Published in final edited form as:

Nanomedicine. 2014 August ; 10(6): 1175–1183. doi:10.1016/j.nano.2014.02.016.

Mycobacteria inactivation using Engineered Water Nanostructures (EWNS)

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Abstract

Airborne transmitted pathogens such as *Mycobacterium tuberculosis* (*Mtb*) cause serious, often fatal infectious disease with enormous global health implications. Due to their unique cell wall and slow growth, mycobacteria are among the most resilient microbial forms. Herein we evaluate the ability of an emerging, chemical-free, nanotechnology-based method to inactivate *M.*

parafortuitum (*Mtb* surrogate). This method is based on the transformation of atmospheric water vapor into engineered water nano-structures (EWNS) via electrospray. We demonstrate that the EWNS can interact with and inactivate airborne mycobacteria, reducing their concentration levels significantly. Additionally, EWNS can inactivate *M. parafortuitum* on surfaces eight times faster than the control. The mechanism of mycobacteria inactivation was also investigated in this study. It was demonstrated that the EWNS effectively deliver the reactive oxygen species, encapsulated during the electrospray process, to the bacteria oxidizing their cell membrane resulting into inactivation. Overall, this is a method with the potential to become an effective intervention technology in the battle against airborne infections.

From the Clinical Editor—This study demonstrates the feasibility of mycobacterium inactivation in airborne form or on contact surfaces using electrospray activated water nano-structures. Given that the method is free of toxic chemicals, this might become an important tool in the prevention of mycobacterial infections, which are notoriously hard to treat.

Keywords

Engineered water Nanostructures; Mycobacteria; Air disinfection; Nanoparticles; Lipid peroxidation

Despite the discovery of antibiotics, the development of vaccines and the important advances in sanitation and hygiene, infectious diseases continue to kill hundreds of millions of people each year. One of the most serious is tuberculosis (TB),¹ which remains second

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only to HIV as a infectious killer of adults worldwide.² In 2011, there were 8.7 million new cases worldwide, 13% co-infected with HIV and 1.4 million deaths. In 2012, despite great progress in controlling the disease, 9,951 new cases were recorded, largely among the foreign-born, reflecting the global epidemic.³ The toll of infectious diseases, including TB, is further complicated through the rapid evolution of antibiotic resistant bacteria. Cases of multidrug resistant (MDR)^{4,5} and extensive drug resistant (XDR) TB are steadily increasing making treatment very difficult.⁶ Tuberculosis is transmitted via the airborne route by inhalation of minute respiratory droplets of less than 5 μm exhaled by persons with pulmonary disease when coughing, sneezing, shouting or even singing.

Since TB is airborne transmitted and the probability of infection is related to the concentration of mycobacteria in the air, air disinfection is considered an effective means in minimizing the risk of transmission. Current air disinfection technologies, such as the use of upper-room UV irradiation (Ultraviolet 254 nm),⁷ high efficiency particulate (HEPA) air filtration⁸ and photocatalysis,⁹ are restricted by their inherent limitations. UV is associated with health risks,¹⁰ requires the upper room UV fixtures, and relies on a well-mixed air concept. Furthermore HEPA filters remove bacteria and viruses from the air effectively, but there are excessive costs associated with the energy needed to move high volumes of air through the filter and for filter replacement.¹¹ Recently, photocatalysis using UV-A has been used to disinfect surfaces.¹² However, this technology is limited to surfaces as nano aerosols cannot be used for airborne pathogen inactivation due to their possible toxicological effects when inhaled.^{13–15} The use of other disinfection methods based on the use of biocidal gases, such ethylene oxide,¹⁶ hydrogen peroxide,¹⁷ ozone,¹⁸ and chlorine dioxide,¹⁹ also has serious limitations associated with toxicity,²⁰ potential material damage, and, in some instances, downtime of the space or equipment being disinfected in hospital environments. Personal protective equipment (e.g., surgical masks for patients, and N-95 type respirators for healthcare workers), although commonly used to prevent infectious disease transmission, are not completely effective or practical in intervening in the transmission of airborne infectious diseases.²¹ While TB is considered an airborne transmitted disease, effective surface disinfection is important in specialized settings such as in the clinical laboratory working with *Mtb*, to prevent laboratory cross-contamination as *Mtb* is proven to survive in the environment for long periods of time.^{6,22} Surface disinfection usually involves the use of chlorine, 5% chloramine, ethyl and isopropyl alcohol and gaseous hydrogen peroxide and formaldehyde.²³

