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A Green Chemistry-Oriented Sporicidal Cocktail

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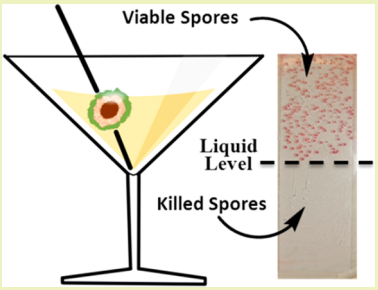
ABSTRACT: The first green chemistry-oriented antispore strategy is herein reported. The tricomponent system of low pH, anionic surfactants, and ethanol kills bacterial spores under relatively mild conditions by sequentially targeting spore resistance and viability, contrasting the current prevailing spore chemical sterilization protocols that invariably require the use of strong oxidizing agents. The sporicidal potency of this system was demonstrated with three spore species both in liquid suspensions as well as on surfaces. Furthermore, the chemical simplicity of this strategy allows it to be easily recreated using common household products.



KEYWORDS: *Green chemistry, Sporicides, Antimicrobial*

INTRODUCTION

Sporulation is the ultimate defense mechanism of many bacterial species, including the clinically important *Bacillus cereus*, a common cause of food poisoning, and *Bacillus anthracis*, a notorious bioterrorism agent. As a result of the dehydrated and therefore metabolically dormant state of spore cores, traditional disinfection procedures, such as UV radiation, antibiotics, detergents, alcohols, and quaternary ammonium compounds, have little effect on spore viability.[1,2] Most known chemical sporicides, including iodine, β-proiolactone, sodium hypochlorite, and glutaraldehyde, have so far only achieved limited application due to serious safety concerns and severe environmental damage.[3] The current standard spore sterilants recommended by the United States Centers for Disease Control and Prevention (CDC) require the use of strong oxidizing agents[4]—ethylene oxide, hydrogen peroxide or peracetic acid—whose general usage are also plagued by chemical instability, corrosiveness, and toxicity.[5] A safer and more environmentally friendly sporicial agent is therefore in great need.

During our recent study, we inadvertently discovered that the surfactant sodium dodecyl sulfate (SDS) under acidic conditions can uniquely and effectively breach the spore chemical resistance at near-physiological temperature, making spores vulnerable to further chemical incursions. The general availability (found in common household products) and the low toxicity (easily neutralizable before disposal) of the chemicals required, particularly in comparison to the existing sporicidal agents, spurred our interest for further research. In this report, we systematically explore and study the efficacy and spectrum, as well as the possible mechanism of this potential solution to a long-standing bioengineering challenge, demonstrating its practicality as a cost-effective and environmentally friendly improvement to conventional spore sterilization procedures.

EXPERIMENTAL SECTION

Chemicals. Na_2HPO_4, citric acid, phenol, urea, sodium chloride, calcium chloride, iron(III) chloride, aluminum chloride, tetrazolium chloride (TTC), sodium dodecyl sulfate (SDS), dodecyltrimethylammonium chloride (DTAC), ethylenediaminetetraacetic acid (EDTA), and sodium hexyl sulfate (SHS) were purchased from Sigma-Aldrich Chemical Co. (MO, U.S.A.). Hand soap (Softsoap Energizing Pomegranate and Mango) was acquired from Colgate Co. (NY, U.S.A.). Lemon juice (Tantillo) was purchase from T&M Imports (NY, U.S.A.). Vodka (100 proof, Monarch) was purchased from Hood River Distillers (OR, U.S.A.). Antispore "cocktail" (per milliliter) contains 200 μL vodka, 700 μL lemon juice, and 100 μmL hand soap. Nutrient Broth No. 1 was purchased from Fluka Analytical (MO, U.S.A.). Bacto agar was purchased from BD (NJ, U.S.A.). *Bacillus megaterium* and *Bacillus cereus* spore stock suspensions were purchased from Mesa Laboratories, Inc. (MT, U.S.A.). *Bacillus atrophaeus* spore suspension was purchased from STERIS Corp. (OH, U.S.A.).

