

# Efficacy of a Rose Bengal-Embedded Antimicrobial Packaging Film in Inactivating *Escherichia coli* under Visible Light Irradiation

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**ABSTRACT:** Antimicrobial packaging reduces the extent of microbial contamination; however, conventional antimicrobial packaging, which releases antimicrobial agents into food, may experience rapid agent depletion and can adversely affect food flavors. In this study, a novel photocatalytic antimicrobial nanofiber film embedded with Rose Bengal (RB) dye that generates reactive oxygen species (ROS) in visible light was designed for inactivating microorganisms. The film's antimicrobial properties under various light intensities and exposure times were evaluated, using *Escherichia coli* as a test microorganism. The results demonstrated that RB generates singlet oxygen as its principal ROS and has potent antimicrobial effects when incorporated into a film, achieving a  $4.4 \pm 0.1$  log CFU reduction in *E. coli* after 45 h under a light intensity of 6500 lx. The film's antimicrobial efficacy was dependent on light intensity, with significant *E. coli* inactivation occurring above 2000 lx. Overall, the RB-incorporated film effectively inactivates *E. coli*, providing a promising alternative to conventional antimicrobial packaging methods.

**KEYWORDS:** antimicrobial packaging, photocatalysis, Rose Bengal, food safety, nanofiber film

## 1. INTRODUCTION

Microbial contamination has led to an alarming increase in the number of foodborne illnesses and the extent of food spoilage in recent decades. The U.S. Department of Agriculture estimates that between 30% and 40% of food goes to waste.<sup>1</sup> Additionally, the U.S. Centers for Disease Control and Prevention estimates that one-sixth of Americans (48 million people) contract foodborne illnesses each year.<sup>2</sup> Subsequently, innovative technologies that control the growth of microorganisms that are harmful for product quality or human health are needed. Among the promising solutions is the incorporation of antimicrobial compounds into food packaging materials. These antimicrobial agents include silver zeolites,<sup>3</sup> organic acids,<sup>4</sup> essential oils,<sup>5</sup> enzymes,<sup>6</sup> peptides,<sup>7</sup> and bacteriocins.<sup>8</sup> However, these antimicrobial agent-incorporated films often fail to provide a broad spectrum of antimicrobial activity. Moreover, these incorporated antimicrobial agents need to be released from packaging films and migrate onto food for bacteria inactivation. This migration process not only causes rapid depletion of the incorporated antimicrobial agents but also raises concerns about the transfer of potentially toxic compounds from packaging to the food, as well as the possible introduction of undesirable flavors.

The photocatalytic antimicrobial packaging technique, which integrates photosensitizers into packaging films, has garnered significant recent interest. Photosensitizers make up a category of chemicals capable of harnessing energy from light to react with surrounding oxygen to generate potent reactive oxygen species [ROS ( $^1\text{O}_2$ ,  $\bullet\text{OH}$ ,  $\bullet\text{O}_2^-$ , and  $\text{H}_2\text{O}_2$ )].<sup>9</sup> The generated ROS can effectively inactivate bacteria via different mechanisms, including the oxidation of intercellular coenzyme A to disrupt bacterial metabolic pathways,<sup>10</sup> oxidative damage

to cell membranes,<sup>11</sup> and harmful oxidative reactions with nucleic acids.<sup>12</sup> The diverse set of pathways that lead to inactivation stymies acquired bacterial resistance against ROS.<sup>13</sup> Consequently, the photocatalytic mechanism can inactivate a broad spectrum of bacteria, positioning it as a promising solution in the antimicrobial packaging industry. Most importantly, during this photocatalytic process, photosensitizers remain fixed within the packaging films, do not migrate to the food, and in fact do not need to contact food directly at all. Rather, the ROS, once created on the packaging film, will travel from the film to the food and inactivate undesirable microorganisms. Studies have been conducted to incorporate  $\text{TiO}_2$ , one of the most used photosensitizers, into a packaging film to inactivate bacteria by producing ROS.<sup>14,15</sup> However,  $\text{TiO}_2$  has a significant limitation because it is responsive to only ultraviolet (UV) light ( $\lambda < 380$  nm) due to its large band gap of 3.0–3.2 eV. In most food storage scenarios, UV light is not easily available.

