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A nano-carrier platform for the targeted delivery of nature-inspired antimicrobials using Engineered Water Nanostructures for food safety applications

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Abstract

Despite the progress in the area of food safety, foodborne diseases still represent a massive challenge to the public health systems worldwide, mainly due to the substantial inefficiencies across the farm-to-fork continuum. Here, we report the development of a nano-carrier platform, for the targeted and precise delivery of antimicrobials for the inactivation of microorganisms on surfaces using Engineered Water Nanostructures (EWNS). An aqueous suspension of an active ingredient (AI) was used to synthesize iEWNS, with the 'i' denoting the AI used in their synthesis, using a combined electrospray and ionization process. The iEWNS possess unique, active-ingredient-dependent physicochemical properties: i) they are engineered to have a tunable size in the nanoscale; ii) they have excessive electric surface charge, and iii) they contain both the reactive oxygen species (ROS) formed due to the ionization of deionized (DI) water, and the AI used in their synthesis. Their charge can be used in combination with an electric field to target them onto a surface of interest. In this approach, a number of nature-inspired antimicrobials, such as H₂O₂, lysozyme, citric acid, and their combination, were used to synthesize a variety of iEWNS-based nano-sanitizers. It was demonstrated through foodborne-pathogen-inactivation experiments that due to the targeted and precise delivery, and synergistic effects of AI and ROS incorporated in the iEWNS structure, a pico- to nanogram-level dose of the AI delivered to the surface using this nano-carrier platform is capable of achieving 5-log reductions in minutes of exposure time. This aerosol-based, yet 'dry' intervention approach using iEWNS nano-carrier platform offers advantages over current 'wet' techniques that are prevalent commercially, which require grams of the AI to achieve similar inactivation, leading to increased chemical risks and chemical waste byproducts. Such a targeted nano-carrier approach has the potential to revolutionize the delivery of antimicrobials for sterilization in the food industry.

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1. Introduction

Safe and nutritious food is an important aspect of public health assurance worldwide (Newell et al., 2010); however, microbiologically contaminated food, across the farm-to-fork continuum, is a constant threat to the agricultural and food processing sector. The annual toll of foodborne disease resulting from the consumption of microbiologically contaminated food is on the rise and has reached an alarming 600 million cases and 420,000 deaths worldwide (World Health Organization, 2015). In the United States, the Center for Disease Control and Prevention (CDC) estimates that 52% of all foodborne diseases are from fresh produce, 22% from meat and poultry, 20% from eggs, and 6% from seafood (Interagency Food Safety Analytics, 2015). Further, it is estimated that, on average, there are approximately 65 outbreaks annually due to fresh produce, more than any other food commodity (Interagency Food Safety Analytics, 2015).

Fruits and vegetables can become contaminated with pathogenic microorganisms anywhere in their production chain starting from the field or orchard, during harvest, transport, processing, distribution, and marketing, or during preparation in food-service establishments and at the home. Possible pathogen sources include contaminated irrigation water (Steele & Odumeru, 2004), animals, birds, insects, soil, manure, and infected workers/food handlers (Berger et al., 2010). In addition, improperly cleaned and disinfected food preparation/contact and storage surfaces can also be important reservoirs of pathogens (Buchholz, Davidson, Marks, Todd, & Ryser, 2012; Kaminski, Davidson, & Ryser, 2014; van Asselt, de Jong, de Jonge, & Nauta, 2008). In recent years, these emerging food safety issues can also be attributed to newly established factors and trends, such as the globalization of the food supply chain (Trienekens & Zuurbier, 2008); growing consumer demand for minimally processed foods, especially fresh produce; and a surge in organic produce consumption (Brackett, 1999; Harvey, Zakhour, & Gould, 2016; Mercanoglu Taban & Halkman, 2011). Modern supermarkets are full of products that are out of season, transported halfway around world to reach the consumer (Schaadt, 2013), usually stacked in containers for weeks (Athukorala & Jayasuriya, 2003), increasing the risk of cross contaminations.

The antimicrobial strategies currently used by the food industry for decontamination mainly include the use of chemicals, such as chlorine-based products, especially sodium hypochlorite (Parish et al., 2003); ozone (Horvitz & Cantalejo, 2014); ultraviolet radiation (Chang et al., 1985; Pearson, Hunt, & Mitchell, 1997); and high-pressure processing (Balasubramaniam & Farkas, 2008; Shearer, Dunne, Sikes, & Hoover, 2000). Other alternative disinfection methods being investigated for fresh produce disinfection include alternative chemical methods, such as vaporized hydrogen peroxide (Ukuku, Bari, Kawamoto, & Isshiki, 2005); electrolyzed water (Izumi, 1999; Koseki, Yoshida, Isobe, & Itoh, 2004); biological methods (bacteriocins, bacteriophages, enzymes and phytochemicals;

(Meireles, Giaouris, & Simões, 2016); physical technologies, such as non-thermal electrical plasma discharges, ionizing radiation, ultrasounds, etc.; and combination methodologies (Vaze, Park, Brooks, Fridman, & Joshi, 2017; Wan, Coventry, Swiergon, Sanguansri, & Versteeg, 2009).