Evaluating Chemical Sporicidal Potency in Liquid Suspensions. *B. megaterium* endospores were acquired through commercial sources. After exposure to various chemicals at 40 °C for 30 min, spore suspensions were immediately washed, diluted, and spread onto Broth No. 1 agar plates. The colony numbers were determined in the following day after overnight incubation at 37 °C. Broth No. 1 agar (10g/L of tryptone, 10g/L of NaCl, 5g/L of yeast extract, and 1g/L of glucose) was verified to completely germinate *B. megaterium* spores without the need for prior heat activation, thus ensuring that the difference in colony number observed comes from loss of viability during chemical exposure and not from incomplete germination in the subsequent incubation.

Evaluating Chemical Sporicidal Potency on Surfaces. All spore suspensions were acquired from commercial sources. The spore suspensions were diluted in sterilized Milli-Q water and subsequently sprayed onto UV-cleaned glass slides, after which the slides were air-dried at ambient temperature for 1 h to ensure spore surface attachment. The dried slides were then half submerged into various testing solutions prewarmed to 40 °C for 30 min. After the 30 min

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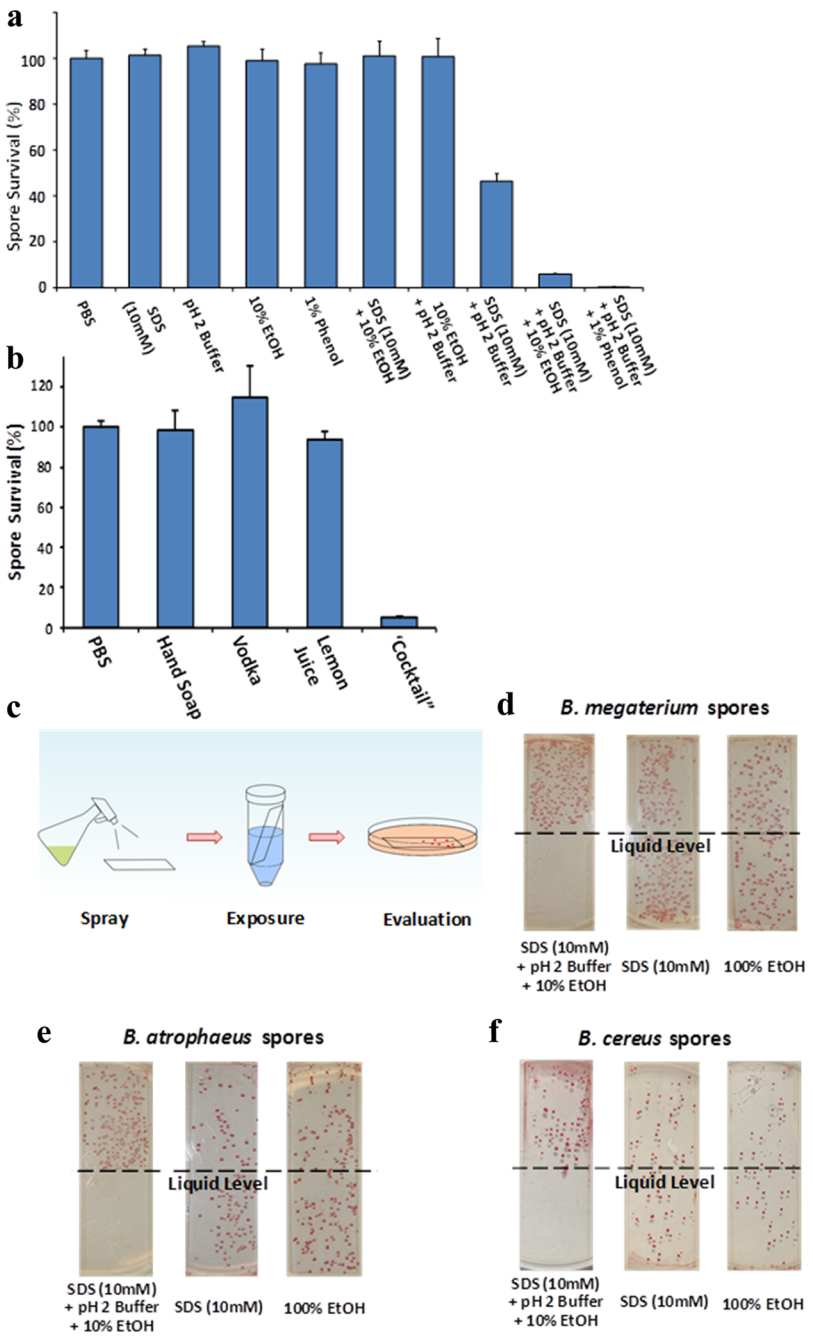

