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Inactivation of Pure Bacterial Biofilms by Impaction of Aerosolized Consumer Products Containing Nanoparticulate Metals

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Abstract

The ability of nanotechnology-enabled consumer sprays to inactivate bacteria has direct health implications. This research investigated the ability of six nanosilver-based consumer sprays to inactivate bacteria. We determined the minimal inhibitory concentrations (MICs) of the products by an agar dilution method, collected particles released from sprays onto bacterial films using impactors, and determined metal concentrations in the products using ICPMS. Also, the size of silver nanoparticles in the products' suspensions was determined using single particle (sp)ICPMS. Two of the six nanoproducts inhibited growth of *Escherichia coli* and *Bacillus atrophaeus* bacteria (MICs of 40,000 and 160,000 ppm). Collection of particles aerosolized from these two products onto films of the same bacteria inhibited bacterial growth; however, the mass concentration deposited onto bacterial films was lower than the MICs. Furthermore, these two nanoproducts had the lowest silver concentrations compared to the other four nanosilver products. Yet, they had the smallest nanosilver particles: mean size of ~20 to 30 nm vs. ~45 nm for the other products. Their suspensions were more acidic (pH ~3–5) and had higher concentrations of zinc and magnesium compared to other products. This research illustrates that some consumer nanoproducts have antibacterial potential and may affect our microbiota. Yet, the inactivation potential cannot solely be presumed based on the nanosilver presence and concentration in the product; the final nanoproduct's form, including its matrix, must be considered. As nanomaterials are increasingly incorporated into consumer goods, this research highlights the need to investigate final-form consumer nanoproducts and their potential to affect our microbial environment.

Graphical Abstract

Nanotechnology-enabled consumer sprays with Ag may have antibacterial potential, but it is not based solely on nano-silver presence and concentration.

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Introduction

Nanotechnology is being increasingly incorporated into everyday consumer products ¹⁻⁴. The Nanotechnology Consumer Products Inventory (CPI) was developed by the Woodrow Wilson International Center for Scholars and the Project on Emerging Nanotechnologies as the first crowdsourced, online inventory of nanotechnology-based consumer products ⁵. According to their most recent data, there have been 1814 consumer products added to the inventory between 2005–2014 under the guidance of inclusion criteria developed by subject matter experts ^{2, 5}. This number is likely higher because this statistic does not include unidentified nanoproducts that have been introduced into the market but not added to the database because product labeling of nanomaterials has not been required in the past; only in 2013, ISO published a document to guide manufacturers on voluntary labeling of consumer nanoproducts ^{6, 7}. However, even for consumer products reported in the CPI, only ~49% (889/1814) provide composition of nanomaterials used, and most products (71%, 1288/1814) do not present enough information to support manufacturer claims that nanomaterials are actually included ⁵.

Because the use and application of nanotechnology-enabled consumer products result in the release of nanoparticles into the environment ^{8, 9}, there is concern that the introduction of nanomaterials into different environmental media is inevitable leading to unanticipated consequences for ecological and human health ^{10, 11}. One of these concerns is potential effects of engineered nanomaterials on environmental microorganisms. So far, most nanotoxicity research in the ecological fields has focused on investigating the effects of pristine nanoparticles on common soil, aquatic and wastewater treatment system microorganisms ^{10, 12}. Research suggests that nanoparticle and nanomaterial concentrations that have been detected in different water and soil systems (e.g., 0.01 to 1 mg-Ag/L in water and 5 to 50 mg-C₆₀ fullerenes/kg in dry soil) may elicit changes in typical microbial community profiles ¹³⁻²¹. Effects of nanoparticles on microbes in the airborne state have also been investigated; Yoon, *et al.* (2008) found up to 76% inactivation of airborne *Bacillus subtilis* when it was mixed with aerosolized, pure water-based colloidal nanosilver in a test chamber for 15 seconds and then sampled onto nutrient agar plates with an impactor ^{22, 23}.

The potential for nanomaterials to influence microbial ecology by inactivating certain microorganisms has direct implications for the health of the people who use these nanoproducts and the environmental composition of microbial flora. For example, rich microbial diversity during early childhood appears to have a protective role against asthma,

atopy and obesity through shaping of the innate immune response^{24–26}. Given the ubiquitous presence of microorganisms in our environment and the increasing use of consumer nanoproducts, we hypothesize that consumer nanoproducts could have significant environmental impacts on microbial ecology and, therefore, human health.