All these methods have their inherent inadequacies and limitations. For example, chlorine-based products used as a “wet” approach in washes and sprays leave behind chemical residues that can be toxic, can have an effect on the sensory quality of food (i.e. color, taste), and generate large chemical waste, which in turn can create environmental problems (Karaca & Velioglu, 2007; Y. Luo et al., 2018; Ventola, 2015). These methods have also been implicated in the loss of nutrients in the treated food items (Z. Chen, Zhu, & Han, 2011). Further, the majority of these chemical-mediated approaches require the tumbling of the produce items in pools of a sanitizer solution or spraying of the produce items with powerful jets of the sanitizer, which is a violent process that can damage delicate produce items, such as berries (Yaguang Luo et al., 2011). Some of these processes can also impart physical damage on the produce, reducing their visual appeal and quality (Rico, Martin-Diana, Barat, & Barry-Ryan, 2007). In addition, many of these chemicals cannot be used with produce that carry the organic label, due to FDA and USDA restrictions.

These aforementioned inadequacies, especially the lack of precision in the delivery of active ingredients (AIs) stress the need for developing new antimicrobial control strategies to supplement or replace existing ones and effectively combat foodborne pathogens at critical control points. Nanotechnology has emerged as an enabling technology to assist our society in combating infections (Eleftheriadou, Pyrgiotakis, & Demokritou, 2017). For example, engineered nanomaterials (ENMs) with antimicrobial properties, such as nanosilver, photocatalytic TiO₂ and ZnO nanoparticles, are being used to produce antimicrobial surfaces (Chaloupka, Malam, & Seifalian, 2010; Espitia et al., 2012); however, these ENMs cannot be implemented for fresh produce disinfection or as an aerosol treatment for airborne disinfection due to associated risks from ingestion and inhalation (H. Chen et al., 2017; Chia, Tay, Setyawati, & Leong, 2015; G. DeLoid et al., 2016; G. M. DeLoid et al., 2017; Giovanni et al., 2015; Lu et al., 2016; McClements et al., 2016; Pirela et al., 2016; Servin & White, 2016; Setyawati et al., 2018; Sohal, O’Fallon, Gaines, Demokritou, & Bello, 2018).

Here, we report the development and evaluation of a nano-carrier platform for the precise and targeted delivery of minute quantities of antimicrobials using the Engineered Water Nanostructures (EWNS) recently developed by the authors (Pyrgiotakis et al., 2016; Pyrgiotakis, McDevitt, Bordini, et al., 2014). In this study, this EWNS platform was expanded and used to incorporate other AIs into the EWNS structure in order to further enhance their antimicrobial potency. These new structures are referred to as iEWNS, where ‘i’ denotes the AI used. As shown, these iEWNS possess unique, active-ingredient-dependent physicochemical properties: i) they are engineered to have a tunable size in the nanoscale; ii) they have excessive electric surface charge, and iii) they contain both the reactive oxygen species (ROS) formed due to the ionization of deionized (DI) water, and the AI used in their synthesis. One of the novel features of these structures is the electrical charge that can be used in combination with an electric field to target them onto the surface

of interest. As a result, the iEWNS deliver the AI with high precision, minimizing the amount required without compromising their effectiveness.

In this study, the focus was on using nature-inspired antimicrobials, such as hydrogen peroxide, citric acid, their combination, and lysozyme. These iEWNS were investigated for their physicochemical and antimicrobial properties and their efficacy was evaluated against foodborne pathogens.

2. Materials and methods

2.1 Synthesis and targeted delivery of iEWNS

Synthesis: Detailed description of the synthesis of the iEWNS is illustrated in Figure 1 and has been explained in great detail in previous publications (Pyrgiotakis et al., 2016; Pyrgiotakis, McDevitt, Gao, et al., 2014).

In brief, an aqueous solution of the utilized antimicrobial is prepared (Figure 1a) by adding the appropriate amount of the AI in DI water. The solution is held in an airtight bottle. High air pressure in the bottle created by an air compressor pushes the aqueous solution through a 0.3-mm ID Teflon tubing to an emitter-capillary (30G stainless steel needle). The emitter is held across an aluminum counter electrode that is grounded. The counter electrode has a 0.64-cm diameter aperture in the center and sits on top of a grounded aluminum funnel that is connected via brass tubing to the various sampling instruments for characterization.

A high voltage source (Spraybase, Dublin, Ireland) is used to apply voltage between the emitter and the counter electrode to form a strong electric field (Figure 1b). As shown in a previous publication (Pyrgiotakis et al., 2016), during this process two distinct phenomena take place: i) electrospray and ii) ionization of the water. The charges accumulated at the water-air interface at the tip of the emitter in combination with the strong electric field causes the formation of the Taylor cone (Taylor, 1964). A strong electrical force, created by the interfacial charge and the strong electric field, results in the emission of highly charged particles (Figure 1c - inset). At the same time, the electric field causes the water molecules to split and ionize, resulting in the generation of ROS, which end up along with the AI in the iEWNS particles (Figure 1c). A digital camera (Point Grey Cameleon, FLIR Integrated Imaging Solutions Inc., Richmond BC, Canada) is used to visually monitor the formation and stability of this Taylor cone. In our current design, three emitters/generation modules are operating in parallel.

It is worth noting that the operational parameters that can be adjusted to control the iEWNS synthesis and particle properties are the emitter-counter electrode distance, h , and the applied voltage, V . The pressure at the bottle is a dependent variable, as it adjusted after the h and V have been set to generate a stable Taylor cone. There is a critical window of operational parameters needed in order for the above-stated phenomena to take place and the EWNS to be formed and optimized in terms of size, electric charge, and ROS content (Pyrgiotakis et al., 2016). These operational parameters were used in the present study with some fine-tuning to accommodate the addition of the various AI and summarized in Table 1.

Targeted delivery of iEWNS nanoparticles: The electric field that was used to synthesize the iEWNS particles can also be used to deliver them on the target surface that is placed below the capillary utilizing their inherent electric surface charge (Figure 1d). The iEWNS, due to their surface charge, exhibit an electrical force that pushes the particles towards the counter electrode.