Figure 1. (a) *B. megaterium* spore survival percentage after exposure to various chemical conditions at 40 °C for 30 min. Spores suspended in phosphate buffer saline (PBS) were included as control. (b) *B. megaterium* spore survival percentage after exposure to common household products at 40 °C for 30 min. (c) Scheme for testing surface sterilizing potency of SDS/low pH plus 10% ethanol against spores from three bacterial species. Note that the upper halves of the slides were not submerged under the antispore solution and were used as references for comparison. (d–f) Surface antispore effects of SDS/low pH plus 10% ethanol against *B. megaterium*, *B. atrophaeus*, and *B. cereus* spores. The incubation conditions were 40 °C 30 min for all experiments. A total of 50 ppm tetrazolium chloride (TTC) was added into the overlaying Lysogeny broth to aid visualization.

incubation, slides were gently rinsed with sterilized phosphate buffer to clean off of various testing solutions. The slides were individually placed into clean Petri dishes and then overlaid with molten Broth No. 1 agar supplemented with 50 ppm TTC at 45 °C. The plates were later incubated at 37 °C (*B. megaterium* and *B. atrophaeus*) or 30 °C (*B. cereus*) overnight before counting the colony number.

Phase Contrast Microscopy. Dormant nutrient-germinated *B. megaterium* spores as well as spores exposed to 10 mM SDS in pH 2 solution were observed directly under a standard phase contrast microscope. The nutrient germination was carried out under a previously reported condition;[6] spores were first heat activated at 60 °C for 15 min, followed by incubation at 30 °C with 10 mM D-glucose in PBS for 60 min to ensure complete germination.

RESULTS AND DISCUSSION

As a starting point, we examine the combined effect of SDS and low pH on *B. megaterium* spore viability with or without additional chemical assistance. From a standard colony formation assay, it was observed that supplementing McIlvaine's buffer (citric acid/Na_2HPO_4) at pH 2 with 10 mM SDS (2.9 mg/mL) decreased the viable spore count to

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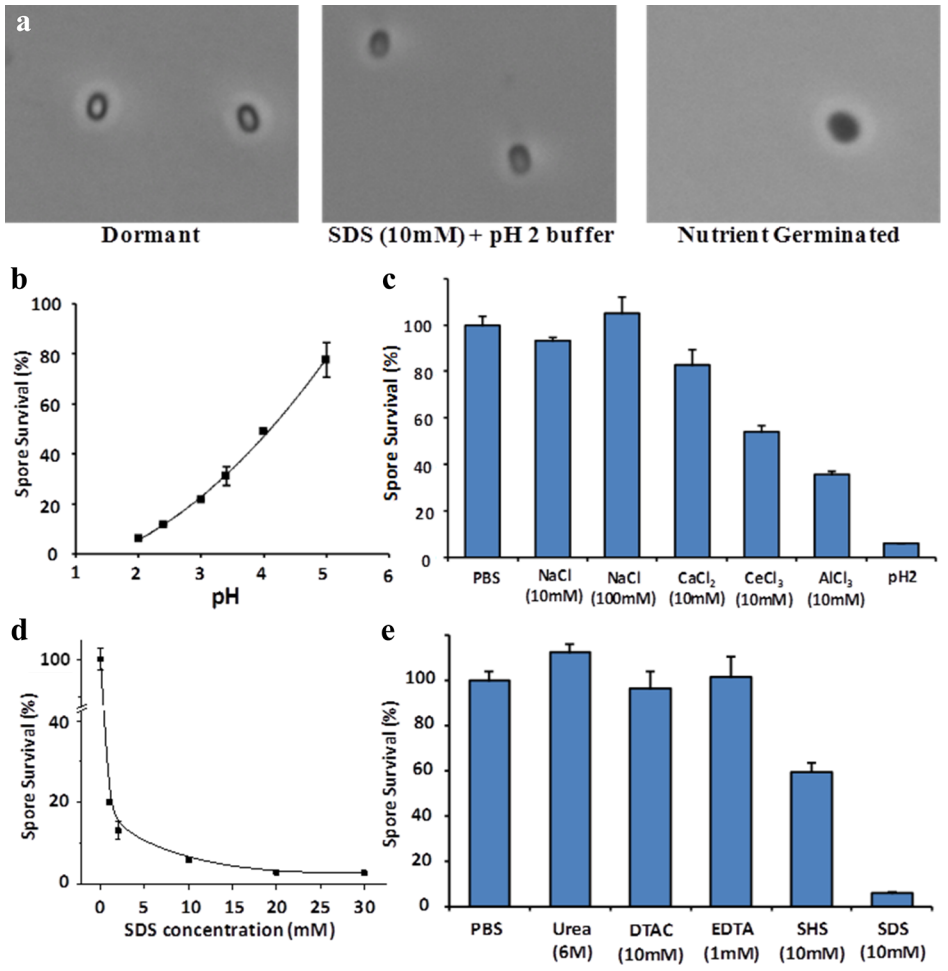

