Synergy between Airborne Singlet Oxygen and a Trisubstituted Olefin Sulfonate for the Inactivation of Bacteria

Rajib Choudhury and Alexander Greer*

Department of Chemistry and Graduate Center, City University of New York, Brooklyn College, Brooklyn, New York 11210, United States

Supporting Information

ABSTRACT: The reactivity of a trisubstituted alkene surfactant (8-methylnon-7-ene-1 sulfonate, 1) to airborne singlet oxygen in a solution containing E. coli was examined. Surfactant 1 was prepared by a Strecker-type reaction of 9-bromo-2-methylnon-2-ene with sodium sulfitte. Submicellar concentrations of 1 were used that reacted with singlet oxygen by an “ene” reaction to yield two hydroperoxides (7-hydroperoxy-8-methylnon-8-ene-1 sulfonate and (E)-8-hydroperoxy-8-methylnon-6-ene-1 sulfonate) in a 4:1 ratio. Exchanging the H2O solution for D2O where the lifetime of solution-phase singlet oxygen increases by 20-fold led to an ~2-fold increase in the yield of hydroperoxides pointing to surface activity of singlet oxygen with the surfactant in a partially solvated state. In this airborne singlet oxygen reaction, E. coli inactivation was monitored in the presence and absence of 1 and by a LIVE/DEAD cell permeabilization assay. It was shown that the surfactant has low dark toxicity with respect to the bacteria, but in the presence of airborne singlet oxygen, it produces a synergistic enhancement of the bacterial inactivation. How the ene-derived surfactant hydroperoxides can provoke ¹O₂ toxicity and be of general utility is discussed.

INTRODUCTION

Although singlet oxygen [¹O₂ (¹Δg)] is an effective toxin for inactivating bacteria, methods to generate it suffer from photosensitizer problems including solubilization, degradation, and bleaching. Turbid solutions can also present problem because light can be blocked from reaching the sensitizers. Because of these issues, there is a need to develop methods for killing bacteria without the physical contact of photosensitizer with the solution. Airborne ¹O₂ offers some promise in this regard.

Figure 1 shows the three-phase apparatus that we used in this study for the delivery of ¹O₂ to the air/water interface of a bacterial solution. By virtue of how the apparatus works, the solution is devoid of any photosensitizers, where gas-phase singlet oxygen diffuses to the solution surface. By analogy, Majima et al. carried out experiments using a sensitizing TiO₂ surface and a terephaladiimide oxygen acceptor adsorbed on another surface that was separated by 1 mm, indicating the formation of a diffusible ¹O₂ species (similar to the Kautsky three-phase test of 80 years ago). The apparatus in Figure 1 leads to ¹O₂ at the air/water interface for E. coli inactivation is not surprising because its design is similar to that of an apparatus invented by Midden. What is new and better (we regard our innovation as an offshoot of the Midden and Majima systems) is the unique function of surfactant 1 in E. coli inactivation by airborne ¹O₂.

Our hypothesis was that a ¹O₂-active surfactant (1) would synergistically enhance bacterial inactivation. Synergy has been found in other branches of singlet oxygen research. It has been found in the photodynamic inactivation of bacteria with biofilm dispersions of a 2-aminoimidazole-triazole conjugate, photodynamic therapy (PDT) with drug additives such as carboplatin, and with the simultaneous reaction of nitric oxide or SO₃, among other ¹O₂ topics. Similar to surfactant 1, there was a report on a 2,5-disubstituted furan surfactant with a cationic tetraalkylammonium headgroup that was oxidized by ¹O₂ to an endoperoxide in a liposome study, but the reaction was not examined for antibacterial activity.

Here we show that an ¹O₂-active surfactant can synergistically enhance microbe inactivation from airborne ¹O₂ through hydroperoxide formation. Our work serves as a starting point where in-situ-generated surfactant hydroperoxides function as secondary toxins to pure ¹O₂ for enhanced bactericidal action. Following the Experimental Section, our results will be presented in four parts: first, the rationale for the selection of surfactant 1; second, measured surfactant photoperoxide formation via airborne ¹O₂; third, measured E. coli killing by ¹O₂ with and without surfactant 1; and fourth, measured E. coli killing by ¹O₂, followed by the addition of hydroperoxides 2 and 3 in the dark.

