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METHODS

Photoactivated Carbon Dots for Inactivation of Foodborne Pathogens *Listeria* and *Salmonella*

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ABSTRACT Foodborne pathogens have long been recognized as major challenges for the food industry and repeatedly implicated in food product recalls and outbreaks of foodborne diseases. This study demonstrated the application of a recently discovered class of visible-light-activated carbon-based nanoparticles, namely, carbon dots (CDots), for photodynamic inactivation of foodborne pathogens. The results demonstrated that CDots were highly effective in the photoinactivation of *Listeria monocytogenes* in suspensions and on stainless steel surfaces. However, it was much less effective for *Salmonella* cells, but treatments with higher CDot concentrations and longer times were still able to inactivate *Salmonella* cells. The mechanistic implications of the observed different antibacterial effects on the two types of cells were assessed, and the associated generation of intracellular reactive oxygen species (ROS), the resulting lipid peroxidation, and the leakage of nucleic acid and proteins from the treated cells were analyzed, with the results collectively suggesting CDots as a class of promising photodynamic inactivation agents for foodborne pathogens.

IMPORTANCE Foodborne infectious diseases have long been recognized as major challenges in public health. Contaminations of food processing facilities and equipment with foodborne pathogens occur often. There is a critical need for new tools/approaches to control the pathogens and prevent such contaminations in food processing facilities and other settings. This study reports a newly established antimicrobial nanomaterials platform, CDots coupled with visible/natural light, for effective and efficient inactivation of representative foodborne bacterial pathogens. The study will contribute to promoting the practical application of CDots as a new class of promising nanomaterial-based photodynamic inactivation agents for foodborne pathogens.

KEYWORDS carbon dots, foodborne pathogens, photoinactivation

Foodborne pathogens such as *Listeria* and *Salmonella* have long been recognized as major challenges for the food industry and have been repeatedly implicated in food recalls and outbreaks of diseases (1–5). For example, *Listeria monocytogenes* can cause listeriosis, a severe invasive and life-threatening infection, with high risk in pregnant women, newborns, the elderly, and immunocompromised individuals (6, 7). It is among the leading causes of death by a foodborne pathogen in the United States, with case fatality rates from listeriosis as high as ~28%, the highest for all foodborne pathogens (8, 9). The organism contaminates food from a variety of environmental sources and food processing facilities. Some strains of *L. monocytogenes* have been known to persist in the food processing environment for impressively long periods of time, even more than 10 years (10, 11). The genus *Salmonella* comprises a diverse group of organisms,

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among which some strains have been recognized as major foodborne pathogens associated with fresh produce and other food products. *Salmonella* can also persistently colonize on surfaces and equipment used for processing meat and multiple other food commodities (12). Particular strains of *Salmonella* can persist for up to 10 years in food processing environments (13). There have been outbreaks associated with *Salmonella* strains persisting on food equipment and food contact surfaces (14, 15). They also display enhanced survival in nonhost environments, such as soil and water, and may undergo passage through a host into the environment and back into a new host (16).

To control such persistent pathogens and prevent foodborne infections and spread in the food processing ecosystem, new tools and approaches are needed. This study demonstrates the use of a new class of visible/natural-light-activated carbon nanomaterial, called carbon "quantum" dots or carbon dots (CDots) (17, 18), for the inactivation of these persistent foodborne pathogens. CDots are generally small carbon nanoparticles with various surface passivation, each with a carbon nanoparticle core (mostly $<$10 nm in diameter) and a thin shell of soft materials (organic or biological molecules) serving the surface passivation function (17, 18). Upon photoexcitation, there are rapid charge transfers and separation for the generation of electrons and holes, which are trapped in various surface defect sites (17, 19). These separated redox species on the dot surface, stabilized by the surface organic functionalization, must be lethal to microbes (20). The subsequent radiative recombinations of the redox pairs result in the emissive excited states, which are capable of producing classical reactive oxygen species (ROS) (17, 20, 21). We have demonstrated that CDots are potent antibacterial agents under visible/natural light against model Gram-positive and Gram-negative bacteria such as *Bacillus subtilis* and *Escherichia coli* (22), as well as multidrug-resistant (MDR) bacterial pathogens (20, 23). Mechanistically, the uniquely potent antimicrobial function of photoexcited CDots is attributed to the combined action of the highly lethal separated redox pairs and the classical ROS (20).

The optical properties and photoinduced redox characteristics of CDots make them more advantageous than classical photosensitizers and conventional semiconductor quantum dots (QDs) for antimicrobial use. Among the advantages are (i) very high optical absorptivities for ready activation by light over the entire visible spectrum, enabling the photoinactivation function under ambient light conditions, thus eliminating any exposure to hazardous UV irradiation; (ii) a special and uniquely potent inactivation mechanism for the observed superior performance; and (iii) being intrinsically nontoxic *in vitro* and *in vivo* and benign to humans in general living environments (24–28).

This study validates and demonstrates CDots' photoinactivation function under visible light toward the common persistent foodborne pathogens, including 4 strains of drug-resistant *Listeria* and 3 strains of *Salmonella* spp. that were isolated from different foodborne outbreaks.