Als used: The following AI aqueous solutions were prepared and used in the making of various iEWNS nano-sanitizers: A 1% hydrogen peroxide solution was prepared using diluted 3% hydrogen peroxide (w/v) solution (Sigma Aldrich, St. Louis MO) in DI water. A 1% citric acid solution was prepared by diluting 10% citric acid (w/v) solution (VWR Analytical, Radnor PA) in DI water. The 0.1% Lysozyme solution was produced by adding 100 mg of lysozyme powder (Sigma Aldrich, St. Louis MO) to 100-mL DI water.

2.2 Physicochemical characterization of the iEWNS

AI solution properties: The conductivity of the AI aqueous solution was measured with a Zetasizer (Malvern Instruments, Worcestershire UK) and the pH was measured using a pH meter (Si analytics, Weilheim, Germany).

Aerosol size and concentration of iEWNS: The Scanning Mobility Particle Sizer (SMPS, TSI, Shoreview, MN) was employed to measure the size and the particle concentration of the iEWNS. The arithmetic mean size and concentration was obtained from this distribution using the Aerosol Instrument Manager software (TSI, Shoreview, MN). Ten discrete measurements were performed, each for duration of 120 seconds, and the average of the measurements was reported.

Electric charge: The details of the measurement have been described elsewhere (Pyrgiotakis et al., 2016; Vaze et al., 2018). A Faraday aerosol electrometer (Model 3068B, TSI, Shoreview, MN) was used to measure the aerosol current concurrently with the SMPS during particle concentration measurement. The sampling flow rate was 0.5 L/min for both instruments. Ten concurrent measurements were performed, each for a duration of 120 seconds. The equation for this calculation is as follows:

$$q = I_{EI} / (N_{SMPS} \times \phi_{EI}) \quad (1)$$

where I_{EI} (amperes) is the measured current, N_{smgs} ($\#/m^3$) is the number concentration measured with the SMPS, and ϕ_{EI} (m^3/s) is the flow of the aerosol into the electrometer. The average charge (q) was calculated in units of the fundamental charge (e^-) per iEWNS particle.

Chemical assessment: The ROS content of each of the iEWNS nano-sanitizers was measured using the Trolox method as described by the authors in detail in a previous publication (Vaze et al., 2018). In summary, 5 mL of solution of Trolox (0.1 mM, in 0.05 M pH 7 phosphate buffer) was placed in an impinger (SKC Inc., Eighty Four, PA) and connected to the output of the iEWNS generation system. The iEWNS aerosol was bubbled through this solution for five minutes with a sampling rate of 0.5 L/min. Potential losses inside the impinger were calculated separately and taken into consideration. Two aliquots of

1 mL were taken from the impinger after the reaction time. The first aliquot was processed without further modification to detect the short-lived ROS, and the second aliquot was spiked with HRP (100 unit/mL final concentration) for the detection of H₂O₂. Samples were incubated for 30 minutes at 37°C prior to analysis. The values of ROS were reported as mol equivalents of H₂O₂/particle.

For the cases of the lysozyme and citric acid, the potential chemical transformation and chemical byproduct formation, caused by the electric field and/or interactions with the iEWNS ROS, were assessed with HPLC and UV/VIS absorption spectroscopy. The levels of AI were estimated in terms of nanograms per iEWNS nanoparticle.

2.3 Microbial inactivation experiments

Exposure protocol and setup: Stainless steel coupons inoculated with bacteria were used to evaluate the iEWNS inactivation. For the bacteria inactivation experiments, a three-emitter system was used (Figure 2). Each iEWNS emitter functions exactly as the single emitter that was described earlier. All emitters share the same voltage source and have the same emitter-counter electrode distance but have independent liquid feed to ensure the stability of the cone.

The target surface was placed concentrically directly below the emitter. The iEWNS were accelerated towards the inoculated coupons due to the electric field and their inherent electric charge (Figure 1d). One inoculated coupon was placed under each emitter. The bacteria were exposed to the iEWNS for an appropriate amount of time. The exposure time was optimized for each AI to be able to measure a 5-log reduction, which is the limit of detection for the method. Upon completion of the exposure, the stainless steel coupons were removed and were processed for enumeration.

As controls, three more coupons were kept at the same environmental conditions away from the EWNS in order to determine the natural decay of the inoculated microorganisms. During all exposures, temperature and relative humidity (T, RH) were recorded to ensure that all inactivation experiments were executed under similar conditions.

Bacterial protocols: *Escherichia coli* (ATCC #25922) and *Listeria innocua* (ATCC #33090) were acquired from ATCC (Manassas, VA). The microorganism cultures were maintained on Tryptic soy agar (Hardy Diagnostics, Santa Maria, CA) plates. For each experiment, the cultures were grown overnight in Tryptic soy broth (Hardy Diagnostics, Santa Maria, CA) inside a shaker incubator at 37°C. This overnight culture was then centrifuged at 3000 rpm for 5 minutes and the pellet was re-suspended in DI water. The final concentration of the inoculum was adjusted to 10⁸ cfu/mL, through O.D.600 measurement. All the protocols are described in great detail in previous publications (Pyrgiotakis et al., 2016; Vaze et al., 2018).

For the stainless steel coupon inoculation, 10 µL of this bacterial inoculum was distributed on stainless steel coupons (stainless steel 304, diameter 1.82 cm, Stainless Supply, Monroe, NC), by adding ten 1-µL droplets in a concentric manner near the center of the coupon. The effective concentration of bacteria on the coupons was 10⁶ cfu/coupon. The coupons were

then placed inside a petri dish and the inoculum was allowed to dry, while placed inside a biosafety cabinet.