In order to identify and understand how nanomaterials may impact microbial ecology, it is necessary to complement research on isolated, pristine nanoparticle-microbe interactions with studies investigating actual final-form consumer nanoproducts that are used in daily lives. Final forms of commercial nanoproducts are typically complex, heterogeneous suspensions; a research framework using these real products will help to understand realistic exposure scenarios that are already present in the public^{6, 27}. Additionally, there are often unwanted, residual manufacturing components used in the synthesis and purification of nanoparticles that are seldom removed with 100% efficiency from the final product²⁸. The inactivation of microorganisms has been shown to be affected by the size, shape, chemical composition and surface charge of nanoparticles^{29–31}, all of which may be affected by how the product is formulated and manufactured⁶.

In addition, the total product matrix and the mode of applying and using the product are expected to affect the ways in which exposures to these nanomaterials occur³². A subset of 771 of the CPI identified consumer products were evaluated for their expected routes of exposure; while the most common route of exposure to the consumer products is expected to be dermal, about 25% of these evaluated products (~200) contain nanomaterials that may be inhaled during use, such as personal care/cosmetic powders, mists and sprays⁵. Research indicates that the use of nanospray products results in aerosolization of particles ranging in size from nano to super coarse particles with inhalation resulting in particle deposition throughout all sections of the respiratory tract^{33–35}. Thus, application of consumer products through spraying/misting is common and has to be taken into account when considering environmental and health effects of using such products.

Therefore, not only may sprayed and eventually deposited nanoproducts influence microbial communities external to the product user (both airborne and surface-borne microbes), inhaled nanoparticles/nano-agglomerates released from these products during their use may impact microflora inside the human body. At least a portion of particles in the upper airways is cleared through the mucociliary elevator and then travels into the gastrointestinal tract where they can interact with the gut microflora that provides important human ecosystem services, such as nutrient metabolism³⁷. Nanoparticles deposited in the lower airways may interact and change the composition of bacteria there leading to potential health effects; for example, evidence suggests that differences in the types of bacteria comprising the lung microbiome of asthmatics versus non-asthmatics may contribute to asthma severity and medication response³⁸.

Given the concerns described above for consumer nanoproduct effects on microbial ecology, the main goal of this research was to investigate inactivation of common environmental and health-related bacteria by six nanotechnology-enabled consumer spray products. Our hypothesis was that released product particles and product matrix will inactivate bacteria, and the efficiency of such inactivation will depend upon each nanoproduct's unique

aerosolized particle size distribution and chemical composition. The focus of this paper is on silver-based products since silver compounds have been commonly used as antimicrobial agents, and silver is the most commonly reported type of nanomaterial in consumer products^{5, 23}. Also, as mentioned above, it was found that aerosolized, pure water-based colloidal nanosilver inactivated environmentally relevant bacteria²². To the best of our knowledge, this is the first research to investigate how actual commercially-available nanoproducts may impact user's microbial microenvironments. This research is the first stage of a larger project investigating how the increasing presence of nanotechnology-enabled consumer products may be impacting the environment and human health by influencing microbial ecology and flora.

Materials and Methods

Selection of Consumer Nanospray Products

Commercially available nanospray products were chosen from the CPI which is continuously updated via crowdsourcing through a registered online community of users^{2, 5}. Nanoproducts chosen for inclusion in this study had to satisfy three criteria. 1) The product had to be advertised as silver-based. Silver is the most common type of nanoparticles used in consumer products, and it has widely reported antimicrobial features^{5, 23}. 2) The product had to have intended application by spraying. The focus was on spray products because it is a major consumer product category², and previous research indicates the potential for these products to become widely dispersed across air and surfaces with a wide range of resultant particle sizes and properties. 3) The product had to be obtainable through consumer purchase, either online or in-store. The final six nanoproducts were also chosen to represent different categories of use, like cleaning products, cosmetics, and alternative medicine supplements. All selected products are part of our larger study investigating potential consumer exposures to silver and zinc based nanotechnology-enabled consumer products³⁹. As this research is the first step investigating microbial impacts of nanotechnology-enabled consumer products, we did not include all of the consumer nanoproducts we have collected over the course of this larger research project. Table 1 describes the six nanospray products chosen for this study. Hence, the nanoproduct codes are not in a consecutive order for this paper but reflect our total collection of nanoproducts under study for various projects³⁹.