RESULTS AND DISCUSSION

Effects of photoexcited CDots on *Listeria* and *Salmonella* cells in suspensions. Figure 1A and B show the results of viable cell reductions in the 4 individually tested *Listeria* strains and 3 individually tested *Salmonella* strains, upon treatment with different concentrations of CDots from 2 to 10 μg/ml for 1 h under visible-light illumination with a 60-W-equivalent daylight LED. For the tested *Listeria* strains, *L. monocytogenes* 10403S is one of the three primary pathogenic strains that are commonly used by researchers in studying the pathogenesis of *L. monocytogenes*. It belongs to serotype 1/2a and is streptomycin resistant. *L. monocytogenes* 2858 and *L. monocytogenes* H7858 are isolates from different foodborne outbreaks, belonging to serotypes 1/2b and 4b, respectively. *Listeria innocua* UAM003-1A is nonpathogenic but is streptomycin and tetracycline resistant. It can serve as an important reservoir for resistance determinants that can transfer to *L. monocytogenes* (29) and can be particularly concerning if there is transmission of antibiotic resistance to a pathogen of major human health significance. As shown in Fig. 1A, CDot treatments were effective for inactivating cells of

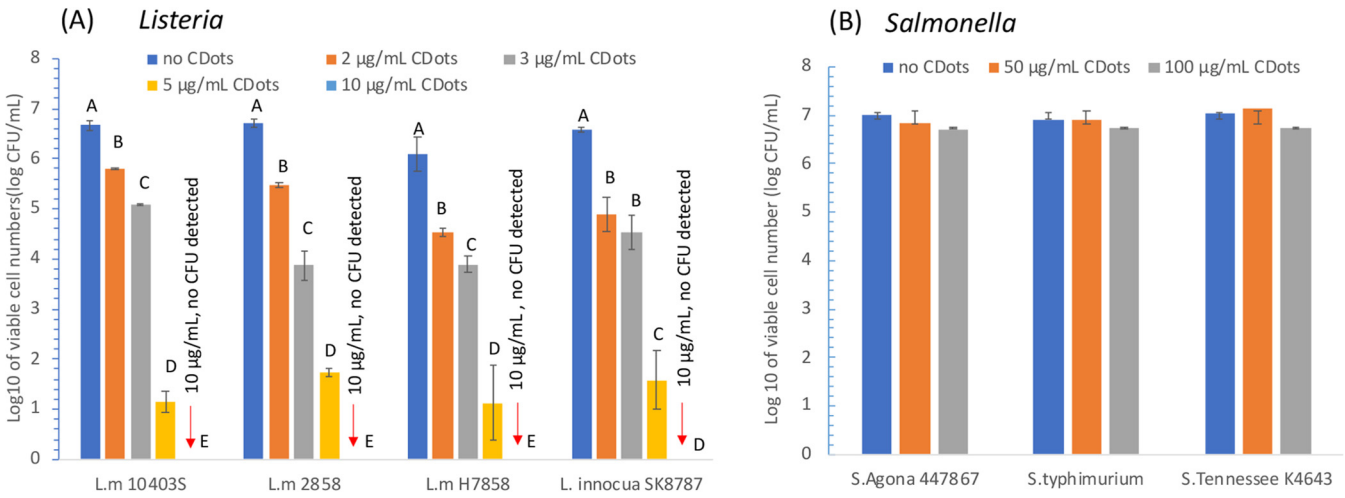

FIG 1 (A) Log_{10} viable-cell numbers of 4 individually tested *Listeria* strains upon treatment with concentrations of CDots ranging from 2 to 10 μg/ml for 1 h under visible-light illumination from a 60-W daylight equivalent LED. (B) Viable-cell numbers of 3 individually tested strains of *Salmonella* cells upon treatment with CDots at 50 and 100 μg/ml for 1 h under the same light condition. Statistical analysis was performed within each cell line. Different letters on columns indicate significant differences between the results ($P < 0.05$); columns with the same letter showed no significant difference between the results (A). No significant difference was found in the results of the 3 *Salmonella* lines tested (B).

all 4 *Listeria* strains, with the viable-cell numbers being reduced by the treatments in a CDot dose-dependent manner. With 2 μg/ml CDot treatment under light for 1 h, all 4 strains had significant reductions in viable cells compared to the samples without CDot treatment, ranging from an ~0.9-log reduction in *L. monocytogenes* 10403S to ~1.3-, ~1.5-, and ~1.7-log reductions in *L. monocytogenes* 2858 and H7858 and *L. innocua*, respectively. When the CDot concentration was increased to 3 μg/ml, the viable-cell reductions became more substantial for 3 strains but not for *L. innocua*. However, at a higher CDot concentration, 5 μg/ml, there were significantly greater viable-cell reductions in all 4 strains, reaching approximately a 5-log reduction in all strains. The treatment with 10 μg/ml CDots was able to completely inactivate all cells in the samples, achieving >6-log viable-cell reductions in all tested strains. Figure 1B shows the viable-cell numbers for 3 strains of *Salmonella* cells after the CDot treatment under a 60-W-equivalent daylight LED for 1 h. Unlike *Listeria* cells, all 3 *Salmonella* strains were not affected by the CDot treatment, even with a 10×-higher concentration of CDots than that used in the treatment of *Listeria* cells.

The results indicated that CDot treatment under visible light was highly effective for inactivating *L. monocytogenes* cells but was not effective for *Salmonella* cells. This observation seemed to agree with other observations suggesting that Gram-positive bacteria are more sensitive to many different dye photosensitizers, whereas Gram-negative bacteria are more resistant (30). However, the different effects of CDot treatment on *Listeria* and *Salmonella* may not be due simply to the known structural differences between Gram-positive and Gram-negative bacterial cells, as it was found previously that CDots could effectively inactivate *E. coli*, another Gram-negative bacterium, both in suspension and on agar plates (22). There must be more factors contributing to the observed different effects of CDots on the two bacterial types, such as the properties of the CDots used in the treatments and the microenvironments and interactions between CDots and bacterial cells. While significant progress has been made in the mechanistic understanding of CDots' photoinactivation of bacteria, more details on the mechanisms are yet to be explored and revealed. Specifically, regarding the different effects of the CDot treatment on *Listeria* and *Salmonella*, experimental comparisons were performed on the generation of intracellular ROS, lipid peroxidation, and the leaking of nucleic acids and proteins from the treated cells. The results are presented below.