EXPERIMENTAL SECTION

Reagents and Instrumentation. Porous Vycor glass (Corning 7930) was purchased from Advanced Glass and Ceramics (Holden, MA).

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Silicon phthalocyanine dichloride, aluminum(III) phthalocyanine chloride tetrasulfonic acid, 9-bromo-2-methylnon-2-ene, sodium sulfite, triphenyl phosphine (PPh₃), benzoic acid, dimethylsulfone, DMF, CH₂Cl₂, ethanol, D₂O, and DMSO-d₆ were purchased from commercial suppliers and were used as received. Dichloromethane was distilled over phosphorus pentoxide prior to use. Deionized water was purified with a U.S. Filter Corporation deionization system (Vineland, NJ). Nuclear magnetic resonance (NMR) data were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz for ¹H NMR and at 100.6 MHz for ¹³C NMR. UV-vis data were collected on a Hitachi UV-vis U-2001 instrument. FAB-mass spectrometry data were collected on a JEOL JMS-HX110 spectrometer using a m-nitrobenzyl alcohol matrix, a 10 kV acceleration voltage, and a Xe beam FAB gun (6 kV) on the MS-1 ion source. Infrared spectra were recorded on a Nicolet iS10 FT-IR spectrometer. Solution temperatures were measured with a digital pyrometer (Thermo Scientific). An Olympus FluoView FV10i confocal fluorescence microscope was used to analyze stained *E. coli* and assess membrane permeability following singlet oxygen exposure.

**Sensitizing Glass Plate.** Using a Pasteur pipet, 50 μL of methanol containing $8 \times 10^{-4}$ M aluminum(III) phthalocyanine chloride tetrasulfonic acid (Pc) was deposited onto one side of PVG (disk shape 14.0 mm × 1.0 mm or square shape 2.25 cm² and 1.0–1.5 mm). Most of the methanol had evaporated after 12–24 h at 26 °C, at which point the sample was used. The result was PC sensitizing glass loaded on one side with $1.1 \times 10^{-5}$ mols of Pc/g of PVG with the penetration of the sensitizer into the glass core and edges.

**Apparatus.** A three-phase apparatus was constructed for airborne $¹O₂$ delivery to the air/water interface of a solution (Figures 2). The sensitizing glass plate was placed sensitizer face down, above a short quartz cuvette (1.0 × 1.0 × 0.7 cm³) containing 0.60 mL of water (from a micropipet, precision ±0.005 mL) and illuminated perpendicularly from a 3.0 cm distance with 669 nm light (383 mW) from a diode laser (model 7404, Intense, North Brunswick, NJ). The light from the laser overlapped well with the Pc absorption. The 669 nm light was passed through an FT-400-EMT optical fiber (Thorlabs, Newton NJ), which produced a Gaussian distribution of incident photons on the sensitizing glass plate (total dose ≈ 1700 J/cm²). The diameter of the laser spot on the sensitizing glass plate was 0.95 cm (area = 0.71 cm²). The sensitizing glass plate was not in contact with the water. The sensitizing glass plate sat atop the short cuvette above the water interface by 0.4 mm situated at the sides of the cuvette. Moving laterally from the cuvette side to the midpoint of the meniscus, the distance between the sensitizing plate and water was 1.5 mm. These distances were measured with a miniature ruler and a 10X magnifying glass with an uncertainty of ±0.04 mm. Water evaporation was negligible and did not measurably change the volume over the
of a surfactant that can readily form a hydroperoxide product. Thus, we selected terminally branched-chain olefin sulfonate I with an eye toward the ease of formation of allylic hydroperoxides. Trisubstituted olefins\textsuperscript{25-27} are much more reactive with $^1$O$_2$ (∼20–500-fold) than are di- and mono-substituted olefins.\textsuperscript{28} For example, the chemical quenching rate constant ($k_q$) of $^1$O$_2$ with 2-methyl-2-pentene is reasonably high ($6 \times 10^7$ M\textsuperscript{-1} s\textsuperscript{-1}).\textsuperscript{29}

In the case of the detergent concentration, we selected a relatively low 1 mM concentration of I so the hydrophobic group would preferably point away from the surface. Our results show that the cmc of I (C\textsubscript{10}H\textsubscript{19}O\textsubscript{3}SNa\textsubscript{2}\textsuperscript{+}, 9.7 mM at 26°C) is lower than that of straight-chain C\textsubscript{10}H\textsubscript{21}SO\textsubscript{3}Na\textsuperscript{+} (43 mM at 25°C),\textsuperscript{30} but similar to that of straight-chain C\textsubscript{12}H\textsubscript{25}SO\textsubscript{3}Na\textsuperscript{+} (9.8 mM or 12 mM at 25°C).\textsuperscript{30} Although cmc’s generally decrease for branched hydrophobic groups,\textsuperscript{31} this mainly applies to internal rather than terminal unsaturated sites of olefin sulfonates. By running experiments below the micellar concentrations of I, the surfactant tends not to aggregate into environments away from the air/water interface.