For the enumeration of the surviving bacteria, both control and exposed coupons were each added to a 50-mL micro-centrifuge tube containing 5 mL of 1x phosphate-buffered saline (VWR International, Radnor PA). The coupons were vortexed for 30 seconds and the resulting rinsate was utilized in a dilution plate-counting assay.

Evaluation of sensory effects: A preliminary evaluation of the sensory effects on produce after iEWNS treatment was carried out using cherry tomatoes. The nano-sanitizer chosen for treatment was h1EWNS. Cherry tomatoes were acquired from a local shop on the day of the experiment. Since only the sensory effects of the tomatoes were to be analyzed, no antimicrobial pretreatment was performed. For treatment, one tomato was placed under each iEWNS emitter and treated with h1EWNS. A 15-min treatment was performed and the tomatoes were flipped and treated for 15 more minutes. Control tomatoes were placed under the emitter but no treatment was performed. The sensory parameters were analyzed in the following aspects: The redness of the tomatoes was tested by taking pictures and analyzing the red color value with ImageJ software (NIH, Bethesda MD). The pH of tomatoes was tested with pH strips and the firmness of the tomatoes was tested with a penetrometer (Deltatrak, Pleasanton CA). Nine tomatoes were analyzed for treatment and nine were held as controls.

2.4 Data interpretation and statistical analysis

Each bacteria inactivation experiment was repeated in triplicate. Each data point represents the arithmetic mean of three replicates. The standard deviation of the three trials was used as the error.

Accounting for the natural decay of the microorganisms at time t , the log reduction was calculated according to the following equation where, C_0 is the microorganism concentration of the treatment coupon at time '0' while $C_{Exp.}(t)$ is the concentration of the exposed microorganisms at time 't'. The log reduction (LR) is defined as:

$$LR = \text{Log}10((C_{Exp.}(t))/C_0) \quad (2)$$

The log reduction rate (LRR) was calculated by fitting the log reduction (LR) against time with a linear equation as follows:

$$LR(t) = LRR \times t \quad (3)$$

where LRR (logs/min) is the log reduction rate and t is the time (mins). The LRR was used to compare the different iEWNS activity (Hamilton, 2010).

2.5 Transmission electron microscopy of exposed bacteria

Stainless steel coupons inoculated with the two bacteria were exposed to iEWNS according to the protocol described above. After treatment, the coupons were rinsed with phosphate-buffered saline (VWR International, Radnor PA) and the recovered rinsate was further

centrifuged at 300 rpm for five minutes. The resulting supernatant was removed, and the pellet was used for fixation. A 2× solution of routine fixative (2.5% Glutaraldehyde 1.25%, Paraformaldehyde, and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4) was added to the pellet in a 1:1 manner. The pellet was fixed for at least two hours at room temperature in the above fixative, washed in 0.1-M cacodylate buffer, post-fixed with 1% Osmium tetroxide (OsO_4)/1.5% Potassium ferrocyanide (KFeCN_6) for one hour, washed 2x in water and 1x in Maleate buffer (MB), and incubated in 1% uranyl acetate in MB for one hour followed by two washes in water and subsequent dehydration in grades of alcohol (10 minutes each; 50%, 70%, 90%, 2× 10 minutes 100%). The samples were then put in propylene oxide for one hour and infiltrated ON in a 1:1 mixture of propylene oxide and TAAB Epon (Marivac Canada Inc. St. Laurent, Canada). The following day, the samples were embedded in TAAB Epon and polymerized at 60°C for 48 hours.

Ultrathin sections (about 60 nm) were cut on a Reichert Ultracut-S microtome, picked up onto copper grids stained with lead citrate, and examined in a JEOL 1200EX transmission electron microscope or a TecnaiG² Spirit BioTWIN, and the images were recorded with an AMT 2k CCD camera.

2.6 Estimation of AI delivered per unit surface area of treatment

For the comparison of the inactivation data, it is important to estimate the delivered dose for each iEWNS nano-sanitizer as a function of the particle number concentration and the size of the iEWNS aerosol. The geometry of the electrospray plume is shown in Figure S4. According to the work of Zhou et al., the cone of the plume has an average angle of approximately 15° (S. Zhou, Edwards, Cook, & Van Berkel, 1999). Given the distance of the target surface from the needle h (cm), the area of exposure can be calculated as

$$S = 4\pi \times (\tan(15^\circ) \times h)^2 \quad (4)$$

Further, the total delivered-to-surface dose of the iEWNS is calculated based on the SMPS aerosol concentration and size distribution data, the particle size and density. Each iEWNS that has diameter d_i (cm) and density of ρ_0 (g/cc) a mass of $m_i = \pi/6 d_i^3 \rho_0$. Knowing the iEWNS aerosol particle concentration C (#/cc), the sampling aerosol flow ϕ (cc/min) and the total exposure time t (min) the total delivered dose (in terms of particle number) is calculated as:

$$M_{\text{tot}} = C \times \phi \times t \times \rho_0 \times \frac{\pi}{6} \times d_i^3 \quad (5)$$

Assuming homogeneous particle distribution across the exposed surface, combining equations 4 and 5 the dose normalized to the surface area (DNS) can be calculated in terms of delivered g/cm² as follows:

$$\text{DNS} = \frac{C \times \phi \times t \times \rho_0}{24 \times (\tan(15^\circ) \times h)^2} \times d_i^3 \quad (6)$$

This value represents the total mass delivered to the exposed surface that includes the DI water and the AI. Knowing the AI concentration in the stock solution and assuming it is the same in the iEWNS, the exact amount of the AI delivered can be calculated.