Rose Bengal is a dye photosensitizer that can respond to visible light at a wavelength of 518 nm through the type II photocatalytic pathway, generating a particularly potent ROS, known as singlet oxygen.<sup>16</sup> More importantly, RB is non-volatile and non-inhalable and is approved by the U.S. Food and Drug Administration for biomedical use, indicating minimal risks for humans. Moreover, because of its distinctive

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pink color, RB has been embraced by the Japanese food industry as a coloring agent (food red no. 105).<sup>17</sup> While RB's ability to produce ROS and inactivate pathogens in the medical field has been researched, its potential integration into packaging films remains uncertain. Additionally, it is important to identify the antimicrobial properties of the RB-incorporated film under the practical conditions of the commercial environments (e.g., grocery stores) that usually have a light intensity of <3000 lx.<sup>18</sup>

Therefore, this study aims to develop a novel RB-embedded packaging film that inactivates microorganisms upon exposure to visible light. To create the film, poly(vinylidene fluoride) (PVDF) was electrospun with RB to thoroughly integrate the RB dye into the film's nanofibers. The electrospinning method prevents the migration of the dye to the food and maximizes the surface area of the RB-embedded film, which ensures a rapid replenishment of oxygen, sourced from the ambient environment, as oxygen is consumed during photocatalytic reactions. The antimicrobial property of the RB-embedded film was assessed under varied light intensities and time durations, both with and without light exposure, utilizing *Escherichia coli* as a model microorganism, because of *E. coli*'s significance in the context of food safety and its prominent role in public health concerns due to outbreaks in recent years. Additionally, the concentration-dependent antimicrobial efficacy of RB was gauged by altering the RB concentration in aqueous solutions in the presence of *E. coli*. The predominant ROS generated by RB, singlet oxygen, was confirmed and quantified over time using furfuryl alcohol as a singlet oxygen quencher and high-performance liquid chromatography (HPLC) for analysis. Broadly, this investigation introduces an innovative antimicrobial packaging film with the promising ability to decrease the extent of microbial contamination, reduce food spoilage, and reinforce food safety.

## 2. MATERIALS AND METHODS

**2.1. Materials.** Polyvinylidene fluoride (PVDF) (KYNAR 761), Rose Bengal (RB, 80%), *N,N*-dimethylformamide (DMF, ≥99%), and acetone (≥99.8%) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Tryptic soy broth (TSB), tryptic soy agar (TSA), and peptone powder were purchased from BD DIFCO (BD Corp., Franklin Lakes, NJ). Phosphate-buffered saline (PBS) powder was purchased from VWR International (Radnor, PA). Aluminum foil was purchased from Thermo Fisher, Inc. (Waltham, WA).

**2.2. Preparation and Quantification of *E. coli*.** An *E. coli* (ATCC 25922) culture was prepared by inoculating the seed bacteria into a test tube with TSB and incubated temporarily in an incubator (Thermo Fisher, Inc.) at 37 °C for 12 h. The culture was serially diluted 10-fold using sterile 1× PBS to yield a bacterial concentration of 10<sup>5</sup> colony-forming units (CFU)/mL. After dilution, the number of colony-forming units of *E. coli* per milliliter was quantified via plating 0.1 mL of the solution onto TSA plates using a spiral plater (Autoplate 4000, Spiral Biotech Inc., Norwood, MA). The TSA plates were incubated at 37 °C for 24 h, photographed with the spiral plate grid, and counted digitally using *ClickMaster 2000* with manual confirmation.

**2.3. Determination of the Antimicrobial Efficacy of the RB Solution versus *E. coli*.** A 1 mM stock RB solution was prepared by dissolving 51 mg of RB in 50 mL of deionized water. Serial dilutions were performed to achieve RB concentrations of 100, 50, 20, 10, 5, and 1 μM. Then, 1.8 mL of the previously prepared *E. coli* suspension (Section 2.2) was aliquoted into each well of two 24-well cell growth plates (VWR), which have a well volume of 3.7 mL (diameter of 1.66 cm and height and 1.72 cm). To each well was added 0.2 mL of RB at varying concentrations [0 (control), 1, 5, 10, 20, 50, and 100 μM] to attain final RB concentrations of 0, 0.1, 0.5, 1, 2, 5, and 10 μM,

respectively, in the *E. coli*/RB mixtures. Each concentration was conducted in triplicate across both plates.