**Airborne Singlet Oxygen Attack on a Partially Solvated Surfactant.** The apparatus brought airborne $^1$O$_2$ in from above onto the H$_2$O or D$_2$O solution for an ene reaction\textsuperscript{33,34} with 1 mM detergent I. The two hydroperoxides that were formed (2 and 3) have a shift of the double bond relative to that of I, which is a fingerprint reaction\textsuperscript{35} for singlet oxygen. We monitored the disappearance of I and the appearance of surfactant peroxides 2 and 3 (mass balance 91%), where 2 and 3 were stable enough for characterization as a mixture but began to decompose after 1 to 2 days at 25°C.

Figure 3 shows that the airborne $^1$O$_2$ oxidation of I led to hydroperoxide products (2 and 3) at double the efficiency in

![Figure 3](image-url)
with 1 below its cmc) likely as a result of the micellar protection of the alkene site from incoming airborne $\cdot$O$_2$ at the air/water interface. Below the cmc, the results point to surface activity where airborne singlet oxygen attacks 1 in a partially solvated state. Partially solvated 1 may relate to the observed stereo-selectivity of hydroperoxides 2 and 3 because the ratio was 4:1 (Table 1). Hydrogen abstraction proceeds mostly from the methyl groups. Thus, the hexyl sulfonate chain in 1 is not acting as a bulky allylic group as could have been expected from methyl groups. We do not think that electronic repulsion$^{47}$ may be key, where the methylene allylic hydrogens of the hexyl sulfonate chain are more restricted to rotation and thus less conformationally accessible (higher barrier to rotation) than the methyl groups. We do not think that electronic repulsion$^{47}$ takes place between the distal peroxy group and the sulfonate anion to explain methyl rather than methylene H-abstraction regioselectivity.

We now turn our attention to the bacterial killing results.

**Top-Down Approach to Bacterial Killing with Airborne $\cdot$O$_2$ and Surfactant 1.** Here, we make a case that detergent 1 synergistically enhances the bactericidal action of incoming $\cdot$O$_2$. Table 2 shows that the apparatus produces airborne $\cdot$O$_2$ at levels toxic to bacteria (entries 1–3). Samples containing 50, 30, and 15 $\mu$g/mL E. coli were inactivated by 25, 38, and 41%, respectively, after 1 h. Table 3 (entry 5) shows that the inactivation of 50 $\mu$g/mL E. coli when followed in 10 min increments led to 27% killing after 1 h.

However, synergistic E. coli inactivation was seen when combining airborne $\cdot$O$_2$ and surfactant 1 (Table 2, entries 4–6). That is, the number of E. coli killed increased by 1.7- to 2-fold compared to $\cdot$O$_2$ treatment without 1. The inactivation by 1 was 2.6% (entry 7) and by airborne $\cdot$O$_2$ was 25% (entry 1), which adds up to 27.6%, not the 50% seen with airborne $\cdot$O$_2$ in the presence of surfactant 1 (entry 4). The synergism was not restricted to the 50 $\mu$g/mL E. coli concentration but was also seen at 30 and 15 $\mu$g/mL.