Summary of Investigated Nanoproduct Features

This research investigated the bacterial inactivation efficiency of six silver-based consumer nanospray products. This research provides the first investigation into the potential for consumer nanoproducts to affect our personal and environmental microflora. In order to understand why a given product did or did not cause inactivation of bacteria, this research investigated not only the inactivation efficiency of the selected products but also the following features of the nanoproducts: 1) their minimal inhibitory concentrations (MICs), 2) inactivation of bacteria by airborne nanoproduct agglomerates, 3) nanoproducts' metal concentrations, 4) dissolved versus nanosilver content in the nanoproducts' liquid suspensions, and 5) size of silver particles within the products' suspensions.

Minimal Inhibitory Concentration (MIC) Tests

The agar dilution method was used to determine the MIC of each nanoproduct. The MIC test is the most commonly used standard method to determine the susceptibility of bacteria to antimicrobial agents. The agar dilution MIC method has been previously used to investigate antibacterial properties for different substances besides traditional antimicrobial agents, including essential oils⁴⁰ and other plant extracts⁴¹. The agar dilution method allows for standardization and direct comparisons of antimicrobial efficacies across different types of substances. Wiegand, *et al.* (2008) provides a detailed explanation of the protocol. Briefly, Tryptic Soy Agar (TSA) (Thermo Scientific, R455002, Waltham, MA) was prepared and mixed with each nanoproduct suspension in its received form in doubling amounts from 20,000 to 320,000 ppm of nanoproduct suspension to agar. The maximum tested ratio of nanoproduct liquid to agar was limited to 320,000 ppm because any ratio exceeding 32% kept the agar from properly solidifying. Agar plates were then poured to have a final volume of 20 mL per plate and left to solidify and dry overnight in a biological safety cabinet (Nuair, Class 2, Type A2, Plymouth, MN) at room temperature.

Two bacterial species were used throughout this study: *Bacillus atrophaeus* (ATCC 49337, Manassas, VA), a hardy, gram-positive bacterium representative of environmental species⁴³, and *Escherichia coli* (ATCC, 15597), a sensitive, gram-negative bacterium representative of a species endogenous to the internal flora of the gut⁴⁴, and prepared according to established protocols⁴⁵. Briefly, active *B. atrophaeus* cultures were inoculated in Nutrient Broth (Thermo Scientific, CM0001B) and incubated at 30°C for 18 h; active *E. coli* cultures were inoculated in liquid Trypticase Soy Broth (Thermo Scientific, R455052) and incubated at 37°C for 18 h. Cells were harvested from microbial suspensions by centrifugation for five minutes at 7000 rpm (BR-4 centrifuge, Jouan, DEC Inc., Lorton, VA). Cells were then washed three times by resuspending pellets in sterile deionized water and repeating centrifugation.

Bacterial suspensions were adjusted visually against a 0.5 McFarland Standard (Thermo Scientific R20421) to achieve a cell concentration of approximately 1.5×10^8 CFU/mL and then diluted ten-fold. Bacterial suspensions were then plated in triplicate across all of the MIC plates (plates with different investigated concentrations) using a Boekel 96-pin replicator (Boekel Industries 140500, Feasterville-Trevose, PA) to achieve about 10^4 CFU/inoculation spot. The latter was confirmed by spread plating serial dilutions of the inoculation suspensions on agar plates: the inoculum used in the MIC tests contained 4×10^4 CFU/spot and 5×10^4 CFU/spot for *E. coli* and *B. atrophaeus*, respectively. MIC plates were then incubated at 37°C (*E. coli*) or 30°C (*B. atrophaeus*) for 20 hours, and the MICs of each nanoproduct were determined as the lowest nanoproduct/agar ratio for which no visible colony growth could be seen.

Inactivation of Bacteria by Aerosolized Nanoparticle Agglomerates

Evidence suggests that the physical and chemical properties of product particles may change after their aerosolization⁴⁶; for example, once spray droplets from nanoparticle agglomerates containing a high amount of propellant or carrier fluid are released, droplets transition to smaller sized particles as the product matrix evaporates⁴⁷. Accordingly, this stage of experiments

investigated bacterial inactivation caused by nanoparticle particles and their agglomerates after desiccating the initial spray to remove volatile compounds. Figure 1 illustrates the experimental setup.

