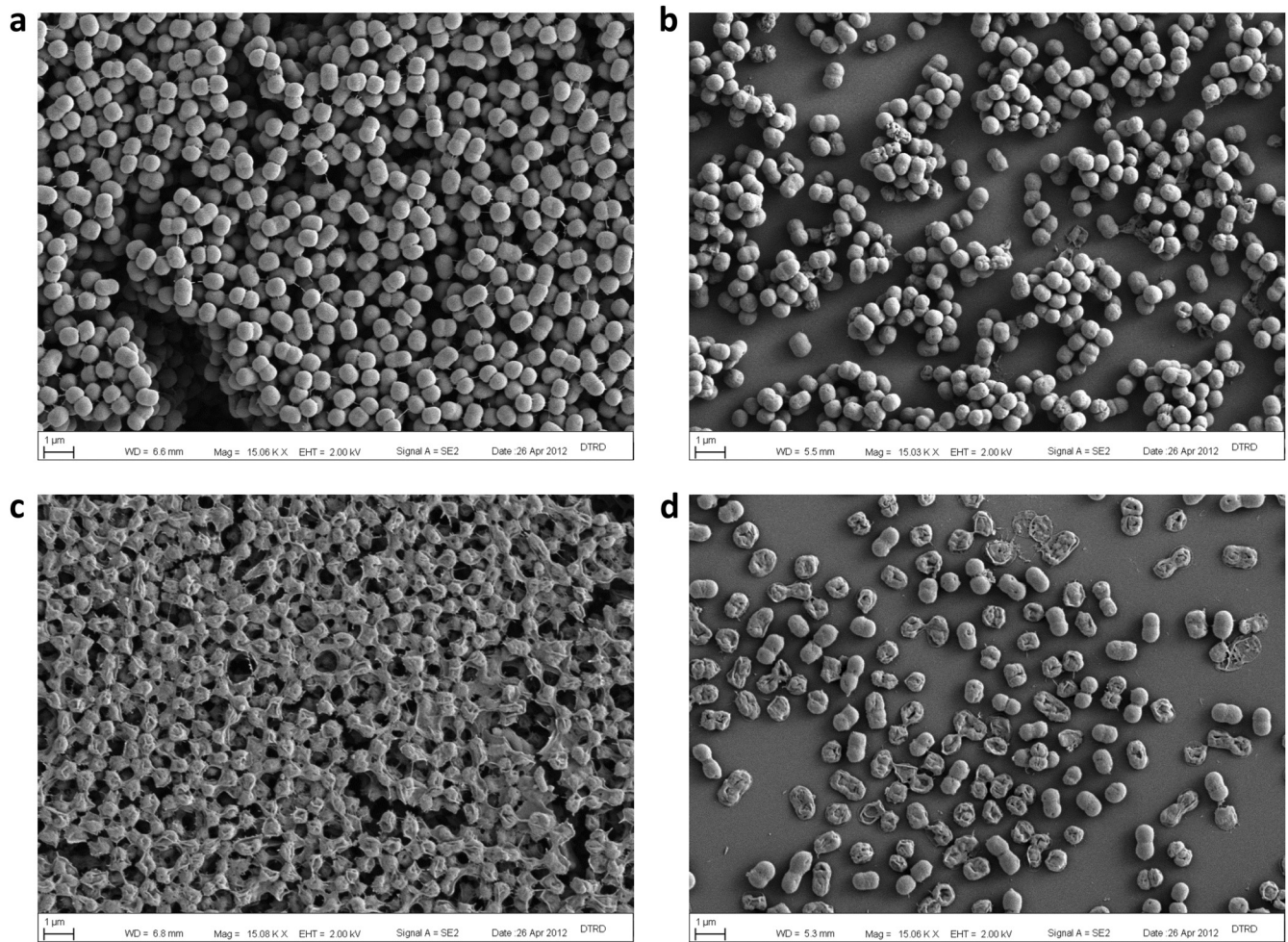
FIG 6 Scanning electron microscopy (magnification, ×15,000) of multidrug-resistant *A. baumannii* strain ATCC BAA-1605 biofilms formed on acrylic slides. All cells were cultured in NB at 37°C for 72 h. a, control; b, N5 without 1% CPC; c, 1% CPC alone; d, N5, including 1% CPC. Biofilm slides were incubated for 1 h in NB containing each agent.

planktonic form ([32]). Their resistance against most therapeutic approaches comes from multiple mechanisms, including an exopolysaccharide matrix, which functions as a barrier against penetration of antimicrobial agents ([20]). Therefore, for an antimicrobial agent to be effective against bacteria such as *A. baumannii*, it is

TABLE 2 Effect of environmental exposure on MIC, MBC and particle size of N5

Environmental conditions[a]	Effective diam (nm)	Polydispersity[b]	MIC[c]	MBC[c]
TS	291	0.142	1:2,187	1:729
HH	494	0.005	1:2,187	1:729
HL	442	0.029	1:2,187	1:729
LL	280	0.238	1:2,187	1:729
Lab	213	0.136	1:2,187	1:729

[a] TS, thermal shock; HH, high humidity/high temperature; HL, high humidity/low temperature; LL, low humidity/low temperature.
[b] Polydispersity is a measure of the distribution width of the droplets provided by the manufacturer of the size analyzer. The dilution of each step was 1:3, and the final dilution was 1:531,441.
[c] MIC and MBC were tested on *A. baumannii* BAA-1605.

important for the compound to possess properties which lead to the dispersion and/or disruption of biofilm structures, leading to access to bacteria, such as that seen when bacteria are in the drug-sensitive planktonic form ([33]). To this end, we evaluated the effects of N5 on the metabolic activity of *A. baumannii* in biofilms via scanning electron microscopy and observed extensive dispersion and disruption of the biofilm structure compared to untreated controls. These data were similar to results reported for nanoemulsion effects on *Streptococcus mutans* ([34]).

This anoemulsion potentially represents a suitable alternative to standard antibiotic and antimicrobial compounds for the treatment against antibiotic-resistant bacteria and those which form impenetrable biofilms ([35]). Our nanoemulsion, N5, which contains 1% CPC and has a mean diameter of 213.9 nm, has effective antimicrobial activity against several *A. baumannii* strains evaluated at high dilutions and may be an ideal candidate for the topical treatment of *A. baumannii* infections and/or instrument/surface decontamination. N5 can effectively reduce the metabolic activities of *A. baumannii* in biofilms at high dilutions and showed consistent antimicrobial activity after exposure to extreme environmental conditions, proving its suitability for field utilization.

Additional studies are necessary and ongoing to improve the antimicrobial activity of N5 by introducing additional antimicrobial agents, such as antimicrobial nanoparticles, chelating agents, and peptides.

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