To assess the role of light in RB's antimicrobial capability, a comparative analysis was conducted between two environmental settings: dark and light. The first 24-well plate was shielded from light in a dark chamber, enclosed within a box wrapped with thick aluminum foil. In contrast, the second 24-well plate was subjected to a luminous intensity of 6500 lx, emanating from a light-emitting diode (LED) light panel (GVM 800D, B&H Photo & Electronics Co., New York, NY). Both plates were then placed in the incubator at 4 °C for 4 h. After incubation, a 1 mL aliquot was taken from each well and serially diluted 10-fold using sterile PBS, and then 0.1 mL of these dilutions was plated onto TSA plates to count the bacteria, as described in section 2.2.

### 2.4. Determination of the Antimicrobial Efficacy of the RB-Embedded Film against *E. coli*.

**2.4.1. Preparation of the RB-Embedded Film.** The RB-embedded PVDF film was prepared following a method outlined in a prior study.<sup>16</sup> Briefly, a homogeneous solution containing 15% PVDF and 0.3% RB was prepared using a solvent blend of DMF and acetone in a 7:3 volume ratio (Table S1). The solution was then electrospun onto a layer of aluminum foil attached to the rotating drum collector for 20 min. The conditions for electrospinning included a solution feed rate of 0.6 mL/h, an applied voltage of 10 kV, and a 10 cm distance between the spinneret tip and the drum. The resultant material was denoted as a PVDF-RB film.

**2.4.2. Determination of the Time-Dependent Antimicrobial Efficacy of the Film.** To assess the antimicrobial efficacy of the RB-embedded PVDF film, 30 circular samples, each with a diameter of 1.6 cm, were cut from the film. These circles were placed at the bottom of the wells of two 24-well plates, with each well subsequently being filled with 2.0 mL of the PBS-diluted *E. coli* suspension (10<sup>5</sup> CFU/mL), as detailed in section 2.2. One plate was shielded from light in a dark chamber, enclosed in a box wrapped with aluminum foil, while the other was subjected to a light intensity of 6500 lx using the same LED panel. Both plates were incubated at 4 °C. The bacterial viability was assessed at time points of 0, 12, 18, 24, and 45 h by sampling from the wells containing the film circles and counting bacteria as specified in section 2.3. Additionally, after a 45 h incubation, control wells without film circles were also enumerated to establish baseline bacterial levels.

**2.4.3. Effect of Light Intensity on the Antimicrobial Properties of the Film.** The same circular film samples were placed in the wells of a 24-well plate. Each well was then filled with 2.0 mL of the PBS-diluted *E. coli* suspension, prepared as described in section 2.2. The 24-well plate was then exposed to different light intensities [6500, 2000, 1500, 400, and 0 lx (as a dark control)] in an incubator at 4 °C for 45 h. After incubation, the surviving *E. coli* in each well was counted as specified in section 2.3.

### 2.5. Determination of Singlet Oxygen Generated by RB.

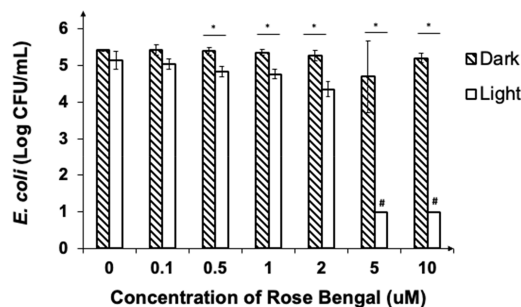
Singlet oxygen (<sup>1</sup>O<sub>2</sub>) generated by RB was quantified by the furfuryl alcohol degradation (FFA) method.<sup>19</sup> Briefly, 1.5 mL of 3 mM FFA and 0.5 mL of a 1 mM RB solution were added to each well across two 24-well plates. One plate was placed in a dark environment inside a light-fast box, while the other was exposed to 6500 lx from the LED light source. Both plates were incubated at 4 °C. Samples were collected in triplicate at four time points: immediately (0 min) and 30 min, 1 h, and 12 h after exposure. FFA degradation was analyzed using reverse phase HPLC equipped with a C18 column (Agilent, Inc., Santa Clara, CA), using a mobile phase composed of methanol, water, and a small amount of phosphoric acid (15:85:0.1). The sample injection volume was 5 μL, and the flow rate of the mobile phase was set at 0.4 mL/min. Detection was set at a wavelength of 218 nm by using a UV light detector.

**2.6. Statistical Analysis.** All experiments were conducted in triplicate. Comparisons between two treatments were conducted using a Student's *t* test, and comparisons among multiple treatments were conducted using one-way analysis of variance (ANOVA), followed by Tukey's HSD test. All statistical analyses were conducted

by using JMP Pro 16. A  $p$  value of  $<0.05$  was considered statistically significant.