The acquired spray products came with their own sprayers. However, using those hand-activated sprayers for aerosolization was not practical because of the time involved and the fluctuation in the produced aerosol concentration. To standardize the aerosolization process across the different products and to produce a steady and repeatable particle size distribution, nanoparticle products and controls (summarized below) were aerosolized using a 3-jet Collison nebulizer (Mesa Labs, Butler, NJ). The Collison was operated with an air flow rate of 5 L/min, 20 psig operating pressure, and a suspension volume of 25 mL.

Aerosolized particles entered the 85 cm long, 8 cm wide stainless steel test chamber through conductive copper inlets to reduce particle loss due to electrostatic effects. Dry, filtered dilution air of 160 L/min was introduced simultaneously and carried the produced particles toward the end of test chamber, where particle number size distributions were measured isokinetically through conductive probes with a combination of instruments: Scanning Mobility Particle Sizer (SMPS 3080) with Differential Mobility Analyzer (DMA 3081), Aerodynamic Particle Sizer (APS 3321) and Condensation Particle Counter (CPC 3786) (all instruments from TSI Inc., Shoreview, MN); these instruments used in combination provided a particle electrical mobility/aerodynamic size range of 14 to 20,000 nm. The dry, filtered dilution air flow rate and the particle residence time in the desiccation chamber were sufficient to remove the aqueous and volatile components of the aerosolized particles. This was verified by aerosolizing autoclaved Milli-Q water (EMD Millipore, Billerica, MA) and verifying that no particles were detected at the chamber outlet. Between every tested nanoparticle product, the nebulizer was rinsed with 70% ethanol and autoclaved Milli-Q water. Then, Milli-Q water was aerosolized by the Collison nebulizer, and the aerosol instrumentation was used to ensure that no particles could be detected in the test chamber while Milli-Q water was aerosolized.

The resulting aerosol particles were sampled from the chamber outlet onto agar plates covered with spread plated bacterial film using a BioStage impactor (SKC Inc.), a single-stage Andersen N6-equivalent⁴⁸ (Figure 1). The sampling time was 1 min, and the sampling flow rate was 28.3 L/min. In a separate set of experiments described below, a 6-Stage Viable Impactor (Tisch Environmental Inc., Cleves, OH) was used to sample aerosolized spray products from the chamber. Since the air velocity in the chamber was 0.55 m/s and a particle size range of the aerosolized product was from 0.65 μm (cut-off diameter of the impactor) to $\sim 10 \mu\text{m}$, the still air sampling criteria were assumed⁴⁹. The sampling probe diameters used for both particle number size distribution measurements and impactor sampling (1 cm and 0.5 cm, respectively) satisfied the still air sampling criteria for unbiased sampling that may otherwise be caused by particle settling and inertia^{50–52}. Due to the relatively small size of the chamber, the BioStage impactor was operated with its calibration adapter, and the sample was drawn via the adapter. *E. coli* or *B. atrophaeus* bacteria were spread plated as a film onto TSA agar plates using 100 μL of prepared bacteria suspension to achieve $\sim 10^8$ to 10^9 CFU total per plate; this allowed for the formation of a uniform film of bacteria across the entire plate. Thirty-five mL of agar was used in the plates as this has been shown to

provide the highest collection efficiency of sampled particles while minimizing impaction stress to microorganisms⁴⁵. Films were spread plated onto the agar immediately prior to loading new plates into the BioStage. At least three repeats were performed for each combination of aerosol particles (i.e., nanoparticle and control) and bacterium.

It was not practically feasible to randomize every repeat for nanoparticle/control type due to the necessary cleaning of the nebulizer. Therefore, the overall tested order of nanoparticles and controls was randomized, and all of the repeats for a given nanoparticle or control were completed with a randomized order of *E. coli* and *B. atrophaceus* plates. Table 2 describes the four types of controls that were used.

After impactor sampling, agar plates were incubated for 24 hours at 30°C (*B. atrophaceus*) or 37°C (*E. coli*), and inactivation was determined by observing holes (voids) in the bacterial film corresponding to the impactor jet spots. For any nanoparticle causing bacterial inactivation, the experiment was repeated in the exact same manner using a 6-Stage Viable Impactor (Tisch Environmental Inc., Cleves, OH) to investigate the influence of sampled particle size on inactivation of bacteria. The 6-Stage Viable Impactor was also operated at 28.3 L/min, and sampling was also performed through its calibration adaptor for 1 min.