To compare the efficiency of the different iEWNS-based nano-sanitizers for various AIs used in the study, the delivered-to-surface mass dose required to achieve 3-log reduction was calculated for each case based on the inactivation data. The 3-log reduction dose is a value that is commonly used to compare effectiveness of antimicrobial technologies (World Health Organization, 2008).

3. Results and Discussion

3.1 Synthesis of iEWNS

It should be noted that under these operational conditions used in the synthesis, our studies have shown that the generated current needed for the synthesis is in the order of several nA, which results in low consumed energy that does not exceed 5 mW/emitter.

It should also be highlighted that very low starting concentrations of AI solutions were used (concentrations never exceeding 1%). More specifically, hydrogen peroxide, which is a known antibacterial, is used in cellular processes and leaves no residue as it dissociates into water (Klapes & Vesley, 1990). Here, the concentration of H₂O₂ utilized was 1% w/v, which is the concentration approved by the FDA for processing various foods, such as starches. Citric acid, a major constituent of citrus fruits, is a well-known antimicrobial, extensively used for food safety applications (In, Kim, Kim, & Oh, 2013). A 1% w/v solution of citric acid was utilized in this study to produce the iEWNS, which is less than the concentration of citric acid found in lemons (5.75%; (Penniston, Nakada, Holmes, & Assimios, 2008). We also investigated lysozyme, which is a natural enzyme that is found in egg white, tears, and breast milk (Hughey, Wilger, & Johnson, 1989). Lysozyme is known to have antibacterial activity and 0.1% w/v solution of lysozyme was utilized for producing iEWNS, which is in the range of the concentration of lysozyme in human tears from 750–3300 mg/L (0.075 to 0.330 %; (Aine & Mörsky, 1984). We also explored a combination of hydrogen peroxide and citric acid, 1% w/v of each for producing iEWNS particles (Ukuku et al., 2005). These concentrations of the AI used were exploratory, and not optimized in terms of the AI concentration.

3.2 Physicochemical characterization of iEWNS

As discussed extensively in previous publications (Pyrgiotakis et al., 2016; Pyrgiotakis, McDevitt, Bordini, et al., 2014), the iEWNS possess a set of very unique, tunable properties that are vital for their stability and their ability to interact and inactivate bacteria. For the iEWNS used here, these properties are summarized in Table 1 and are compared with the baseline-iEWNS (EWNS synthesized using purely DI water) that have been studied extensively in previous publications (Pyrgiotakis et al., 2015, 2016).

Size of iEWNS: The baseline EWNS generated using only pure DI water with no AI were observed to have a mean diameter of 12.1 ± 0.1 nm, which is in agreement with earlier studies (Pyrgiotakis et al., 2016). The h1EWNS particles had the smallest size at 11.9 ± 0.3 nm,

while the c1h1EWNS particles had the largest size at 48 ± 3 nm. The lysozyme and the citric acid iEWNS were in the middle range with 13.1 ± 0.3 nm and 36.0 ± 0.3 nm respectively. This variation is expected and it relates primarily to the properties of the utilized solution pH and conductivity (Tang & Gomez, 1994). These properties of the citric acid and hydrogen peroxide solutions and their mixture, are summarized in Figures S1 and S2. The iEWNS particle properties are summarized in Table 1 and indicate that for a starting solution, as the pH decreases and the conductivity increases, the size of the iEWNS particle produced by that solution increases as well. However, the mixture of hydrogen peroxide and citric acid does not seem to follow the same trend, due to potential interactions between the AIs utilized that can affect the synthesis of such iEWNS.

Electric charge of iEWNS: The lowest charge was observed for the EWNS and the h1EWNS with $13 e^-$ /particle and $11 e^-$ /particle respectively. L0.iEWNS particles were found to have electric $15 e^-$ /particle. The highest charge was observed for the c1EWNS and c1h1EWNS with $23 e^-$ /particle and $77 e^-$ /particle respectively. The charge results seem to follow a similar trend with the size in terms on the dependency on the starting solution properties.

It is worth noting that the electric charge is a very important property of iEWNS particles and linked to the lifespan in environmental conditions. A droplet of nanoscale dimensions would be expected to evaporate in a few milliseconds in room air conditions (Robert E. Williamson & E. Dale Threadgill, 1974). However, Nielsen et al. (Nielsen, Maus, Rzesanke, & Leisner, 2011) showed that the electric charge of water droplets retards their evaporation time significantly. As was shown in earlier studies with EWNS nanoparticles, their lifespan can reach up to several hours in indoor environmental conditions and their size does not change (Pyrgiotakis et al., 2016; Pyrgiotakis, McDevitt, Gao, et al., 2014).

This link between size and charge extends beyond the iEWNS stability and lifetime; it also imposes limitations in the way size is measured. In a previous publication, we showed that the Scanning Mobility Particle Sizer (SMPS) utilized to measure iEWNS size can underestimate their size (Pyrgiotakis et al., 2016). The SMPS uses a Kr-85 neutralizer to bring particles towards the Boltzmann electric charge equilibrium (approximately $\pm 1 e^-$ per particle depending on the particle size). This imposes two limitations when measuring the size with the SMPS. Firstly, by reducing the charge of the iEWNS particles, their “actual” size is also affected, and secondly, these neutralizers are not effective for highly charged particles (Ji, Bae, & Hwang, 2004). In previous studies with the EWNS (baseline iEWNS, with DI water only), we addressed this issue by estimating the EWNS size with atomic force microscopy (AFM) and it was shown that their actual size was larger than the size measured by SMPS by a factor of ~ 2 (12 nm as measured by SMPS vs 25 nm by AFM) (Pyrgiotakis et al., 2016; Pyrgiotakis, McDevitt, Gao, et al., 2014).