Sublethal injury of cells by CDot treatment. In the experiments with *L. monocytogenes* 10403S as a representative strain, it was noticed that a significant portion of

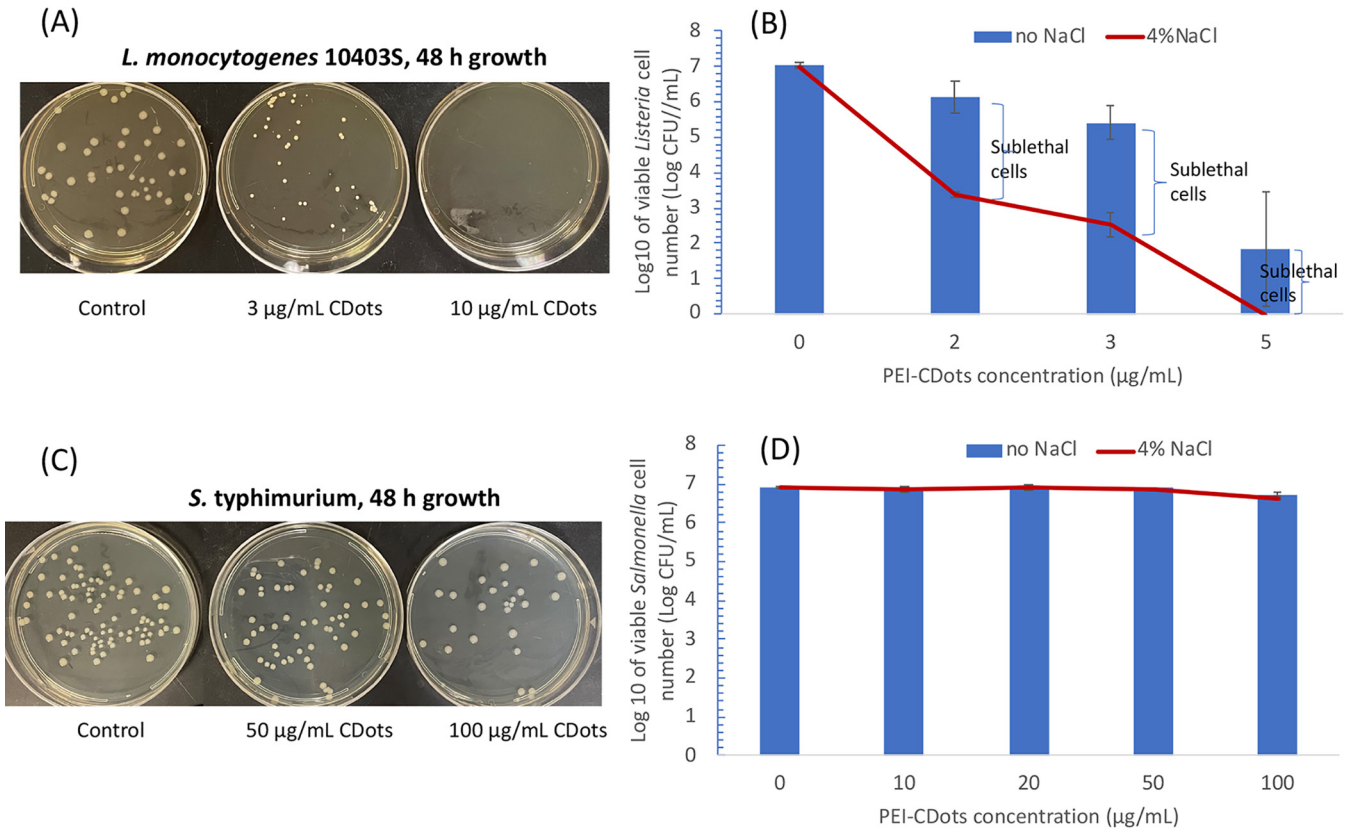

FIG 2 (A) Colonies formed by untreated and CDot-treated *L. monocytogenes* 10403S cells on BHI agar plates; (B) $\log_{10}$ CFU/ml of *L. monocytogenes* 10403S obtained with colonies formed on BHI agar and BHI agar containing 4% NaCl. (C) Colonies formed by untreated and CDot-treated *S.* Typhimurium cells on LB agar plates; (D) $\log_{10}$ CFU/ml of *S.* Typhimurium obtained by the colonies formed on the LB agar and the LB agar containing 4% NaCl, showing no differences.