Figure 2. (a) Representative phase contrast microscopy photos of dormant and nutrient-germinated *B. megaterium* spores as well as spores exposed to pH 2, 10 mM SDS solution at 40 °C for 30 min. Darker color at the spore core region is indicative of a higher water content. (b) *B. megaterium* spore survival percentage after exposure to 10 mM SDS solution with 10% ethanol under different pH. (c) *B. megaterium* spore survival percentage after exposure to 10 mM SDS solution with 10% ethanol at neutral pH but supplemented with various metal cations. A 10 mM SDS solution with 10% ethanol at pH 2 was included as a control experiment. The actual concentrations of Ca^{2+}, Ce^{3+}, and Al^{3+} are expected to be lower than the 10 mM originally prepared, as the solutions turned partially turbid upon mixing due to the ion pair formation between multivalent cations and SDS. (d) *B. megaterium* spore survival percentage after exposure to pH 2 buffer with 10% ethanol at various SDS concentrations. (e) *B. megaterium* spore survival percentage after exposure to pH 2 buffer with 10% ethanol supplemented with anionic surfactant SDS and SHS, cationic surfactant DTAC, metal chelating agent EDTA, and protein denaturing agent urea. All incubation conditions from b−e were identical at 40 °C and 30 min.

below 50% after 30 min at 40 °C (Figure 1a). Although not particularly potent as a sporicide by itself, the SDS/low pH treatment evidently breached the spore chemical resistance, as creating a more biologically challenging milieu with additional 10% ethanol caused the surviving spore number to plummet below 6%. Confirming this observed synergy separately or in other combinations of SDS, low pH and ethanol showed no impact on spore viability (Figure 1a). The addition of ethanol is to facilitate the killing of defense-compromised spores that would otherwise be immune to these organic compounds. As such, 10% ethanol can be readily replaced by other common antiseptics, e.g., 1% phenol solution in combination with SDS under acidic condition saw the complete annihilation of viable *B. megaterium* spores. As a reference, a previous study indicated that to impair spore viability with acid alone required the use of HCl above 500 mM,[7] 50 times more acidic than the condition used in this work. The complete lack of antispore activity of its individual components demonstrated in this experiment as well as the specificity of the chemicals needed in this antispore combination (see below) suggests a concerted action of defense-weakening (SDS/low pH) followed by inactivation (ethanol or phenol), rather than merely imposing a universally destructive environment that indiscriminately exterminates all life forms within. This mechanistic aspect shall be further explored in a later part of this report.