Chemical and Size Characterization of Nanospray Products

The total metals (dissolved + particulate) present in the consumer nanoparticles were determined using inductively coupled plasma mass spectrometry (ICPMS). For digestion, 1.0 mL of the nanoparticle was added to 1.0 mL nitric acid (TraceMetal™ Grade) (Fisher Chemical, Pittsburgh, PA). In a pressurized TFM-PTFE (thin film membrane polytetrafluoroethylene, or Teflon) microwave digestion vessel (Milestone Inc., Shelton, CT), the samples were heated to 180 °C for 10 min. After digestion, the samples were diluted to 20 mL with 18.2 MΩ-cm water and analyzed by ICPMS (Agilent 7700, Agilent Technologies Inc., Santa Clara, CA), equipped with a collision cell and using indium as an internal standard. For accurate analysis of some high-concentration elements, samples were diluted 1:100 by using 100 µL of digested sample and diluting to 10 mL using HNO₃ (to maintain acid matrix) and 18.2 MΩ-cm water.

Single particle ICPMS (spICPMS)⁵³ was then used to measure the particle number size distribution of silver nanoparticles within each investigated nanoparticle. spICPMS is a relatively new application of ICPMS, and the general operating principles of ICPMS are explained by Laborda, *et al.* (2013). Briefly, spICPMS takes advantage of the superior elemental specificity and sensitivity of quantitative ICPMS. However, instead of using one relatively long dwell time (~ 100 ms), spICPMS utilizes sufficiently dilute samples (~50 ng analyte L⁻¹) and records large numbers of individual measurements at very short dwell times. Here, we used a 10 ms dwell time, which provided 20,000 data points collected in 200 sec. 100 nm gold particles were used as a standard to obtain the ICPMS nebulization efficiency for each run and dissolved silver standards (0.0, 0.1, 0.5, 1.0, 1.5, 2.0, 5.0 ppb) were analyzed prior to any nanoparticle suspensions to discern dissolved background silver from actual silver nanoparticles. When the ICPMS signal was analyzed, silver nanoparticles were counted as present if their signal was greater than the background mean signal plus three times the standard deviation of the background. In order to limit particle coincidence,

un-acidified nanoproducts were diluted in Milli-Q water so that only 1 of 10 measurements were nanoparticle events or “hits.”

The pH of the liquid suspensions of the nanoproducts (15 mL of each) were also measured three times, then averaged using an Accumet AB15 Basic (Fisher Scientific) at a stable temperature of 24.8°C.

Results

Minimal Inhibitory Concentration (MIC) Tests

As described above, the MIC for each nanoproduct was determined using the agar dilution method to establish the lowest ratio of nanoproduct to agar for which no visible *E. coli* or *B. atrophaeus* growth could be observed. Only nanoproducts S3 and S13 caused any growth inhibition (i.e., inactivation) with MICs of 160,000 ppm (16%) and 40,000 ppm (4%), respectively. The same MIC was found for both bacteria for both nanoproducts. The results were the same across all triplicate repeats. Nanoproduct S13 provided bacterial inactivation at 4x lower concentration compared to nanoproduct S3, despite the manufacturer of S13 recently claiming that S13 product is not nanosilver-based.

Nanoproducts S1, S7, S8 and S11 were completely ineffective for bacterial inactivation up to 320,000 ppm (32%) concentration of nanoproduct in agar. As this was the highest tested nanoproduct concentration in MIC tests, follow-up tests with undiluted products were performed. Here, spread plated films of 10^8 CFU for each bacterium were exposed to ten 3 μ L drops of each nanoproduct S1, S7, S8, and S11 in undiluted form, in triplicate. This drop size was chosen to allow for easy visual observation of potential inactivation without flooding bacterial film on the agar. However, same as in MIC tests, these nanoproducts were completely ineffective against the tested bacteria. It should be noted that these four products (S1, S7, S8, and S11) were advertised as being nanosilver-based, and nanoproduct S7 was also advertised as being an antimicrobial spray (antifungal).