Table 2 shows that the surfactant 1 toxicity in the dark is low. For example, for 50 $\mu$g/mL E. coli, 2.6% was killed by 1 mM 1, and for 15 $\mu$g/mL E. coli, 7.3% was killed (entries 7–9). The addition of a 4:1 mixture of 2 (0.144 mM) and 3 (0.036 mM) (similar to the amount generated in situ in Figure 3) in the dark was also relatively nontoxic, and the mixture led to 5–8% E. coli inactivation (Table 2, entries 10–12). Entry 13 shows a control reaction of the E. coli viability of 1.5% in the dark without surfactant 1 or hydroperoxides 2 and 3. The red light emitted from the device was also mostly nontoxic to E. coli, and the inactivation ranged from 3.7 to 8% for E. coli concentrations of 50 to 15 $\mu$g/mL (Table 2, entries 14–16). These observations point to low levels of 3–8% E. coli inactivation based on additives 1–3 in the dark or in red light alone. Next, we explored the effects of incubating hydroperoxides 2 and 3 with $\cdot$O$_2$-pretreated cells.
Table 2. E. coli Inactivation by the Airborne Singlet Oxygen Treatment as a Function of Additives and Other Conditions

<table>
<thead>
<tr>
<th>entry</th>
<th>condition</th>
<th>E. coli (μg/mL)</th>
<th>surfactant 1 added (mM)</th>
<th>4:1 mixture of hydroperoxides 2 and 3 added (mM)</th>
<th>% killed after 1 h</th>
<th>number of cells killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>airborne 1O₂⁴⁺</td>
<td>50</td>
<td></td>
<td></td>
<td>25 ± 5</td>
<td>7.5 x 10⁶</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>38 ± 5</td>
<td>6.8 x 10⁶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>41 ± 4</td>
<td>3.7 x 10⁷</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>airborne 1O₂⁴⁺</td>
<td>50</td>
<td>1.0</td>
<td></td>
<td>50 ± 6</td>
<td>1.5 x 10⁷</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>71 ± 3</td>
<td>1.3 x 10⁸</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>70 ± 3</td>
<td>6.3 x 10⁹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>dark</td>
<td>2.6 ± 0.5</td>
<td>5.2 x 10⁴</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td>6.3 ± 1.1</td>
<td>1.2 x 10⁵</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>7.3 ± 2.0</td>
<td>1.4 x 10⁵</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>dark</td>
<td>5 ± 1</td>
<td>1.0 x 10⁴</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>30</td>
<td>7 ± 3</td>
<td>1.4 x 10⁵</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>8 ± 3</td>
<td>1.6 x 10⁵</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>dark</td>
<td>1.5 ± 0.5</td>
<td>3.0 x 10⁴</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>669 nm light (no 1O₂)</td>
<td>50</td>
<td></td>
<td></td>
<td>3.7 ± 0.5</td>
<td>7.4 x 10⁴</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
<td>6.3 ± 0.6</td>
<td>1.2 x 10⁵</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>15</td>
<td>8 ± 2</td>
<td>1.6 x 10⁵</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⁴⁺Airborne 1O₂ is generated and crosses an intervening gap to the H₂O solution. bError bounds were obtained from three or more measurements.

Table 3. Percent of E. coli Killed after Treatment with Airborne 1O₂ in the Presence and Absence of Hydroperoxides 2 and 3

<table>
<thead>
<tr>
<th>entry</th>
<th>irradiation time (min)</th>
<th>% E. coli killed by airborne 1O₂</th>
<th>surfactant 1 added (mM)</th>
<th>4:1 mixture of hydroperoxides 2 and 3 (mM)</th>
<th>% E. coli killed⁴⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>10 ± 2</td>
<td>0.01</td>
<td>15 ± 2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>16 ± 3</td>
<td>0.03</td>
<td>27 ± 3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>21 ± 2</td>
<td>0.08</td>
<td>30 ± 3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>26 ± 3</td>
<td>0.12</td>
<td>42 ± 2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>27 ± 5</td>
<td>0.15</td>
<td>46 ± 3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>28 ± 3</td>
<td>1.0</td>
<td>27 ± 4</td>
<td></td>
</tr>
</tbody>
</table>

⁴⁺Airborne 1O₂ is generated and crosses an intervening gap to the H₂O solution. bError bounds were obtained from three measurements. E. coli cells were treated with airborne 1O₂ for 1 h. Hydroperoxides 2 and 3 (in a 4:1 ratio) were added to the cells in the dark for 2 min. E. coli cells were treated with airborne 1O₂ for 1 h. Surfactant 1 was then added to the cells in the dark for 2 min.