A major motivation behind our study of the SDS/low pH antispore effect is that it offers a considerably cheaper and milder procedure than the currently prevailing spore sterilization protocols. To emphasize this point, we effectively recreated the same chemical mixture (SDS/low pH/ethanol) used in our previous experiments with a combination of common household products: hand soap (typically containing 0.1−0.4% w/v anionic surfactants), lemon juice (5% w/v citric acid, pH naturally at 2), and 100 proof vodka (50% v/v ethanol). While, as expected, these components individually display no antispore activity, the combined "cocktail" (10% v/v hand soap, 70% v/v lemon juice, and 20% v/v vodka) eliminates over 95% viable spores after an incubation time of 30 min at 40 °C (Figure 1b).

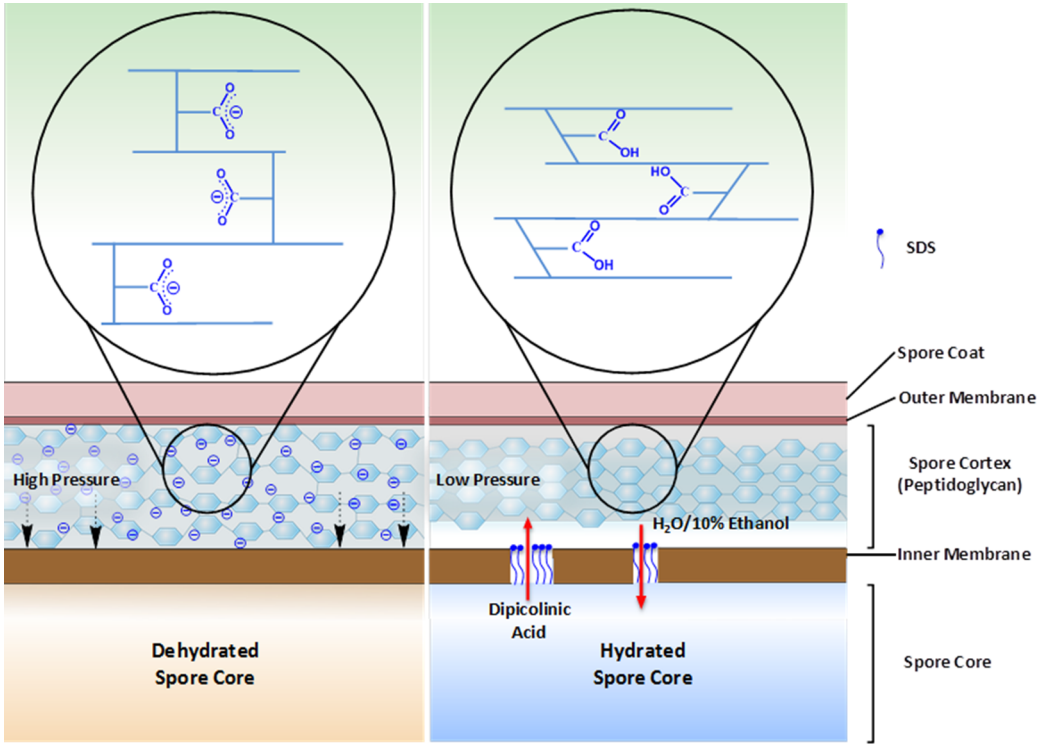

Figure 3. Proposed antispore mechanism of low pH and SDS, in which reduced cortex osmotic pressure due to carboxylate protonation and inner membrane instability triggered by SDS work in synergy, leading to the diminishment of spore chemical resistance and the eventual inactivation by ethanol.

We further test the utility of this approach by mimicking the common clinical scenarios in which substrate surfaces are to be sterilized and rid of all viable spores. To this end, spore suspensions of *B. megaterium*, *B. atrophaeus* (*B. anthracis* simulant), and *B. cereus* (a common culprit for food poisoning) were sprayed onto glass surfaces. After air-drying for 1 h to allow for spore surface attachment, the glass slides were half submerged into a solution containing 10 mM SDS and 10% ethanol at pH 2. Following 30 min incubation at 40 °C, the slides were removed from the solution mixture and gently rinsed with sterilized phosphate buffer, and the surviving surface-bound spores were quantified in a colony formation assay (Figure 1c). The SDS/ethanol mixture at pH 2 lead to a complete elimination of viable spores from all three bacterial species, while no sporicidal effect was observed in samples exposed to 10 mM SDS solution or 100% ethanol separately, evincing its high efficacy and general applicability (Figure 1d–f).