### 3. RESULTS AND DISCUSSION

**3.1. Antimicrobial Properties of the RB Solution against *E. coli* at a Light Intensity of 6500 lx.** The ability of RB to inactivate *E. coli* under light exposure is significantly influenced by its concentration (Figure 1). In the control



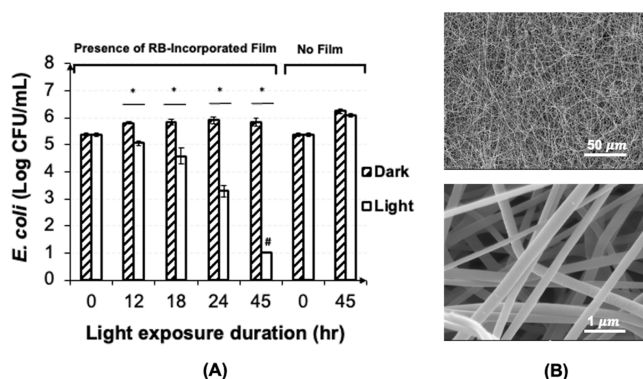
**Figure 1.** Effect of RB concentration on the CFU/mL of *E. coli* under two conditions: with 6500 lx light exposure and without light exposure. Asterisks indicate a significant difference between the light and dark treatments ( $p < 0.05$ ). Number signs indicate the viable bacterial load is below the detection limit (1 log CFU/mL) of the surface plating method; because no enrichment experiment was conducted to confirm the zero bacterial number, it was conservatively labeled as 1.0 here.

group without RB, a minor difference (0.3 log CFU/mL) was observed between the light-exposed samples and the dark control, but statistically insignificant ( $p > 0.05$ ). Similarly, at an RB concentration of 0.1  $\mu\text{M}$ , no significant difference in *E. coli* CFU/mL was observed between light and dark conditions, suggesting the negligible impact of RB at this concentration against a bacterial load of 5 log CFU/mL. We speculate that the ROS generated from such a low concentration (0.1  $\mu\text{M}$ ) of RB is not sufficient to overwhelm *E. coli*'s natural defense and repair mechanisms. It is reported that *E. coli* can undergo elaborate mechanisms in response to ROS attack, including utilizing powerful antioxidant compounds and repairing cellular components to counteract oxidative DNA damage.<sup>18</sup> In the future, it would be interesting to quantify the generation of ROS from different concentrations of RB solutions and investigate how bacteria respond to different levels of ROS.

However, significant reductions in *E. coli* CFU/mL were observed at RB concentrations of 0.5, 1, and 2  $\mu\text{M}$  under light exposure ( $p < 0.05$ ), with decreases of  $0.54 \pm 0.16$ ,  $0.59 \pm 0.13$ , and  $0.92 \pm 0.33$  log CFU/mL, respectively. These reductions suggest that ROS production by RB at these concentrations was sufficient to overpower *E. coli*'s cellular defenses, leading to cell death. At high RB concentrations of 5 and 10  $\mu\text{M}$ , RB demonstrated the most antimicrobial activity, with no viable *E. coli* cells detected after a 4 h exposure (CFU/mL was below the detection limit of the surface plating method), underscoring the effectiveness of RB-generated ROS in inactivating *E. coli*.

**3.2. Antimicrobial Properties of an RB-Embedded Film against *E. coli* under Different Light Exposure Durations.** The antimicrobial efficacy of the RB-embedded films was assessed by immersing the film circles in *E. coli* suspensions and subjecting them to varying durations of light and dark conditions. In the negative controls (without film),

there was no reduction in *E. coli* CFU/mL, regardless of light exposure (Figure 2A). Instead, a slight increase in the *E. coli*

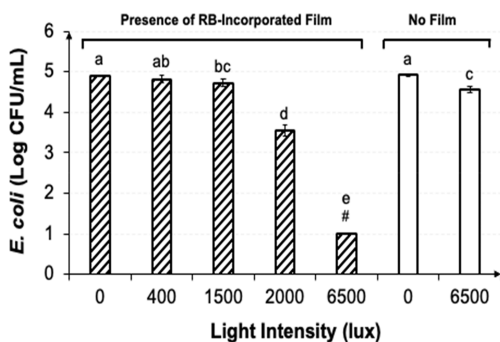


**Figure 2.** (A) Log CFU/mL of *E. coli* under visible light irradiation and in the dark when merged with and without the RB-embedded film at different time points of 0, 12, 18, 24, and 45 h. (B) Scanning electron microscopy (SEM) images of the RB-embedded electrospun nanofibrous film at 500 $\times$  magnification (top) and 2500 $\times$  magnification (bottom). Asterisks indicate a significant difference between the light and dark treatments ( $p < 0.05$ ). Number signs indicate that the bacterial concentration is below the detection limit (1 log CFU/mL) of the surface plating method.