Recently, our group is experimenting with a novel, nanotechnology-based method for inactivating bacteria in the air and on surfaces/fomites. The method relies on generating engineered water nano-structures (EWNS) through electrospraying. The EWNS possess a unique set of physical and biological properties which we have fully characterized in previous publications.^{24,25} In brief, our studies revealed that EWNS have an average of 10 electrons per structure and have an average nanoscale size of 25 nm.²⁴ It was demonstrated that the electric charge increases the particle surface tension, thus reducing the evaporation rate and resulting to long lifetime (~hours) in indoor environmental conditions.²⁵ In addition, electron paramagnetic resonance (EPR) showed that the EWNS contain a large number of reactive oxygen species (ROS), primarily OH• and superoxides (Figure 1, C).²⁴

In our previous studies, the EWNS bactericidal potential was investigated using airborne *S. marcescens* and was demonstrated that the EWNS can interact with and significantly reduce the airborne bacteria under indoor environmental conditions, in indoor environmental conditions.²⁴ In other experiments, we have demonstrated that EWNS are equally effective against both gram-positive and gram negative bacteria on surfaces.²⁵ What is quite important, however, is that preliminary toxicological studies have shown that these water made nanostructures are toxicologically benign when inhaled at levels found to exhibit bacteriocidal properties.²⁴

Here we evaluate the potential of the EWNS to inactivate in air, a medically important and unique class of bacteria, the mycobacteria. Although surface transmission is not relevant to tuberculosis, there are other pathogenic members of the mycobacteria family that can be transmitted via surfaces, such *M. ulcerans*.²⁶ In addition, surface decontamination is important in the diagnostic and research laboratory working with *Mtb*.²³ It is therefore important to evaluate the ability of the EWNS to inactivate mycobacteria on surfaces as well. Finally, the mechanism of bacteria inactivation was also assessed.

Methods

EWNS synthesis and characterization

EWNS synthesis—The EWNS are synthesized via electrospray, a method used widely to aerosolize particles and fibers from liquid suspensions. Electrospray relies on a strong electric field to aerosolize a liquid, that is typically contained in a fine metal capillary.²⁷ The required strong electric field is generated by the application of a high voltage (most often negative voltage) between the metal capillary and a counter electrode positioned in a fixed distance from the capillary. The strong electric field causes the liquid to break into highly charged droplets.²⁷ This phenomenon, known widely as Rayleigh effect, states that a liquid droplet with high surface charge density is unstable. While the surface charge density increases, the distance among the charges becomes smaller which increases the electrostatic interactions. This results in a non-favorable energy increase of the system forcing the droplet to break into smaller droplets with smaller surface charge density (the overall surface area increases, while the charge remains the same). The only force that counters the breaking is the surface tension. There is a critical diameter, known as Rayleigh diameter, for which the surface tension is not enough to counter the electrostatic interactions and the droplets break into smaller droplets.²⁸

Figures 1, A and B, illustrate the electro-spray module used in this study and the process for the synthesis of EWNS respectively. In brief, a gold plated electrode is cooled down to 6 °C via a Peltier element. The atmospheric water vapor, condensed on the electrode, becomes the source of water for the electrospray. High voltage of approximately 5 kV is applied between the Peltier electrode and a grounded counter electrode causing the water to break into small droplets as it is described above.^{24,25,29} For this particular experimental setup, the operational environmental conditions were maintained at 20–25 °C and 45–55% Relative Humidity (RH).

EWNS characterization—In each experiment, the EWNS particle number concentration was measured with a Scanning Mobility Particle Sizer (SMPS, TSI, Shoreview, MN).