More importantly, however, the surface charge can be used in combination with an electric field to direct the iEWNS to a target surface. In the past, we took advantage of this property with the use of an electrostatic precipitation exposure (EPES) system, which utilizes another electric field in series, in order to direct the EWNS aerosol to the target surface (Pyrgiotakis et al., 2015). Although this is an effective method, it requires a complicated series setup that can result in losses of the EWNS in the sampling apparatus and in the EPES. In the current

approach, the delivery mechanism was simplified by utilizing the electric field that was used to synthesize the EWNS to deliver them on the target surface. This methodology of treatment leads to the majority of the particles being targeted towards the surface of interest with essentially no losses. Further, the electric field ensures that all of the iEWNS are delivered to the surface with minimal diffusion into the surrounding air minimizing any environmental and occupational exposure risks.

Chemical properties of iEWNS: In addition to their unique physical properties, these iEWNS possess very unique chemical properties. As explained in previous studies (Pyrgiotakis et al., 2015, 2016; Pyrgiotakis, McDevitt, Yamauchi, & Demokritou, 2012; Pyrgiotakis, McDevitt, Gao, et al., 2014; Vaze et al., 2018), during the synthesis of the baseline EWNS particles, ROS are generated from the aqueous phase, contained within the iEWNS, and play a significant role in the inactivation of microorganisms. Here in this study, as explained in the methods section above, the ROS levels in various iEWNS were quantified with respect to H₂O₂ equivalent units (Table 1). As expected, the results indicate that the various iEWNS nano-sanitizers produced in this study contained 10⁻¹⁵ to 10⁻¹⁷ mol H₂O₂ equivalent levels of ROS/per iEWNS particle. The highest quantity of short-lived ROS species (superoxide and hydroxyl radicals) was detected in the baseline EWNS, with 1.03×10⁻¹⁵ mol/particle. Interestingly, the highest levels of H₂O₂ were detected in the L0.1EWNS, with 9.85×10⁻¹⁶ mol/particle, which was greater than the H₂O₂ levels detected in the h1EWNS particle (5.5×10⁻¹⁶ mol/particle).

The collected iEWNS were also compared to the original aqueous solution to assess whether or not the organic AIs were altered by the strong electric field and potential interactions with ROS. Regarding the L0.1EWNS particles, HPLC showed no detectable differences between the stock solution used in the synthesis and the lysozyme in the collected L0.1EWNS, which indicates no detectable alterations in the lysozyme structure or byproducts in the iEWNS (Figure S2). Similarly, no evidence of chemical transformation or the formation of byproducts was observed for citric acid in the case of c1EWNS and c1h1EWNS nano-sanitizers (Figure S3). Typical oxidation byproducts of citric acid are aconitic and itaconic acid (Steiger, Blumhoff, Mattanovich, & Sauer, 2013), none of which were detected. These results conclusively show that there are no detectable changes in the chemical changes of the AI, or if there are detectable changes, they are below the limit of detection of the HPLC that is in the order of 50 fmol.

Although the exact structure of the iEWNS is very challenging to de-convolute in terms of whether the AIs are on the surface or within the structure of EWNS particles, for the case of hydrophilic compounds used in this study, it is believed that they are homogeneously distributed within the structure. However for hydrophobic AIs, it is difficult to predict or visualize using analytical techniques their localization in the AIs. It is worth noting though that for such tiny, nanoscale particles with high surface to volume ratios, AIs are primarily on the surface or vicinity of the surface of the particles.

The quantification of the mass of AI per particle of iEWNS was carried out and the details are presented in Table 1. The levels of citric acid per particle of c1EWNS and c1h1EWNS were found to be 4.14×10⁻⁷ ng/particle and 1.27×10⁻⁶ ng/particle respectively. Similar

calculations were performed for lysozyme. Here, an even lower concentration of 0.1% was utilized to produce these particles and the levels of lysozyme detected per particle were found to be 2.56×10^{-8} ng/particle. These results confirm the miniscule presence of the AI in their corresponding iEWNS. The mass of the AI delivered to the target surface during the treatment period is calculated in the next section.

3.3 Microbial inactivation and AI-delivered dose to the target surface area

Figure 3 summarizes the results for the bacteria inactivation. Figure 3a illustrates the inactivation of *E. coli* as a function of exposure time for the various iEWNS nano-sanitizers. The control decay samples indicated that there is a 1-log reduction in the concentration of viable *E. coli* after 45 minutes, which is in accordance with other reports (Wani, Maker, Thompson, Barnes, & Singleton, 2015). The baseline EWNS (produced only with DI water) resulted in a 2.4-log reduction after 45 minutes of exposure resulting to an inactivation rate of 0.05 logs/min, which is in agreement with previous data obtained by the authors with the EWNS (Pyrgiotakis et al., 2015).

However, when the nature-inspired AIs were added in the structure of iEWNS along with the ROS generated from the aqueous phase, the inactivation potential increased dramatically. For the c1EWNS nano-sanitizer, there was a 5-log reduction observed after 15 minutes of treatment resulting to an inactivation rate of 0.35 logs/min. Further increase in the antimicrobial inactivation rate was observed for h1EWNS reaching the limit of detection (5-log reduction) in just 5 minutes with an inactivation rate of 1.05 logs/min. Similar results were obtained for the L0.1EWNS, with a 5-log reduction observed in 5 minutes resulting also to an inactivation rate of 1.05 logs/min. The most significant result, however, came from the c1h1EWNS, which are made from a combination of citric acid and hydrogen peroxide that resulted in a 5-log reduction within two minutes (a rapid rate of 2.5 logs/min inactivation). This inactivation was more effective than the additive rates of the inactivation produced by hydrogen peroxide or citric acid alone, which is an indication of synergistic effects of the two AIs with the inherent ROS produced by the aqueous phase.