Inactivation of Bacteria by Aerosolized Nanoproduct Agglomerates

Figure 2 and Figure 3 present the particle mass and particle number size distributions, respectively, of nanoproducts aerosolized into the test chamber. The different sections on Figures 2 and 3 represent the cut-point diameters for the different stages of the 6-Stage Viable Impactor. Nanoproducts S3 and S13 had the highest overall number and mass concentration of aerosolized particles. Thus, products S3 and S13 had the highest mass of nanoproduct to impact onto the agar plates covered by bacterial films. It can also be seen that the control suspension of 1 μ m PSL particles closely matched the particle size distributions for nanoproducts S3 and S13. All other controls and nanoproducts had lower particle number and mass concentrations compared to S3, S13, and the aerosolized 1 μ m PSL particles; however, they had similar particle mass and particle number size distributions among themselves.

Of the six investigated nanoproducts, aerosolized particles of S3 and S13 caused inactivation of both test bacteria when these were sampled onto bacterial films by impactors. An example of the inactivation of *E. coli* bacteria caused by airborne nanoproduct S13 particles

impacting onto the bacterial films in the BioStage is shown in Figure 4. The dry air and aerosolized 70% ethanol controls did not have any effect on bacterial films (Figure 4a and 4d). The dark dots in Figure 4b show accumulation of 1 μm PSL particles on the bacterial film and no inactivation of bacteria was observed. The white circles in Figure 4c, on the other hand, represent the voids (holes) in the bacterial film where nanoparticle S13 impacted the agar; i.e., the areas where bacteria were inactivated by the impacting material from the S13 product. Similar observations were made for nanoparticle S3, while no inactivation was observed for the rest of the tested nanoparticles.

In the follow-up experiment, the nanoparticles and controls were sampled out of the test chamber by the Six-Stage Viable Cascade Impactor to determine the effect of nanoparticle size on bacterial inactivation; here, inactivation of both bacteria was observed by product S13 for impactor stages N4–N6 (cut sizes 0.65 μm to 3.30 μm); the product S3 inactivated bacteria in impactor stages N5–N6 (cut sizes 0.65 μm to 2.10 μm) (Figure 5). As in the MIC tests, no inactivation was observed by nanoparticles S1, S7, S8, and S11. The mass of nanoparticles hitting the surface of agar plates in different impactor stages in the six-stage impactor and the BioStage impactor is presented in Figure 5 and compared with the amount of nanoparticle per surface area applied during MIC tests. The amount of nanoparticle hitting the agar plates was estimated based on the nanoparticle's airborne mass concentration (Figure 2) in the size range for each impactor stage according to the cut-point diameters and assuming a total sampled air volume of 28.3 L (28.3 L/min, 1 min sample). The deposited surface concentration for S13 was 0.84 $\mu\text{g}/\text{mm}^2$ for 6-Stage Viable Impactor stages 4–6 (cut sizes 0.65 μm to 3.30 μm). For product S3, the deposited surface concentration was 0.87 $\mu\text{g}/\text{mm}^2$ for stages 5 and 6 (cut sizes 0.65 μm to 2.10 μm).

The equivalent nanoparticle mass per agar surface area for S3 and S13 during MIC tests are shown by reference lines in Figure 5; these were calculated by dividing the volume of nanoparticle used to achieve the MIC by the agar height of 3.14 mm (90 mm diameter plates) and the agar plate total surface area of 6362 mm^2 , and then multiplying the result by the measured nanoparticle densities (0.79 g/cm^3 for S3 and 1.05 g/cm^3 for S13)³⁹. It is important to mention that the surface mass concentrations of S3 and S13 product impacting the agar plates never reached the same concentration per surface area that were achieved in the MIC tests, and yet both test bacteria were inactivated in the impactor tests. The result suggests that aerosolized and desiccated nanomaterials have different properties compared to liquid nanoparticles that enabled their inactivation of bacteria at concentrations more than 10x lower compared to those in MIC tests. The potential factors for this difference are discussed below.

Chemical and Size Characteristics of Nanospray Products

Figure 6 presents concentrations of the three primary metals (Ag, Mg and Zn) detected in the liquid suspensions of the nanoparticles, while all other detected metals are included as “other.” More details about the ICPMS-detected metal concentrations are provided in Table 3. Interestingly, nanoparticles S3 and S13 had the lowest silver concentrations compared to the other nanoparticles, yet they were the most effective at inactivating bacteria. Zinc was the dominant metal present in nanoparticle S3, while magnesium and zinc were the most

dominant metals present in nanoproduct S13. The metal with the highest concentration in the products S1, S7, S8, and S11 was silver, yet they were ineffective against the test bacteria.