Test microorganism

For all experiments, *Mycobacterium parafortuitum* was used due to structural similarities with the *Mycobacterium tuberculosis*.³⁰ *M. parafortuitum* is an aerobic, non-motile, rod-shaped bacterium measuring 2–4 µm long and approximately 0.5–1 µm in diameter.³¹ It grows rapidly (~3 days) on standard bacterial culture medium and produces very characteristic smooth, pale yellow colonies that disperse readily in water. This bacterium has been used previously as a nonpathogenic surrogate for *Mtb* and other pathogenic mycobacteria in other bioaerosol and fomite studies.^{32–34}

Bacteria inactivation experiments

Airborne *M. parafortuitum* inactivation experiments—Figure S1 illustrates the experimental setup used in the airborne bacteria inactivation experiments. It contains the following components:

EWNS aerosol generation system: The EWNS aerosol was synthesized using an EWNS aerosol generator that was recently developed by our group (Figure S1). In brief, the generator contains twenty electrospray modules which can be operated in series. It is worth mentioning that the generator can produce variable concentrations of EWNS aerosol up to 500,000 #/cm³ at flows up to 15 lpm (liters per minute). It is also equipped with an ozone scrubbing system based on glass honeycomb denuders previously developed by the authors, in order to reduce ozone levels.³⁵ The construction and overall operation of the EWNS generator are described in great detail elsewhere.²⁴

Bioaerosol generation system: A standard CN-6 Colison nebulizer (BGI Inc., Waltham, MA) operated with HEPA-filtered dry air at 103 kPa (15 psi) was used to aerosolize a *M. parafortuitum* (ATCC # 19686) suspension. The concentration of the stock suspension was reduced to 10^{4.5} CFU/ml with PBS to produce bioaerosol concentrations of 5–7 CFU/l, which is adequately to obtain reliable air concentration data. In order to avoid large PBS droplets with suspended bacteria and bacteria clusters, the nebulizer output was mixed with an equal flow of dry air in a 20 l drum prior to delivery to the exposure chamber.

Airborne bacteria inactivation protocol: The aforementioned EWNS aerosol generator and *M. parafortuitum* bioaerosol generation system were connected to 1 m³ polyacrylic environmental chamber that was lined with grounded aluminum panels to minimize particle losses. The two aerosol streams are mixed in the environmental chamber, and the number of culturable airborne bacteria was monitored as a function of time. The mixing was facilitated with two mixing fans. The input and output airflows in the chamber were fully controlled to create a ventilation scenario of 3 Air Change per Hour (ACH). The experimental setup and the flows used in the experiment are shown in detail in Figure S1. Throughout all experiments, the temperature and RH in the chamber were monitored and maintained at 21–23 °C and 45–55%, respectively. A negative pressure of 0.2–0.3 inH₂O (49.768–74.652 Pa) was continuously maintained in the chamber in order to comply with biosafety requirements.

Ozone levels in the chamber were also monitored with the 205 Dual Beam Ozone Monitor™ (2B Technologies, Boulder, Co).

The above experimental setup was used to perform the following experiments:

Bioaerosol size distribution experiments: Since tuberculosis is primarily transmitted with droplets smaller than 5 μm ,³⁶ the bioaerosol size distribution was measured to evaluate the size distribution of the generated bioaerosol. The size distribution and the total concentration of the culturable bacteria in the environmental chamber were measured in the absence of the EWNS (all the flows were kept the same, as shown in Figure S1, but with the EWNS aerosol filtered with a HEPA filter). The *M. parafortuitum* aerosol size distribution was measured using the Six Stage Viable Sampler (BGI Inc., Waltham, MA), loaded with TSA plates and operated at 28.3 lpm flow rate. Prior to each air sample, a dummy plate was used for 30 s to void the dead space of the sampling line. The air sampling time was optimized at 3 minutes.

Statistical analysis: The obtained data were fit with a log normal distribution to estimate the mean, the mode and the standard deviation of the airborne bacteria size distribution. The parameter χ^2 was used to evaluate the quality of the fit. The curve fit was calculated with ProFit™ (QuantumSoft, Uetikon am See, Switzerland).