The h1EWNS was also evaluated against the gram-positive *Listeria innocua* (Figure 3b). The data show a 5-log reduction within 15 minutes. This is less effective compared to the h1EWNS against *E. coli*, which is due to the difference between the gram-positive (*L. innocua*) and gram-negative (*E. coli*) bacteria. Gram-positive microorganisms tend to have a thicker cell wall and are generally more resilient than the gram-negative bacteria.

These bacteria inactivation results were also confirmed with TEM imaging (Figure 4). Figure 4a shows control (unexposed) *E. coli* cells that have their membrane intact. On the contrary, the h1EWNS-treated *E. coli* cells (Figure 4b) showed rupture of the membrane and leakage of cellular components. This observed effect on microbial cells is in agreement with what is known of the mechanism of inactivation of hydrogen peroxide in liquid or vapor form (Finnegan et al., 2010). The c1EWNS-treated *E. coli* (Figure 4c) showed mostly intact cell membrane, indicating that the primary mechanism of action of the c1EWNS is not through membrane damage, but rather through intracellular processes, such as interference with the respiratory chain of the bacterial cell. This mechanism of inactivation of the citric acid is the interference in the cellular respirator by disturbing the citric acid cycle (Ukuku et

al., 2005). The most interesting results were observed for the c1h1EWNS-treated cells, where stretching and deformation of the cell membrane was observed with some leakage of the cellular components, indicative of potential synergistic effects (Figure 4d). A possible explanation for this effect would be that hydrogen peroxide is not producing complete peroxidation but causes changes to the membrane permeability, thus facilitating the entry of the citric acid into the cell for the inactivation. This suggests synergistic effects for certain combinations of two or more AIs can result in inactivation rates greater than those of the individual AIs can. This multi-mechanism approach is especially important for defending against the generation of any antimicrobial resistance. The L0.1EWNS-treated *E. coli* cells are indicative of lysis and loss of their cellular shape (Figure 4e). This effect has been observed in earlier studies related to the effect of lysozyme on microorganisms such as *E. coli* and *Pseudomonas*. Here, the hydrolysis of peptidoglycans in the cell wall has been implicated as the major inactivation mechanism for lysozyme (Masschalck, Van Houdt, Van Haver, & Michiels, 2001).

The most significant feature of the iEWNS platform is the miniscule dose of AI delivered to the target surface. To illustrate this, the dose required for 3-log bacterial removal was estimated based on the acquired data. Figure 5 illustrates the required delivered dose to achieve a 3-log reduction for each iEWNS nano-sanitizer.

For the baseline EWNS that contain no AI, the delivered dose of iEWNS aerosol per treated area required to produce a 3-log reduction was 2.0625 ng/cm² of iEWNS mass. The c1EWNS required 114.53 ng/cm² of iEWNS mass to produce a 3-log reduction, which translates to approximately 11.5 ng/cm² of citric acid delivered. In the case of the L0.1EWNS, the dose per treated area required to produce the 3-log reduction was estimated to be 2.81 ng/cm² of iEWNS mass or to inactivate *E. coli* less than 0.3 ng/cm² of AI, which is significantly lower than what is in a tear drop (Aine & Mörsky, 1984). The efficacy of such miniscule dose of lysozyme delivered to the target surface area is an indication that such enzymes with known antimicrobial activity can be delivered effectively through the iEWNS-targeted delivery approach, opening up an array of possibilities to target specific microorganisms with very low quantities of other nature-inspired enzymes and peptides of known antimicrobial properties (Voss, 1964).

For h1EWNS, the delivered dose was 0.078 ng/cm² of iEWNS mass, which translates to 8 pg/cm² of AI. This is in sharp contrast to conventional treatments with aqueous solution of hydrogen peroxide at 5% and 10% concentrations that resulted in 2.2- and 3.5-log reduction respectively in the same amount of time (R. Zhou et al., 2015).

For the citric acid and H₂O₂ combination (c1h1EWNS), it was shown that the required dose per treated area to produce a 3-log reduction was 32.79 ng/cm². This translates approximately to 1.6 ng/cm² for each AI. This synergistic additive effect is in agreement with results from studies using the combination of the two AIs in 'wet' type of treatments where the produce typically are dunked and tumbled in large pools of AI solutions (Ukuku et al., 2005). Such results are highly encouraging and illustrate that the iEWNS carrier platform can be utilized to deliver a combination of AIs with each one suitable to target specific mechanisms and families of microorganisms on surfaces (Ukuku et al., 2005).

As was shown in earlier studies with EWNS nanoparticles, their lifespan can reach up to several hours in indoor environmental conditions due to their excessive electric charge which increases surface energy and tension and reduces their evaporation (Pyrgiotakis et al., 2016; Pyrgiotakis, McDevitt, Gao, et al., 2014). It is also worth noting though that in this study, their synthesis and delivery to the surface of interest happens at the same time utilizing the electric field and their electric charge Figure 1(d)). This means that the delivery happens in milliseconds after their synthesis, therefore their lifespan is not a critical parameter for such surface inactivation applications.