After demonstrating the effectiveness and practicality of this antispore strategy, we aimed to probe into its potential mechanism. The ability of bacterial spores to withstand environmental adversities is resulted from the dehydrated state of the spore core.[8] Consistent with this consensus, the core section of dormant *B. megaterium* spores after exposure to a pH 2, 10 mM SDS solution at 40 °C for 30 min showed an increase in water content under phase contrast microscope (significant darkening in the central region of the spore), thus providing a morphological account of the previously observed loss of spore chemical resistance (Figure 2a).

On a molecular level, though the details for maintaining spore core dehydration are not completely clear, it is generally accepted that both the high pressure from spore cortex[9] and the low permeability of inner membrane toward small molecules (including water and H^+)[10] are the both essential in maintaining this dehydrated state. It has been further suggested that the role of the cortex layer in core dehydration is at least in part resulted from the high osmotic pressure created by the anionic carboxylate groups on the cortex peptidoglycan matrices.[11] Under acidic conditions (which protonate the carboxylate anions, hence lowering the matrix osmotic pressure), the loss of spore chemical resistance reported in this work (Figure 2b) and the previously noted reduction in heat resistance[12] are both consistent with this cortex osmoregulatory model. Furthermore, the low pH used in the antispore cocktail can to a certain extent be functionally substituted by multivalent metal cations (Figure 2c), which are known to bind strongly to the anionic polymer matrices, including the spore cortex layer, through chelation. Therefore, one plausible explanation for the role of low pH in this antispore combination lies in its ability to quench the negatively charged peptidoglycan matrix, thus dampening the cortex high osmotic pressure critical for core dehydration.

Meanwhile, SDS is hypothesized to weaken the inner membrane as a permeation barrier based on the surfactant's well-known function in cell biology. It is worth noting that this speculated membrane interaction is specific for anionic surfactants, including SDS (Figure 2d) and, to a much lesser degree, sodium hexyl sulfate (SHS) (Figure 2e). In contrast, a representative cationic surfactant (dodecyltrimethylammonium chloride, DTAC), strong protein denaturing agent (urea), or metal chelating agent (ethylenediaminetetraacetic acid, EDTA) displays no antispore effects when similarly mixed with 10% ethanol under pH 2 (Figure 2e). The overall proposed mechanism is summarized in Figure 3. Under this scheme, the low pH condition and SDS interact with spore cortex and inner membrane, respectively, leading to partial rehydration of

spore core and an increase in spore susceptibility to organic compounds including ethanol.

It is worth noting that a significant portion of SDS molecules at 10 mM may exist in the self-assembled micelle form under the experimental condition employed in this study (10% ethanol, pH 2 at 40 °C), as 10 mM is comparable to the previously reported SDS critical micelle concentration (CMC) in a similar ethanol/water solvent system and under the same temperature.[13] Furthermore, the acidic condition used in the antispore assays is known to protonate the SDS sulfate head groups and reduce the electrostatic repulsion, and as a result, considerably lower the surfactant CMC.[14] However, this micelle formation is unlikely to be a prerequisite for the cooperative sporicidal effect of SDS due to the known size exclusion property of the spore coat[15] (the micelle structure in its entirety can hardly reach spore cortex and inner membrane) as well as the observed SDS concentration dependence of the tricomponent mixture antispore potency (Figure 2d; increasing SDS concentration beyond 10 mM, which increases surfactant micelle concentration, only marginally lowers the surviving spore count).

In summary, we herein present the first green chemistry-oriented antispore strategy via spore sensitization (low pH and anionic surfactants) followed by elimination (ethanol, phenol, or other regular bactericidal agents). We demonstrated its capability to inactivate spores from different bacterial species under relatively mild chemical conditions (careful handling and proper eye protection may still be needed during sterilization, while the low solution pH can be easily neutralized prior to disposal). The advantages of this approach over the existing spore sterilization methods—with regard to safety, cost, ease of implementation, and low environmental impacts—make it a promising new sporicidal procedure to be used in biodefense, food industries, and various clinical applications.

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Notes

The authors declare no competing financial interest.

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