Steady State *M. parafortuitum* inactivation experiments: The inactivation of the airborne bacteria was investigated under steady state conditions in the environmental chamber, a study design routinely used in evaluation of intervention technologies. In particular, in health care facilities the ventilation rate varies in different areas between 1 and 6 Air Changes per Hour (ACH).³⁷

In this study three ACH was used as a ventilation rate while 21 °C and 55% relative humidity were maintained in the environmental chamber during the experiments. The aerosol flows in the chamber were maintained constant for the equivalent time to reach steady state conditions (equivalent time required for three air changes, 60 minutes). Three air samples were obtained at 20 minute intervals, using the N6 Single Stage Viable Impactor (BGI Inc. Waltham, MA) loaded with Tryptic Soy Agar (BD, Franklin Lakes, NJ) plates and operated at 28.3 lpm flow rate. Prior to each air sampling, a dummy plate was used for 30 s to void the dead space of the sampling line. The air sampling time was optimized at 1 minute. The cultures were incubated for 72 h at room temperature prior to colony counting. Positive hole corrections were applied to all plate counts.³⁸

As control experiments, EWNS were filtered out of the air using a HEPA filter, while all other procedures for sampling and culturing protocols were kept the same. Pre and post air samples were taken directly from the Colison jar to ensure that the *M. parafortuitum* stock solution was viable throughout the experiments.

Statistical analysis: All experiments were done in triplicate. The standard deviation of the number concentration was used as the measurement error. At steady state, the data were compared based on the Bacteria Viability (BV):

$$BV = \frac{C_{EWNS}}{C_{Control}} \quad (1)$$

Where C_{EWNS} is the number concentration of the bacteria in air during the EWNS exposure and $C_{Control}$ the number concentration of the bacteria in air during the control experiment at the same time point. In addition the removal rate (RR) was calculated as:

$$RR = \frac{C_{Control} - C_{EWNS}}{C_{Control}} \quad (2)$$

ANOVA was used to analyze the data and the value P -value was used to determine the statistical significance of the observed differences. The P -value was calculated with Mathematica™ (Wolfram Research, Champaign, IL).

Bacteria inactivation experiments on surfaces—Although there is little relevance for transmission of tuberculosis via fomites, it is still important to evaluate the effect of EWNS on surface mycobacteria, as this is relevant to the clinical and research laboratory where the risk of cross-contamination exists. Furthermore the mycobacteria family contains also pathogenic environmental mycobacteria such a *M. ulcerans* that are transmitted via surfaces.²⁶

Exposure system: Figure S2 illustrates the experimental setup used in the surface bacteria inactivation experiments. A 45 l rectangular chamber with one side open was used to expose the bacteria to the EWNS aerosol generated by 4 electrospray modules. The modules were monitored with a digital data logger ensuring proper function of electrospray process during the experiments.²⁵ The chamber humidity was maintained at approximately 50% with filtered air that had been humidified by passing through a bubbler with deionized water. The mycobacteria-inoculated surfaces were placed at a distance of 5 cm from the modules.

Experimental protocol: For all the surface *M. parafortuitum* inactivation experiments, an in-house developed, optimized, and well-documented protocol was followed.^{25,39} Due to the relevance to nosocomial/lab environments, stainless steel (304 Steel, 2B finish) was selected as a representative test surface. Stainless steel coupons (size 2.5×7.5 cm) were used in all the surface experiments.

Prior to each experiment, the coupons were cleaned with soap and water. Then they were placed in 50 ml centrifuged tubes, covered in a solution of water and labware grade detergent (5% v/v) and sonicated for 3 h to remove any residual organic matter from previous experiments. The coupons were then rinsed sequentially with water, acetone, ethanol, and a copious amount of de-ionized water. These steps are essential as they guarantee the proper spreading of the bacteria solution on the stainless steel surfaces. Prior to each use, the coupons were autoclaved. In total, 7 coupons were used for each experiment.

A vial of 1 ml stock solution of *M. parafortuitum* of initial concentration 10^8 CFU/ml was thawed, and the concentration was adjusted to approximately 10^6 CFU/ml with PBS. One hundred microliters of the *M. parafortuitum* solution was spread on each coupon in a circular shape of approximately 2 cm diameter. The coupons were then left in a laminar flow hood to dry (approximately 45 minutes). One coupon was used as a control for time $t = 0$ min, three coupons were exposed to the EWNS for 30, 60 and 90 minutes, and the remaining three were left in the laminar flow hood for the same amounts of time as controls. The environmental conditions for both exposed and control coupons were kept the same at 50% RH and 21 °C. Immediately following exposure, each coupon was recovered and placed in 50 ml centrifuge tubes and rinsed 20 times repeatedly with 1 ml PBS.