colonies formed on brain heart infusion (BHI) agar by the CDot-treated *Listeria* cells were not visible at 24 h of incubation but could be seen at 48 h of incubation and were much smaller than those formed by the control cells (Fig. 2A), suggesting that some cells were sublethally injured during the treatment with CDots at low concentrations. Sublethal injury is defined as a consequence of exposure to a chemical or physical process that damages but does not kill a microorganism. It is not surprising to observe sublethally injured *Listeria* cells during treatment, as there have been a number of reports on the observation of sublethally injured *Listeria* cells by various food preservation strategies and antibacterial strategies (31, 32), especially in the investigation of novel antibacterial applications (32). Since selective agars are usually unable to support the growth of sublethally injured cells, yet nonselective nutrient agars are able to support the recovery of the injured cells, the portion of sublethally injured cells can be estimated or determined by plating the treated samples on nonselective and selective agars to quantify the difference (32). It can also be determined by plating the treated *Listeria* samples on nonselective BHI agar and BHI agar containing 4% NaCl, where the injured cells are not capable of repairing damage and surviving (31). Figure 2B shows the viable numbers of *L. monocytogenes* 10403S cells calculated based on colonies formed on BHI agar and BHI agar containing 4% NaCl after the samples were treated with polyethylenimine (PEI)-CDots at different concentrations under visible light for 1 h, as the maximum noninhibitory NaCl concentration for native cells was determined to be 4% in control tests. The differences in the viable cell numbers from the two types of agars for the same CDot-treated samples indicated the portion of sublethally injured cells. There were approximately 2.76, 2.87, and 1.83 log of sublethally injured *Listeria* cells after the treatment with 2, 3, and 5 μg/ml PEI-CDots under visible light for 1 h, whereas the untreated control samples gave the same number of CFU on

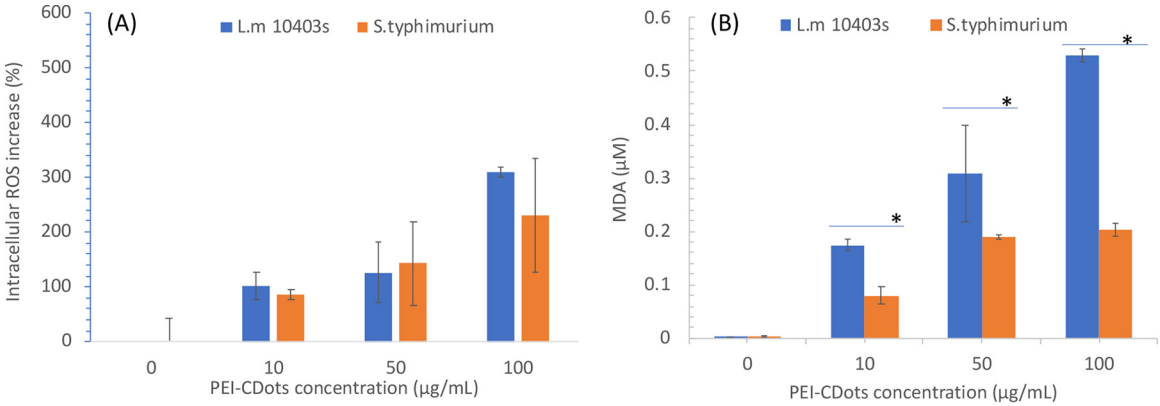

FIG 3 (A) Increases in intracellular ROS levels; (B) detection of the lipid peroxidation product MDA in *L. monocytogenes* 10403S and *S.* Typhimurium cells treated with various concentrations of CDots for 1 h under visible light, along with control cells without CDot treatment. *, $P < 0.05$.

BHI agar and BHI agar containing 4% NaCl. However, when the CDot concentration was sufficiently high (e.g., 10 μg/ml under our test conditions), no colonies were formed on both types of agar, suggesting that all treated cells were completely inactivated or nonculturable. Therefore, in the determination of the antibacterial effect of the CDot treatment using nonselective agar, described in the section above, the sublethally injured cells were not included; thus, there was no overestimation of the antibacterial effects.

On the other hand, in the experiments with *Salmonella enterica* serovar Typhimurium as a representative strain, there was no morphological difference in the colonies of CDot-treated and untreated *Salmonella* cells (Fig. 2C) and also no difference in the viable cell number of *Salmonella* cells counted on LB agar and LB agar containing 4% NaCl (Fig. 2D), indicating that the treatment with PEI-CDots did not inactivate or sublethally injure *Salmonella* cells, even with the 100×-higher concentration of CDots used than in the treatment of *Listeria* cells. Such observations led to the subsequent experimental investigations on other consequences to the cells due to the different effects of the CDot treatment on *Listeria* and *Salmonella*.

CDots induced intracellular ROS generation and lipid peroxidation in *Listeria* and *Salmonella* cells. As demonstrated in our earlier studies, the photoactivated antimicrobial function of CDots can be attributed to the combined actions of the initially photogenerated separated electron-hole redox pairs in CDots and the classical ROS produced by the emissive excited states (19, 20, 33). To further examine the responses of *Listeria* and *Salmonella* cells to the CDot treatment, the levels of intracellular ROS in the two types of cells upon the same CDot treatments were determined using *L. monocytogenes* 10403S and *S.* Typhimurium (Fig. 3A). Surprisingly, at all tested CDot concentrations, the intracellular ROS levels in the two types of cells increased by similar magnitudes. Upon treatment with 10 μg/ml CDots, the intracellular ROS levels in both *L. monocytogenes* and *S.* Typhimurium cells increased 100%, with the level of ROS in the cells without CDots as the baseline. Further increases in ROS levels were observed at higher CDot concentrations used in the treatments, reaching ~300% with the CDot concentration of 100 μg/ml. However, the outcomes with respect to the inactivation were very different between the two types of cells. At 10 μg/ml CDots, for example, all *L. monocytogenes* cells were completely inactivated, yet *S.* Typhimurium cells survived. The latter could apparently survive the very high level of intracellular ROS (~300%) when treated with CDots at 100 μg/ml (Fig. 1B).