CFU/mL was observed. Although there were residual nutrients from the tryptic soy broth in the PBS-diluted *E. coli* suspension, *E. coli* usually cannot grow at the low incubation temperature at 4  $^{\circ}\text{C}$ . It is probable that this slight increase in *E. coli* CFU occurred while the samples were prepared at room temperature ( $\sim 1$  h) before they were held at 4  $^{\circ}\text{C}$ . In contrast, the RB-embedded film demonstrated pronounced antimicrobial capability under light exposure. Notably, upon exposure to light, the RB-incorporated film led to a significant reduction in *E. coli* after 12 h ( $p < 0.05$ ), with this trend continuing in subsequent time points (Figure 2A). After light exposure for 45 h, the *E. coli* CFU/mL was reduced by  $4.4 \pm 0.1$  log CFU/mL, from  $5.4 \pm 0.1$  log CFU/mL to a nondetectable level (1.0 log CFU/mL), highlighting the potent antimicrobial effect of the RB-embedded film under light exposure. This antimicrobial effect is likely due to the large surface area of the prepared electrospun nanofibrous film, as shown in Figure 2B, which enhances the exposure of RB to light and oxygen for optimal ROS generation and ensures a rapid replenishment of oxygen for the photocatalytic reactions. On the contrary, in the absence of light, the film did not show any bactericidal activity (Figure 2A). This is because that RB necessitates external light to generate ROS, vital for bacterial inactivation, further highlighting the necessity of light for RB to undergo any antimicrobial reactions and produce crucial ROS. The increased efficacy in microbial inactivation observed with the films after prolonged light exposure ( $>12$  h) is beneficial in real-world scenarios. Within the food supply chain, the extent of bacterial contamination on food products is typically low initially; however, these bacteria can proliferate and reach high concentrations during the later exponential growth phase, which leads to food spoilage and potential health hazards. In contrast to traditional antimicrobial packaging, which exhibits peak efficacy initially that diminishes over time, our innovative RB-incorporated RB packaging solution has increased microbial inactivation efficiency over time. This feature makes it more effective at inhibiting bacterial growth and preventing

food spoilage, offering a more practical approach for ensuring food safety.

**3.3. Antimicrobial Efficacy of RB-Embedded Films under Different Light Intensities.** The antimicrobial efficacy of the RB-embedded film was further evaluated under varying light intensities, from complete darkness (0 lx) to 6500 lx, for 45 h. As illustrated in Figure 3, the antimicrobial



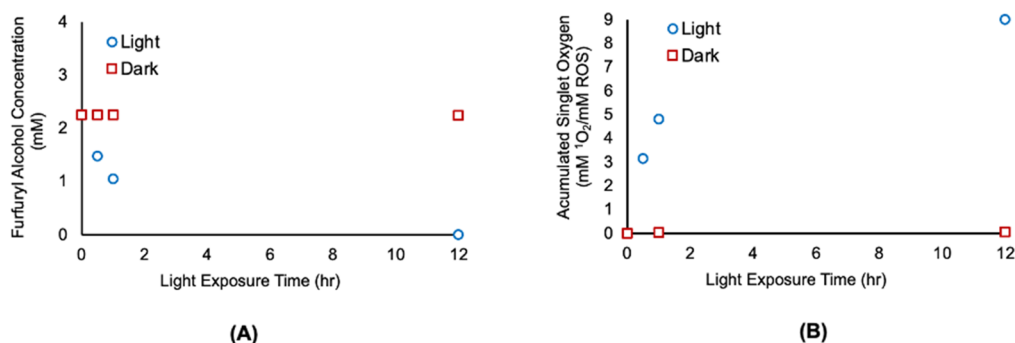
**Figure 3.** Effect of light intensity on the effectiveness of the antimicrobial properties of the RB-embedded film. Means denoted by a different letter indicate significant differences between treatments ( $p < 0.05$ ). Number signs indicate the concentration of the bacteria is below the detection limit (1 log CFU/mL) of the surface plating method.