The rinsate was then serially diluted to yield a target concentration of approximately 100 colonies per plate. All dilutions were then spread on TSA and were incubated at room temperature for 72 h before colonies were counted.

Statistical analysis: All the experiments were done in triplicates and the standard deviation of the concentration was used as the measurement error. Bacteria log-reductions were computed for a given condition according to:

$$\text{Log Reduction} = \log_{10} \left(\frac{C_n}{C_0} \right) \quad (3)$$

Where C_0 is the bacteria concentration of the control coupon at time 0 (i.e. after coupon was dried, but before placed into a chamber) and C_n is the bacteria concentration of the coupon after n minutes of exposure. To calculate the removal rate, the data were fit with an exponential decay equation:

$$\frac{C(t)}{C_0} = e^{-t/\tau} \quad (4)$$

Where the C_0 and $C(t)$ are the bacteria number concentration at time 0 and time t respectively and the τ is the time required to remove 63.21% of the bacteria. This was further converted to Log Removal Rate (removal rate in terms of Logs/hr.):

$$\text{Log Removal Rate (LRR)} [\text{Logs/hr}] = 0.039 \tau \quad (5)$$

The curve fit was calculated with ProFit™ (QuantumSoft, Uetikon am See, Switzerland). The parameter χ^2 was used to evaluate the quality of the fit.

Investigation of the inactivation mechanism—In previous publications we have shown that the EWNS contain a number of ROS.²⁴ Furthermore, transmission electron microscope images of the exposed bacteria clearly showed membrane damage that was attributed to the ROS.²⁵ Same was also demonstrated quantitatively using a membrane permeability assay.²⁴ In this study, the hypothesis that the primary mechanism of destruction

of the membrane of the EWNS exposed bacteria is the lipid peroxidation due to the delivery of ROS by the nanostructures, was assessed.

A Lipid Hydroperoxide assay (LPO, Item Number 705002, Cayman Chemicals, Ann Arbor, MI) was used to assess the oxidation of the lipids in bacteria exposed to EWNS. The Hydroperoxide assay was performed according to the supplier's protocol and the adsorption was measured with the Beckman DU-640 UV/VIS spectrophotometer (Beckman, Miami, FL). Control experiments were performed with a) *M. parafortuitum* exposed to room air only, b) *M. parafortuitum* treated with vitamin-C (ascorbic acid, VWR, 341-5343) and exposed to EWNS, c) *M. parafortuitum* treated with vitamin-C and exposed to room air, and d) *M. parafortuitum* exposed to 650 ppb of ozone. The vitamin-C was added and readily dissolved directly to the bacteria stock solution to give a final concentration of 1 mM.⁴⁰

A large concentration of bacteria ($\sim 10^8$ CFU/ml) is required for the successful execution of the Lipid Hydroperoxide assay. Hence, we restricted experiments to the protocol for the surface inactivation, which allows exposure and collection of large numbers of bacteria. The assay signal was further increased by loading each coupon with 500 μ l of *M. parafortuitum* solution of 10^8 CFU/ml. The preparation of the stainless steel coupons was done according to the aforementioned protocol. The bacteria solution was spread to cover the entire area of the coupon and left for 2 h in a laminar flow hood to dry. The coupons were then exposed to 8 EWNS modules for 90 minutes. The SMPS was used to monitor the number of particles and the Ozone Monitor to measure the ozone levels. The ozone control experiments were done based on these conditions. Similarly prepared surfaces were left in the biosafety hood and used as controls. The bacteria were recovered from the coupons using the same protocol described earlier. In order to increase the concentration of the bacteria in the solution, the solutions were centrifuged at 20,000 rpm for 15 minutes. The supernatant was removed and the bacteria were re-suspended in 200 μ l of deionized water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$). The samples were then flash frozen with liquid nitrogen until the assay execution. The experiment was done in triplicate and the results were analyzed with ANOVA. The *P*-value was used as a quality control.