In general, the ROS species generated strongly interact with various cell components and cause severe damage to the cell (34–36). Both intracellular and extracellular ROS are able to disrupt cell membranes (37, 38), and the membrane damage is often associated with lipid peroxidation (39). Lipid peroxidation is a process in which lipids' carbon-carbon double bonds are attacked by free radicals and nonradical oxidizing

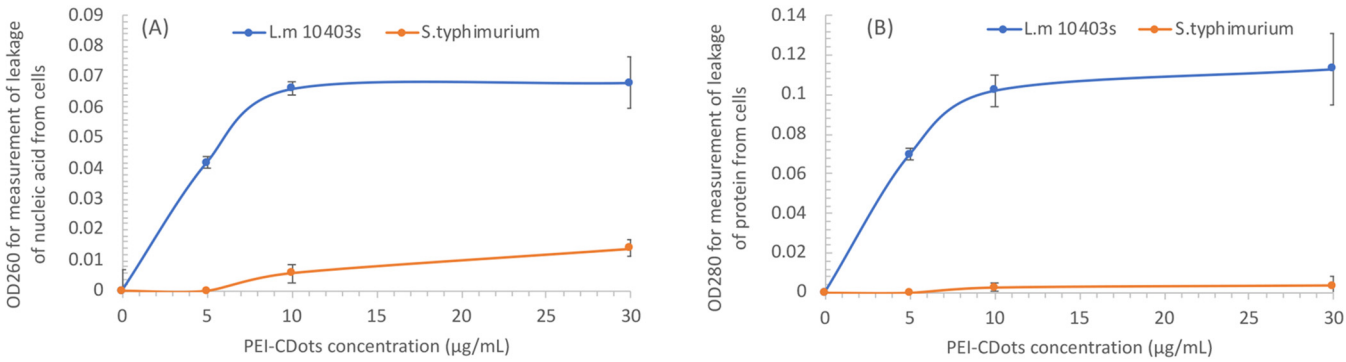

FIG 4 Measured OD of the cell supernatants for detecting the leakage of (A) nucleic acid and (B) proteins from *L. monocytogenes* 10403S and *S.* Typhimurium cells treated with various concentrations of CDots under visible light for 1 h.

species (40–42). To determine the potential damage caused by the generated ROS, the lipid peroxidation in *L. monocytogenes* and *S.* Typhimurium cells after CDot treatments was examined. The secondary product of lipid peroxidation, malondialdehyde (MDA), is commonly used as an indicator for the lipid peroxidation due to its ability to react with thiobarbituric acid (TBA) to form red fluorescent adducts for easy detection and quantification (43, 44). In this study, the measurement of lipid peroxidation was performed using the thiobarbituric acid reactive substances assay (TBARS). The reaction of TBA with MDA forms MDA-TBA adducts that can be detected colorimetrically at 532 nm or fluorimetrically at 532 nm excitation for 553 nm emission. Figure 3B shows the levels of MDA in the tested *L. monocytogenes* 10403S and *S.* Typhimurium cells treated with 10, 50, and 100 μg/ml of PEI-CDots for 1 h with visible light illumination. For all three tested CDot concentrations, MDA levels in CDot-treated *L. monocytogenes* and *S.* Typhimurium cells increased significantly from those in their respective untreated cells. Between the two types of cells, the MDA levels in *L. monocytogenes* cells were significantly higher than those in *S.* Typhimurium cells for the same treatment, nearly doubled the MDA level in *S.* Typhimurium cells. The results suggested that *L. monocytogenes* cells were more susceptible to the ROS generated in the CDot treatment, leading to a high level of lipid peroxidation in the cell membrane, and *S.* Typhimurium cells were more tolerant of the generated ROS, with much less lipid peroxidation in cell membranes.

Leakages of nucleic acid and protein from CDot-treated *Listeria* and *Salmonella* cells. To further examine the membrane damage in *L. monocytogenes* and *S.* Typhimurium cells, the leakages of nucleic acid and protein from the CDot-treated cells were compared. Figure 4 shows the leakages of nucleic acid and protein from *L. monocytogenes* 10403S and *S.* Typhimurium bacteria that were treated with 0, 5, 10, and 30 μg/ml PEI-CDots and visible light for 1 h. After the treatment, the samples were centrifuged at 12,000 $\times$ *g* for 2 min, and the supernatants were used for optical density (OD) measurement. OD at 260 nm was used to detect leakage of nucleic acid from the bacterial cells (Fig. 4A), and OD at 280 nm was used to detect the leakage of proteins (Fig. 4B). The samples before treatment (at time zero) were used as blanks. Obviously, the leakages of nucleic acid and protein from *L. monocytogenes* cells increased with the increasing concentration of CDots used in the treatments. The treatment with 10 μg/ml CDots resulted in a saturated level of leakages of nucleic acid and protein from *L. monocytogenes*, while there were no obvious leakage of nucleic acid and protein from *S.* Typhimurium cells with the same treatment. These results again suggested that the CDot treatment caused severe damage in the membranes of *Listeria* cells but did not cause detectable damage in membranes of *Salmonella* cells, consistent with the detected lipid peroxidation levels in the two types of cells, although the total detected ROS levels in both bacteria were similar during the CDot treatments.