Effect of Added Hydroperoxides. The above data suggest that airborne 1O₂ with surfactant 1 enhanced singlet oxygen toxicity by an increase in oxidative stress (e.g., partial loss of cell membrane integrity). Evidence supporting this idea is shown in Table 3. Airborne 1O₂ exposure was followed with the postreaction addition of a 4:1 mixture of 2 and 3 in the dark (entries 1–5). Entries 1–5 ranged from 0.01 to 0.15 mM to mimic the hydroperoxide concentrations that form in situ for the reaction of 1 with airborne 1O₂ in H₂O in Figure 3. Airborne 1O₂ treatment for 1 h followed by the addition of 0.15 mM hydroperoxides 2 and 3 in the dark produced a similar inactivation of 50 μg/mL E. coli (46%, Table 3, entry 5) compared to that of airborne 1O₂ with surfactant 1 (50%, Table 2, entry 4). The measured inactivation by hydroperoxides 2 and 3 was 5%, and by airborne 1O₂ it was 25%, whereas the amount from the combination of airborne 1O₂ and surfactant 1 was 50%, fully 20% greater inactivation. Pre-exposure to airborne 1O₂ with the postreaction addition of 1 in the dark did not enhance the E. coli inactivation (Table 3, entry 6). We believe that this enhanced inactivation is relevant to synergy, where air 1O₂-predamaged cells in the presence of 2 and 3 provoke cell killing. Thus, we sought to gain insight into whether membrane damage was significant in 1O₂-treated cells.

We find evidence for cell permeabilization after 1O₂ treatment in the presence or absence of 1 based on fluorescent labeling with a commercially available LIVE/DEAD BacLight bacterial viability kit (Figure S17, Supporting Information). With SYTO-9 and propidium iodide stains added to 50 μg/mL E. coli samples after treatment and centrifugation, the propidium iodide staining of cells indicated damaged membranes. Consequently, we propose that airborne 1O₂ causes permeabilization but that some cells can recover. However, the presence of hydroperoxides 2 and 3 may impede such a recovery by further destabilizing the cell. In a similar vein, Redmond et al. attributes signaling and bystander effects to diffusing species such as H₂O₂ for the killing of neighboring cells adjacent to those photodynamically damaged.

The results of this work show a heightened E. coli sensitivity to hydroperoxides produced in situ or added after airborne 1O₂ treatment. We know that 1O₂ exposure in the presence or absence of 1 leads to compromised cell membranes. We do not know the relative toxicities of 2 and 3, for example, whether hydroperoxide 1 will cause greater membrane damage after the initial 1O₂ reaction. Our work also does not resolve whether 1 interacts with the cell membrane of the bacterium by adsorption or intercalation of its hydrophobic chain, but we believe that such sorption processes play a minor role as a result of the submicellar requirement mentioned earlier for hydroperoxide 2 and 3 formation. Our interest in a relatively low 1 mM detergent concentration was to potentially aim the hydrophobic group toward the surface, rather than aggregated it into a micelle away from the air/water interface. It turns out that reactive species preceding hydroperoxide formation are not likely to contribute to the toxicity because intermediates in the 1O₂ ene reaction are usually not thought to form. In the absence of E. coli, we find no NMR evidence for facile hydroperoxide self-degradation, such as through hydroperoxide pair Russell reactions, although we have not scrutinized...
hydroperoxide samples after 2 days when decomposition takes
place.

In summary, E. coli oxidation was carried out with airborne 1O2 and with the addition of 1 or hydroperoxides before and after 1O2 exposure to examine the mechanistic aspects. A Majima–Menden-like apparatus,9,10,12 as used here, exposes 1O2 to bacteria free from the effects of sensitizer pigmentation, bleaching, and degradation. Here, the sensitizer glass plate was physically isolated from water as a means to inactivate bacteria. Offering an innovative feature, the combination of airborne 1O2 with an oxidizable surfactant is promising. A ∼2-fold 1O2 toxicity enhancement was found in the presence of surfactant 1.

■ CONCLUSIONS

The arrival of airborne 1O2 to a water interface was used instead of its generation by a solvated photosensitizer. The apparatus has the advantage of being a source of gaseous singlet oxygen, otherwise the characteristics of surfactant solutions can change has the advantage of being a source of gaseous singlet oxygen, of its generation by a solvated photosensitizer. The apparatus

^ 2

■ ASSOCIATED CONTENT

Supporting Information

Description of the synthesis of 1 and generation of oxidized products 2–5, spectra of 1–5, a cmc plot, and photographs of the apparatus. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: agreer@brooklyn.cuny.edu.

Notes

The authors declare no competing financial interest.

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■ REFERENCES