Results

Airborne bacteria inactivation

Bacteria airborne size distribution—Figure 2, A, shows the airborne culturable bacteria size distribution and concentration in the environmental chamber. The mean aerodynamic diameter was 1.56 μ m and the airborne concentration was 4.80 ± 0.75 CFU/l. The geometric standard deviation of the distribution is 1.42. At the same conditions, the EWNS number concentration in the chamber was stable at approximately 36,000 $\#/\text{cm}^3$.

Airborne mycobacteria inactivation—Figure 2, B, shows the airborne mycobacteria inactivation results. At the steady state condition (after 3 AC, 60 minutes) EWNS reduced the airborne bacteria concentration to 36% viability of the control concentration (*P*-value = 0.0046), which translates to a removal rate of 63%. The same was observed for the intermediate samples: at 20 minutes the viability is 38% (*P*-value = 0.0029) with removal rate 61% and at 40 minutes it is 31% (*P*-value = 0.0029) with removal rate 68%.

Bacteria inactivation on surfaces

Figure 3 shows the results for the bacteria inactivation on surfaces for two different EWNS exposure levels. Figure 3, A, shows the result for the case of 9,000 #/cm³ EWNS particle concentration level. After 90 min exposure, a 1-log removal was observed with a removal rate of 0.63 Logs/hr. Figure 3, B, shows the results for the case of 32,000 #/cm³ EWNS particle concentration level. The inactivation rate was found higher at 0.83 Logs/hr. The control (no EWNS) showed a natural inactivation of 0.097 Logs/hr. Figure 3, C, summarizes surface inactivation results in terms of survival ratio and removal rate at the end of the exposure as a function of the EWNS particle number concentration level.

Investigation of the inactivation mechanism

Figure 4 shows the data for the peroxidation assay. When mycobacteria are exposed to EWNS aerosol (98,000 #/cm³), there is significant higher lipid peroxidation as compared to the unexposed bacteria (P -value = 0.003, compared to the bacteria exposed to room air). In addition, bacteria treated with vitamin-C prior to their EWNS exposure demonstrated significantly reduced levels of peroxidation and at similar levels seen in the bacteria exposed to room air (P -value = 0.397, compared to the bacteria exposed to room air). Moreover, exposure to ozone alone (650 ppb), at the same levels as the ones occurred during the EWNS exposure experiments, showed no increase in peroxidation with values equivalent to the controls (no EWNS exposures) (P -value = 0.420, compared to the bacteria exposed to room air).

Discussion

Here we evaluated the ability of a novel, nanotechnology-based method, to inactivate *M. parafortuitum*, a non-pathogenic surrogate for *Mtb*, in air and on surfaces using EWNS nanoparticles.

In the airborne *M. parafortuitum* surrogate inactivation experiments, important parameters, such as the bioaerosol size distribution and the bacteria concentration in terms of CFU/l, were characterized. The airborne bacteria mean aerodynamic diameter was found to be approximately 1.56 μ m. This is in accordance with actual dimensions of the airborne bacterium.³¹ It is worth mentioning that this size is in the range that is highly infective by inhalation.³⁶ Furthermore, these size distribution data indicate that the developed bacteria generation/exposure system results in airborne bacteria which consist primarily of single bacteria without large PBS droplets or bacteria clusters.

The airborne bacteria inactivation data indicate that the EWNS reduced the culturable airborne bacteria concentration levels in the chamber, at steady state conditions, by 63% as compared to the control experiments (without EWNS). Reducing the concentration of the airborne bacteria can significantly impact the probability of infection and proves the potential of the proposed intervention technology as an effective approach in the battle against the spread of disease.