CDots photoinactivate *Listeria* and *Salmonella* cells dried on surfaces. *Salmonella* and *Listeria* can survive for impressively long periods of time on dry abiotic surfaces in

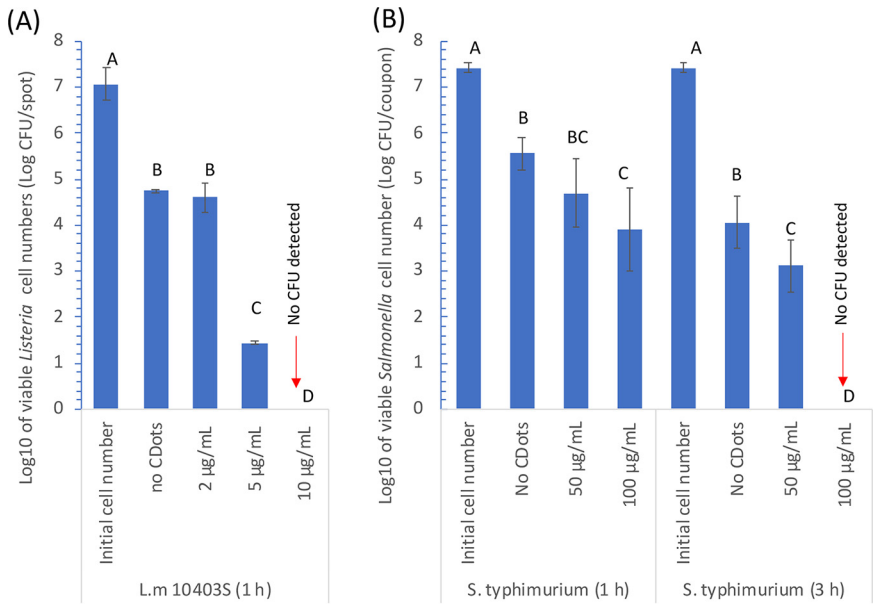

FIG 5 Log_{10} of viable cell numbers of *L. monocytogenes* 10403S cells (A) and *S.* Typhimurium cells (B) in the initial samples and recovered from the samples dried on stainless steel surfaces after treatments with various concentrations of CDots under visible light for 1 h (for *Listeria*) or 1 h and 3 h (for *Salmonella*). Statistical analyses were performed separately for *L. monocytogenes*, *S.* Typhimurium at 1 h, and *S.* Typhimurium at 3 h. Different letters on columns indicate significant differences between the results ($P < 0.05$); columns with the same letter(s) have no significant difference.

the absence of nutrients and growth, with the cells from such surfaces exhibiting increased tolerance to various inactivation strategies (16, 45–51). Cells persisting on dry abiotic surfaces may serve as inocula upon subsequent dispersal or when conditions become permissive of growth. Therefore, it is of great interest to examine whether the photoinactivation by CDots is applicable to cells dried on abiotic surfaces.

Figure 5A shows the effect of PEI-CDots on *L. monocytogenes* cells dried on stainless steel surfaces. Aliquots of 10 μl *L. monocytogenes* 10403S cells were placed on stainless steel coupons. After a 15-min air-drying process, 60 μl of CDot solutions of different concentrations were applied, followed by the illumination with visible light from a 60-W-equivalent daylight LED for 1 h. While there could be cells that died naturally in the samples without CDots, it was clear that the treatment with 10 μg/ml CDots under visible light was effective at killing all of the cells on the surfaces, similar to what was observed in the sample suspensions described above.

Figure 5B shows the results for *S.* Typhimurium cells from a similar experiment but with a longer drying time. Aliquots of 10 μl *S.* Typhimurium cells were applied dropwise to stainless steel coupons. After a 40-min air-drying step, 60 μl of CDot solutions at different concentrations was applied and then illuminated with visible light for 1 h and 3 h. Besides the cells that died naturally, *Salmonella* cells were inactivated to a limited extent by the 1-h treatment at a CDot concentration 10 times that used for *Listeria* cells. However, with a longer treatment time of 3 h and a higher CDot concentration of 100 μg/ml, the treatments were able to inactivate all of the *S.* Typhimurium cells on the stainless steel surfaces.

The results indicated that CDot photoinactivation was more effective on *Listeria* cells and much less effective on *Salmonella* cells, both dried on stainless steel surfaces, similar to what was observed in suspensions. Nevertheless, the inactivation of *Salmonella* cells by CDot treatment is possible, but it requires higher CDot concentrations and longer treatment times than the inactivation of *Listeria* cells. Overall, the study focused on informative assessment and validation on the ability of CDots to effectively inactivate foodborne bacterial pathogens in suspension and on dried

TABLE 1 Bacterial strains tested

Strain	Description
L. monocytogenes 10403S	Serotype 1/2a; commonly used by the majority of investigators in the USA studying the pathogenesis of *L. monocytogenes*; streptomycin-resistant isolate of parental strain 10403. Strain 10403 was first isolated by D. C. Edman in 1968 from a human skin lesion obtained from Montana State University (55).
L. monocytogenes 2858	Serotype 1/2b; implicated in a 2011 multistate outbreak of listeriosis via whole cantaloupe; tetracycline resistant (56)
L. monocytogenes H7858	Serotype 4b; isolated from a multistate outbreak of 1998-1999 in the USA; Quat, crystal violet, and cadmium resistant (57)
L. innocua UAM003-1A)	*L. innocua* is nonpathogenic but can serve as a reservoir for resistance determinants that can be transferred to *L. monocytogenes*. Isolated in May 2017 using the ISO method, which involved culturing the feces of a black bear (*Ursus americanus*) captured in California. Streptomycin and tetracycline resistant (58).
Salmonella Agona strain 447867	Isolate from a 2008 rice and wheat puff cereal outbreak (15)
Salmonella Tennessee strain K4643	Clinical isolate from a 2006-2007 peanut outbreak (59)
Salmonella Typhimurium	Originally purchased from Carolina Biological Supply Co. (Burlington, NC)

stainless steel surfaces. In practical settings in food processing facilities, however, the ecology of *Salmonella* and *Listeria* is complex and remains poorly understood, including that in samples or on surfaces containing soluble solids or food residues. Under those conditions, the CDots with light can in principle still inactivate the pathogens, but the effectiveness and efficiency may be challenged by the interferences associated with the presence of the solids or food residues. Further investigations to address these special challenges are required.