activity of the RB-embedded film was dependent on light intensity. Under low light intensities of 400 and 1500 lx, there was no significant decline in *E. coli* CFU/mL ( $p > 0.05$ ), suggesting inadequate ROS generation to overcome the defense mechanisms of the bacteria. ROS can damage chromosomal DNA and free nucleotides through oxidative reactions, ultimately leading to bacterial death.<sup>12</sup> Nonetheless, at moderate concentrations, ROS can act as signaling molecules prompting bacteria to activate an array of defensive genes.<sup>20</sup> These genes lead to repair pathways that mitigate oxidative DNA damage, thereby protecting the bacterial cell from lethal outcomes.<sup>20</sup> When the light intensity was ramped to 2000 lx, the *E. coli* CFU/mL decreased significantly from  $4.9 \pm 0.01$  to  $3.6 \pm 0.1$  log ( $p < 0.05$ ), demonstrating the film's ability to inactivate *E. coli*. This finding is particularly relevant for practical applications, as the average LED light intensity in retail environments is  $\sim 2000$  lx, reaching 3200 lx in some national chain stores.<sup>18</sup> Notably, at 6500 lx, the surviving *E. coli* CFU/mL was below a detectable level (1.0 log), reflecting a  $3.9 \pm 0.01$  log reduction compared to the dark control. In

absence of the film, although there is a statistical difference between the dark and light (6500 lx) treatment, the difference is practically small ( $< 0.3$  log). These results emphasize that a synergy of RB and a medium to high light intensity (exceeding 2000 lx) is pivotal for the film's bactericidal action against *E. coli*.

**3.4. Quantification of Singlet Oxygen Generated by RB.** The generated  $^1\text{O}_2$  was calculated by measuring the decline in FFA levels, as the reaction between FFA and  $^1\text{O}_2$  occurs instantaneously with a 1:1 stoichiometry.<sup>21</sup> The results confirmed the generation of  $^1\text{O}_2$  (Figure 4a) by RB upon catalysis by light. In the absence of light, minimal FFA degradation ( $< 1\%$ ) was noted in the presence of RB, indicating the absence of FFA degradation by any generated by RB over the 12 h period. Conversely, when the FFA and RB solution was exposed to 6500 lx light, FFA exhibited rapid degradation within the initial hour and was entirely depleted after 12 h. This underscores the proficiency of the RB in generating  $^1\text{O}_2$ , which oxidizes FFA (Figure 4B). The findings further bolster the notion that light exposure is indispensable for RB to generate  $^1\text{O}_2$ . Additionally, a decline in the rate of ROS generation was noted after several hours, as depicted in Figure 4. However, the RB-incorporated film demonstrated superior antimicrobial effectiveness over an extended period, as evidenced by Figure 2. This suggests that the cellular damage caused by  $^1\text{O}_2$  is cumulative rather than transient. Previous studies reported that  $^1\text{O}_2$  can react with intracellular amino acids, leading to cellular inhibition due to the depletion of amino acids.<sup>22</sup> Moreover, these amino acids react with  $^1\text{O}_2$  to form peroxides, which may decompose into toxic intermediates that accumulate within cells to inhibit cells.<sup>22</sup> In addition, continuous damage from the interaction between  $^1\text{O}_2$  and membrane lipids and nucleotides is also reported to inactivate cells.<sup>13</sup>

In summary, this study investigated the novel RB-embedded nanofiber film to inactivate bacteria under a commercially available LED light source. The results revealed that the nanofiber film incorporated with RB can significantly inactivate *E. coli* over time, with heightened light intensities and dye concentrations amplifying the antimicrobial effects. On the whole, RB embedded in a nanofiber film can effectively and overwhelmingly inactivate bacteria and presents itself as a promising solution for antimicrobial packaging. To fully understand the implications and feasibility of incorporating RB into food packaging, comprehensive research of the film on real food samples with a meticulous safety evaluation is essential. Overall, this study paves the way for developing



**Figure 4.** (A) Degradation of furfural alcohol (FFA) by RB under LED light exposure and in the dark and (B) calculated accumulated singlet oxygen ( $^1\text{O}_2$ ) produced by RB.

visible light-driven photocatalytic packaging film to combat microorganism contamination.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsfoodscitech.4c00040>.

Recipe for a photosensitized electrospun nanofibrous PVDF-RB membrane (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

RB, Rose Bengal; ROS, reactive oxygen species; FFA, furfural alcohol; PVDF, poly(vinylidene fluoride); DMF, *N,N*-dimethylformamide; TSB, tryptic soy broth; TSA, tryptic soy agar

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