It is worth noting that the 63% reduction in the airborne bacteria levels observed are a result of maintaining EWNS concentration levels of 36,000#/cm³, in addition to the 3 ACH

ventilation. The airborne bacteria reduction can be probably further increased by increasing the EWNS concentration in the chamber. In previously published work we investigated the effect of the interaction time and EWNS concentration on the bacterial removal.²⁴ It was shown that the potential of EWNS to remove bacteria depends on both the relative ratio of EWNS to bacteria concentration (referenced as r_b) and the residence time in the chamber, determined by the ventilation rate (ACH). In this recently published paper, two different exchange rates (1.7 ACH and 3 ACH) and two different aerosol particle number concentrations (25,000 #/cm³ and 36,000 #/cm³) were used and the airborne bacteria concentration as a function of time was measured. It was observed that, for both longer interaction times and higher particle concentrations, the inactivation rate increased not in a linearly manner though, as expected.

Nevertheless, while this is not an intervention technology meant to sterilize the room air, its advantages compared to upper room UV disinfection, HEPA filtration and biocidal gas vapors make it a powerful tool in the battle against tuberculosis. Upper room UV requires upper room installation of UV fixtures, and relies on a well-mixed air concept, HEPA filtration requires moving the air, and it is associated with high energy and maintenance costs while biocidal gases also have serious limitations associated with toxicity, potential material damage, and downtime of the space or equipment being disinfected. On the other hand, the EWNS intervention method is a chemical-free technology that consumes only a small amount of power (5w per module) and can be used in the presence of people without requiring to move large volumes of air.

Moreover as shown in an animal inhalation toxicological study, EWNS exposures caused no impact on the lungs of acutely exposed animals under concentration levels up to 60,000 #/cm³ (concentrations higher than the 36,000 #/cc levels used in this study for the inactivation experiments).²⁴ The measured biochemical and cellular parameters (both from the nasal and bronchoalveolar region) indicated that a 4-hour exposure to EWNS, did not produce respiratory tract toxicity (the most likely location for an acute response to occur). It should be mentioned that the EWNS concentration levels and lengths of time of the inhalation study were significantly higher than those used here for the bacteria inactivation. It is safe therefore to conclude that the EWNS at this concentration have no acute deleterious effect on the respiratory system (nasal and bronchoalveolar). These results seem to contradict reports for the ROS-mediated toxic effects of other engineered nanomaterials. Solid nanoparticles have the ability to diffuse into the alveolar fluid and being internalized by epithelial cells and macrophages, generating ROS that may cause DNA or membrane damage. On the contrary, when EWNS interact with the alveolar or airway fluid, ROS content gets neutralized by organic molecules before they come in contact with epithelial cells, located beneath the lining fluid. Hence, although the EWNS contain ROS, they cannot cause any lung injury or inflammation. While the acute inhalation study illustrated their biological inert nature, a chronic inhalation study is needed in order to address potential toxicological outcomes from chronic exposures.

This finding confers significant advantage of the method to other traditional intervention methods using gaseous disinfectants, such as ethylene oxide,¹⁶ hydrogen peroxide,¹⁷ ozone,¹⁸ and chlorine dioxide,¹⁹ that have been linked to significant health implications.

Furthermore, this chemical free novel nanotechnology approach is of very low cost requiring only a very low energy consumption.

It is also evident that the EWNS are capable of inactivating *M. parafortuitum* on surfaces. In the presence of EWNS aerosol at the level of 9000 #/cm³, the bacteria removal rate is 0.63 Logs/hr, which is seven times higher than the control conditions (room air conditions, no EWNS). Furthermore, it is demonstrated that at higher EWNS levels (32,000 #/cm³), the inactivation was further improved, resulting in the removal rate of 0.83 Logs/hr, 10 times higher than the control.

It should be mentioned that the removal of the mycobacteria on the surface is a time depended process. EWNS are being delivered to bacteria present on surfaces due to Brownian motion²⁵ (estimated time required for the EWNS to reach the stainless steel by diffusion is 5 minutes). Increasing therefore the interaction time increases the delivered EWNS dose. However, it is observed that the increase in removal does not follow a linear relationship with the aerosol particle number concentration over the stainless steel coupons. This is attributed to the fact that the number concentration of the EWNS above the coupons, used as an exposure dose metric here, does not reflect the actual EWNS delivered to bacteria (delivered dose). The fact that there are more EWNS available in the air does not linearly translate to higher EWNS delivered to bacteria and removal rates.