Conclusions. This study demonstrated the application of visible-light-activated CDots for the inactivation of the common foodborne pathogenic bacteria *L. monocytogenes* and *S.* Typhimurium in suspensions and dried on stainless steel surfaces. The results show that the photoinactivation by CDots is more effective with *L. monocytogenes* cells than with *S.* Typhimurium cells, despite similarly elevated intracellular ROS levels in both cell types. However, the lipid peroxidation induced by the generated ROS in *L. monocytogenes* is higher than that in *S.* Typhimurium cells. Furthermore, the leakage of nucleic acid and proteins from CDot-treated *Listeria* cells suggests damage to the cell membrane, whereas no similar leakage of cellular contents was found for the treated *Salmonella* cells. The antibacterial performance of the CDots at relatively low concentrations coupled with visible-light illumination for the quantitative inactivation of *Listeria* cells in suspension and on dried surfaces is comparable to or better than that of many widely investigated antibacterial agents. The CDot treatment is also able to inactivate *Salmonella* cells on dried stainless steel surfaces, though further investigations on performance improvements and the associated mechanistic understanding concerning *Salmonella* and Gram-negative bacteria in general are needed and will prove rewarding.

MATERIALS AND METHODS

Bacterial pathogens and preparation. Bacterial strains used in this study are listed in Table 1. *L. monocytogenes* strains 10403S, 2858, and H7858, *L. innocua* SKB787 (also called UAM003-1A), and *S. enterica* serovar Agona strain 447867 and serovar Tennessee strain K4643 were in collections in one of our laboratories (S.K.). *S.* Typhimurium, originally sourced from Carolina Biological Supply Co. (Burlington, NC), was kindly provided by Daniel Williams in the Department of Biology at North Carolina Central University. The other two *Salmonella* strains were isolated from outbreaks of foodborne illness. All of the *Listeria* strains are drug resistant. The antimicrobial susceptibility of the strains was determined using the standard agar dilution method. More information on each strain is provided in Table 1.

For our experiments, *Listeria* cultures were grown in 15 ml brain heart infusion (BHI) broth (Oxoid, UK) by inoculating the broth with a single colony from BHI agar plates, and incubated overnight at 37°C with constant agitation at 225 rpm in an Excella E24 incubator shaker (New Brunswick Scientific). Bacterial cultures were centrifuged at 12,000 $\times$ *g* (Beckman Coulter Life Sciences, Indianapolis, IN, USA) for 8 min and washed twice using phosphate-buffered saline (PBS). The pellet was then resuspended in PBS and prepared for further experiments. *Salmonella* strains were cultured in Luria-Bertani (LB) broth, and the cells were harvested and processed using the same protocol.

The actual cell numbers in the cultures were determined using the traditional surface plating

method. Briefly, cell suspensions were 1:10 serial diluted with PBS, and aliquots of 100 μl of appropriate dilutions were surface plated on BHI agar plates for *Listeria* strains or LB agar plates for *Salmonella* strains, and the plates were then incubated at 37°C overnight. The colonies were counted, and the cell concentration was calculated as CFU per milliliter in the cell suspensions.

Sublethally injured cells were able to grow into colonies on BHI or LB agar plates but were not able to grow into colonies on BHI or LB agar containing 4% NaCl, while the growth of healthy cells was not affected on the BHI or LB agar containing 4% NaCl. Therefore, to determine the portion of sublethally injured cells in a CDot-treated sample, the sample was surface plated on both BHI agar and BHI agar containing 4% NaCl (for *Listeria* strains) or both LB agar and LB agar containing 4% NaCl (for *Salmonella* strains). The difference in viable cell numbers obtained between BHI agar and BHI agar with 4% NaCl, or between LB agar and LB agar with 4% NaCl, was the portion of sublethally injured cells of *Listeria* or *Salmonella*, respectively, in the CDot-treated sample.

CDots. PEI-CDots were prepared by the surface functionalization of preprocessed and selected small carbon nanoparticles with oligomeric polyethylenimine (PEI; average molecular weight, ~600). Briefly, the small carbon nanoparticles were harvested from the commercially acquired carbon nanopowders (US Research Nanomaterials, Inc.) in procedures similar to those reported previously (52, 53). In a typical experiment, a sample of carbon nanopowder (2 g) was refluxed in concentrated nitric acid (8 M, 200 ml) for 48 h. The reaction mixture was cooled to room temperature and centrifuged at 1,000 *g* to discard the supernatant. The residue was redispersed in deionized water, dialyzed in membrane tubing (molecular weight cutoff, ~500) against fresh water for 48 h, and then centrifuged at 1,000 $\times$ *g* to retain the supernatant. Upon the removal of water, the small carbon nanoparticles were recovered and used in the functionalization reaction for PEI-CDots. The thermally induced functionalization reaction protocol and conditions were the same as those reported previously (52, 53). The resulting PEI-CDots were characterized by using nuclear magnetic resonance (NMR), microscopy, and optical spectroscopy techniques, results of which were consistent with those for similarly prepared samples reported previously. According to atomic force microscopy (AFM) and transmission electron microscopy (TEM) results, the PEI-CDots were on the order of 5 nm in average diameter (54). The dot samples were stored in the dark at room temperature and used for the antimicrobial experiments.