Collectively, the aforementioned inactivation results show that a miniscule dose per exposed area delivered using the iEWNS nano-carrier platform can be effective and result in complete inactivation in minutes, whereas, in wet approaches, gram-level quantities of such AI will end up on the surface of interest for the same or lower levels of inactivation (ng vs. g respectively) (Akbas & Ölmez, 2007; Eswaranandam, Hettiarachchy, & Johnson, 2006; Sapers & Sites, 2003). This is due to the targeted aerosol delivery of the AI and the high mobility of iEWNS nanoparticles due to their nanoscale features and extensive surface per volume ratio. The miniscule quantities of AIs delivered on surfaces of interest using the iEWNS nano-carrier platform can minimize the risk from chemical residues and eliminate the production of chemical waste. Similarly, the generated, miniscule ROS levels present in the iEWNS structure will be inactivated upon contact with the produce surface due to their short lives and therefore minimize any chemical risk for the consumer. Furthermore, iEWNS have the potential to reduce or even eliminate any negative sensory effects commonly associated with wet approaches used for fresh produce disinfection (Delaquis, Fukumoto, Toivonen, & Cliff, 2004). To assess any potential sensory issues from the iEWNS, a preliminary assessment was carried out following the protocols described in the methods section and supplemental data using cherry tomatoes. The tomatoes treated with h1EWNS indicated no significant changes to the sensory quality of the tomatoes (Figure S5). Larger scale sensory evaluation for other iEWNS nano-sanitizers needs to be performed in future studies in order to fully assess the potential of iEWNS to induce sensory damage.

4. Conclusions

The iEWNS platform developed in this study to deliver various nature-inspired antimicrobials in a targeted manner was shown to be exceptionally effective in producing up to 5-log reductions in the concentrations of two food-related microorganisms in a matter of minutes. This targeted delivery approach makes it possible to utilize miniscule quantities (nanogram levels) of an antimicrobial ingredient to obtain surface microorganism inactivation. The high efficacy of inactivation of a combination of AIs denotes the ability of this platform to take advantage of synergistic effects of various AIs incorporated in the EWNS structure.

The iEWNS platform can potentially address the lack of an established methodology for the precise and targeted delivery of minute quantities of antimicrobials for microorganism inactivation. The low energy and water requirements and the undetectable chemical residues make this technology a green sustainable alternative to current methods. The iEWNS platform has the potential to be scaled up and employed at various critical control points

across the farm-to-fork continuum. Collectively, these features make the iEWNS nano-carrier platform a potentially game-changing approach in the battle against foodborne microorganisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

Synthesis and targeted delivery concept of iEWNS. (a) The active ingredient is added in DI water to result in an aqueous solution. (b) The solution is transferred to the iEWNS emitter using an air compressor. The applied voltage between the capillary and grounded electrode results in the combined electrospray ionization process that produces the iEWNS nanoparticles. (c) The synthesized iEWNS have nanoscale size and high electric charges. They also contain both the AI molecules and the ROS produced during the ionization process. (d) The targeted delivery of these particles to the surface of interest is performed utilizing the electric field and their inherent electric charges.

Figure 2: Bacterial inactivation inoculation experiments. The stainless steel coupons inoculated with pathogen were placed under each emitter (centered) and exposed to iEWNS nanoparticles as a function of time. Inactivation results were derived as a function of exposure time for each iEWNS tested in the study. Non-exposed pathogen-inoculated SS coupons were used as controls.

Figure 3:

Antimicrobial efficacy of various iEWNS developed and tested: (a) Inactivation of *E. coli* produced by various iEWNS (please note that the h1EWNS and L0.1EWNS lines and data points overlap); (b) Inactivation produced by h1EWNS against *L. innocua*; Note: The initial pathogen inoculum concentration was 10^6 cfu. Experiments were performed in triplicate. Error bars represent the standard deviation of the measured value.

Figure 4:
Transmission electron microscopy images of *E. coli* exposed with various iEWNS nano-sanitizers: (a) control (non-exposed), (b) h1EWNS, (c) c1EWNS, (d) c1h1EWNS, (e) L0.1EWNS.

Figure 5:

Required delivered iEWNS and AI dose to produce 3-log reductions in the case of *E. coli*. Note that the EWNS contain no added AI. The only activity is due to the generated ROS in the aqueous phase.

Table 1:

Summary of the operational parameters and physicochemical properties of the iEWNS

iEWNS			Operational Parameters		Physical Properties		Chemical Properties		
Abbreviation	AI	AI Conc. [w/v%]	V [kV]	h [cm]	Diameter [nm]	Charge [(e ⁻)]	Short-lived ROS [mol/iEWNS]	H ₂ O ₂ [mol/iEWNS]	AI [ng/iEWNS]
							H ₂ O ₂ equivalent units		
EWNS	-	N/A	−6.5	4	12.1± 0.1	13±0	1.03×10 ^{−15}	0	n.a.
h1EWNS	Hydrogen peroxide	1%	−6.8	4	11.9± 0.3	11±0	5.22×10 ^{−16}	5.50×10 ^{−16}	n.a.
L0.1EWNS	Lysozyme	0.1%	−6.8	4	13.1± 0.3	15±0	2.79×10 ^{−17}	9.85×10 ^{−16}	2.56×10 ^{−8}
c1EWNS	Citric acid	1%	−6.8	4	36± 2	23±2	3.77×10 ^{−17}	3.45×10 ^{−17}	4.14×10 ^{−7}
c1h1EWNS	Citric acid + Hydrogen peroxide	1% each	−6.8	4	48±3	77 ± 14	7.85×10 ^{−17}	9.37×10 ^{−17}	1.27×10 ^{−6}