Although fomite mediated transmission is not common for tuberculosis, good infection control attempts to keep surfaces in hospitals, clinics and medical laboratories free from bacteria is important. It is worth mentioning here that mycobacteria can survive for months on surfaces.⁴¹ In addition, there are other pathogenic environmental mycobacteria species, like *M. ulcerans*²⁴ and *M. fortuitum*⁴² that can be transmitted via contaminated surfaces and become a burden in wound infections.

Despite the general resilience of mycobacteria to disinfecting agents,⁴³ the results in this study show that the EWNS are effective in inactivating them, overcoming their structural mechanisms of resistance. It is well known, that in contrast with gram-negative bacteria, all *Mycobacterium* species share a characteristic cell wall, unique among bacterial species, consisting mainly of complex lipids, and therefore is hydrophobic and waxy.³⁰ This thick and waxy nature of its cell wall contributes to its survivability⁴¹ and is responsible for the resistance to many commercial disinfectants and inactivation methods.⁴³

The results from the lipid peroxidation assay (Figure 4) clearly confirm that the primary inactivation mechanism is the delivery of ROS to bacteria by EWNS nanoaerosol. The EWNS very effectively protect the otherwise short lived ROS and deliver them due to their highly mobile nature to the bacteria, causing significant lipid peroxidation and destruction of the cell membrane. This peroxidation effect disappeared, when the vitamin-C, a well-known antioxidant that prohibits ROS mediated lipid peroxidation,⁴⁰ is added to the bacteria prior to their exposure. It is important to note that the vitamin-C alone does not seem to have any positive effect to the bacteria since the room air exposed vitamin-C treated bacteria do not show statistically significant peroxidation compared to the control bacteria. These data are in agreement with previous published by the authors work for other bacteria, demonstrating

that the presence of EWNS results in the destruction of the bacteria cell membrane.^{24,25} Collectively, these data conclude that the ROS account for one of the dominant pathways of inactivation of bacteria exposed to EWNS.

The electrospray-generated EWNS were found effective in inactivating *M. parafortuitum*, an *Mtb* surrogate. This is a promising finding in the fight against the tuberculosis epidemic, offering a cost effective intervention approach, which can be implemented in a variety of indoor environmental settings thus reducing the risk of transmission. Advantages of the proposed control technology include the ease of use, the low cost, and the low energy consumption. Overall, this is a chemical-free, sustainable, and environmentally friendly technology that has the potential to reduce the risk of transmission of diseases, such as tuberculosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to acknowledge Panasonic Ltd. for supplying the electrospray modules, the Center for Nanotechnology and Nanotoxicology at the Harvard School of Public health and the USDA/NIFA (Project Number MASW-2013-01614) for the financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2014.02.016>.

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Figure 1.

The EWNS synthesis and concept. **(A)** The utilized electrospray module with the Peltier cooled electrode, used to condense atmospheric humidity. **(B)** The applied high voltage results in atomizing the condensed water and the Rayleigh effect reduces the size down to the nanoscale level. **(C)** The formed EWNS have a unique structure: an electron rich water shell and contain a number of ROS generated during the electrospray process. **(D)** EWNS due to their nanoscale nature are highly mobile and can interact with airborne pathogens.

Figure 2.

Airborne bacteria inactivation results. **(A)** The culturable airborne bacteria size distribution and concentration. **(B)** Airborne bacteria inactivation after each air change and at steady state conditions. The symbols *, ** and *** denote statistical significant values (P -value < 0.005).

Figure 3.

The surface inactivation results. **(A)** The results for the bacteria exposed to 1 module (9,000 #/cm³) and **(B)** the results for the bacteria exposed to 4 modules (32,000 #/cm³). **(C)** Summary of the results showing the bacteria removal as a function of the EWNS number.

Figure 4.

The hydroperoxide assay results for the various exposures and controls. The EWNS-exposed bacteria appear to have significantly more peroxidation as compared to the bacteria that were exposed to room air. When vitamin-C (ROS scavenger) is added, the lipid peroxidation is reduced to levels comparable to the control (room-air exposed bacteria). The ozone control experiment showed that the ozone levels are not enough to induce any peroxidation. The symbols *, ** denote statistical significant values (P -value < 0.005).