CDot treatments of bacterial cells in suspension. The treatments of cells with PEI-CDots were performed in 96-well plates, and the treatments were performed with each individual strain. Aliquot of 100 μl of bacterial cell suspensions were placed in the wells, and 100 μl PEI-CDots at various concentrations were added to reach the desired final concentrations of PEI-CDots in the wells. The bacterial cell concentration in each well was around 10^6 to 10^7 CFU/ml. The control samples were bacterial suspensions with PBS. The samples for each treatment were prepared in triplicate. The plates were placed on an orbital shaker (BT Lab Systems) at 350 rpm and exposed to visible light from a commercial daylight LED lamp (Cree; 60-W equivalent, omnidirectional, 815 lumens) placed ~10 cm away from the surface of the plate, for 1 h or otherwise-stated times. For all CDot treatment experiments, at least three independent tests were performed.

Determination of the CDots' photoinactivation effect. After treatment, the viable-cell numbers in the treated and the control samples were determined using the traditional plating method described above. The photoinactivation effect of PEI-CDots on the pathogen strains was evaluated by the logarithmic reduction in the viable-cell number in the treated samples compared to the control samples.

Determination of ROS generation and lipid peroxidation in CDot-treated cells. The fluorescence probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) was used to quantify the generation of intracellular ROS in *Listeria* and *Salmonella* strains upon treatment with PEI-CDots. After treatment with CDots at 0, 10, 50, and 100 μg/ml, the cells were washed twice, and the supernatants were removed and then resuspended in 0.85% NaCl. The cells were split between two tubes. The dye H2DCFDA in 0.85% NaCl solution was added to one of the tubes, while the tube without dye received the same volume of 0.85% NaCl and was used as a blank for ROS calculation. At the staining step, cells were kept in the dark for 30 min. After staining, the cells were centrifuged, and the supernatants both with and without dye were removed. The cells were then washed once with 0.85% NaCl and resuspended in 0.85% NaCl. The fluorescence intensity (excitation/emission, 485 nm/528 nm) of the samples were read using a SpectraMax M5 multidetection reader with the software SoftMax Pro5.4.5 (Molecular Devices Corp., CA). For each CDot-treated sample, the fluorescence reading of the sample with dye minus the reading of the well without dye was used for the calculation of ROS generation. The percent ROS increase in the CDot-treated samples compared to the controls (with CDot treatment) is presented.

To quantify the lipid peroxidation in cell membrane by CDot treatments, the concentration of MDA (43, 44) as an indicator of lipid peroxidation was measured using the TBARS assay kit (Cell Biolabs) following the manufacturer's instructions. Briefly, aliquots of 100 μl cell suspension (approximately 10^8 CFU/ml) loaded in the wells of 96-well plates and added with 100 μl of PEI-CDots at the desired concentrations. The plate was exposed to visible light for 1 h. After the treatment, each sample was centrifuged at 9,000 $\times$ *g* (Beckman Coulter Life Sciences, Indianapolis, IN, USA) for 7 min. The cells were washed once with PBS, and the pellets were resuspended in 200 μl PBS. Aliquot of 2 μl of 100$\times$ butylated hydroxytoluene (BHT) was added to each sample to prevent further oxidation. Then, 100 μl of each treated sample, control, and MDA standard (from the kit) was transferred to 1.5-ml microcentrifuge tubes, an aliquot of 100 μl of SDS lysis solution was added, and all samples were mixed thoroughly and incubated at room temperature for 5 min. Then, to each of these treated samples, controls, and MDA standards, 250 μl of TBA reagent was added, and tubes were incubated at 95°C for 50 min. After the incubation, the samples were allowed to cool to room temperature by placing them in an ice bath for 5 min. Then, all of the samples were centrifuged at 1,000 $\times$ *g* for 15 min. A 200-μl aliquot of the

supernatant of each sample was loaded in the wells of a 96-well plate for absorbance measurement at 532 nm using the SpectraMax M5 microplate reader. The absorbances at 532 nm in the treated and control samples were compared to the standard curve, which was obtained separately using the MDA standard provided with the TBARS assay kit.

Determination of nucleic acid and protein leakage from CDot-treated cells. The leakage assay was used to test if CDot treatments could affect cell permeability and cause leakage of intracellular components, such as nucleic acid and protein. *L. monocytogenes* 10403S and *S.* Typhimurium cells were treated with 0, 5, 10, and 30 μg/ml PEI-CDots in 96-well plates under light for 1 h. The cells were then centrifuged (12,000 $\times$ *g*, 2 min), and the supernatants were used for OD measurement. Absorbance at 260 nm was used to detect the leakage of nucleic acid from the bacterial cells, while OD at 280 nm was used to detect the leakage of proteins. The samples before light treatment (time zero) were used as blanks.

CDot treatments of bacterial cells on stainless steel surfaces. The stainless steel coupons (discs 0.5 in. in diameter) were purchased from Biosurface Technologies Corp. (Bozeman, MT) and sterilized with 70% alcohol before use. Aliquots of 10 μl of *L. monocytogenes* 10403S or *S.* Typhimurium cells in PBS were spread on the coupons and air dried for 15 min in a biosafety hood. CDots (60 μl) at various concentrations were placed onto the dry cell spots, which were exposed to a 60-W-equivalent daylight LED for 1 or 3 h. The cells were then eluted from the coupons by vigorously vortexing in 3 ml PBS in a tube, followed by serial dilutions in PBS and plating on BHI or LB agar plates to determine the viable cell numbers, in CFU per milliliter, in the samples.

Statistical analyses. The test results were statistically analyzed using the SAS system 9.2 (SAS Institute Inc., Cary, NC, USA) with the general linear model (GLM), with *P* values of <0.05 being considered statistically significant.

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