The nanosilver particle size distributions in the nanoproduct's liquid suspensions determined using spICPMS are shown in Figure 7. As explained above in the Materials and Methods section, silver nanoparticles were counted as present if their signal was greater than the background mean signal plus three times the standard deviation of the background; therefore, based on the dissolved silver background analyzed prior to each nanoproduct, a cut-off size was determined representing the minimum nanoparticle size that could be detected for each nanoproduct. For S1, S3, S7, S8, S11, and S13, these cut-off diameters were 35.3 nm, 25.9 nm, 33.2 nm, 35.3 nm, 26.0 nm, and 17.3 nm, respectively.

While the total concentration of nanosilver was the lowest in nanoproducts S3 and S13 (Figure 6), the mean size of the nanosilver particles in these two products was the smallest among the investigated products: approximately 31 nm and 22 nm, respectively. The mean size of silver nanoparticles in products S7, S8, and S11 ranged from approximately 43 nm to 50 nm. Nanoproduct S1 was found to contain only dissolved silver, i.e., no silver nanoparticles. This was determined based on the particle number size distribution of S1 in comparison to the dissolved silver background as depicted in Figure S1 of the Supplemental Material. The average pH readings for S1, S3, S7, S8, S11, and S13 were 6.26, 5.12, 5.52, 7.69, 6.47 and 3.27, respectively.

Discussion and Conclusions

All of the investigated products were advertised as nanosilver-based spray products, and silver is known for its antibacterial properties⁵. However, only two of the six tested silver nanospray products (S3 and S13) were found to be capable of inactivating bacteria, while all other nanosprays were ineffective, even when undiluted spray liquid was applied to the bacterial films. The question arises as to why these two nanoproducts are different. Differences between the S3 and S13 nanoproducts and the rest of the studied nanoproducts emerged during our investigation.

First, nanoproducts S3 and S13 were the only tested products capable of inactivating bacteria with similar MICs for both *E. coli* and *B. atrophaeus*. Previous studies have investigated the MICs of pure silver nanoparticles and colloidal nanosilver against bacteria. The antibacterial properties of nanosilver are understood to be due to the release of silver ions which causes membrane permeability, and inhibition of cell wall, protein, and nucleic acid syntheses^{55–57}. MICs for exposure of aqueous nanosilver suspensions to different types of bacteria have generally been reported as 5 to 15 ppm^{58, 59}; Cho, *et al.* (2005) reports an MIC for silver nanoparticles of 10 nm average diameter of 5.4 ppm (0.0054%) by liquid broth dilution method. Using an adjusted version of the liquid broth dilution method, Petrus, *et al.* (2011) used a stock solution of colloidal nanosilver (1–100 nm, 200 ppm), and found MICs ranging from 7 ppm to 25 ppm for different gram positive and gram negative food pathogen isolates.

The MICs found for nanoproducts S3 and S13 were much higher. At the same time, these are not pure nanosilver products. According to the manufacturer, S3 contains 0.001% nanosilver in ethanol by weight, e.g., 10 ppm. Our data showed Ag107 concentration in this product of 12.2 ppb (Table 3). The nanosilver concentration in S13 was 3.6 ppb. Then, based on data in Figure 6 and Table 3 as well as observed MICs, approximately 2 ng/mL of silver for S3 and 0.14 ng/mL of silver for S13 were mixed in with agar to achieve inactivation of bacteria. These concentrations are much lower than pure nanosilver suspension concentrations, e.g., ~1.69 µg/mL silver, observed to inactivate bacteria in other studies⁶⁰. Obviously, there are other factors in play. It could be the presence of other metals in the suspension, and/or the effect of product matrix including its pH level and ionic strength⁵⁷. S3 and S13 had the most acidic pH levels: 5.12 and 3.27, respectively; although the pH of S3 (5.12) was only slightly lower than that of S7 (5.52). pH of S1, S8, and S11 were higher than 6.

Second, nanoproducts S3 and S13 had the greatest number and mass of aerosolized product agglomerates larger than the impactor cut-off size of 0.65 µm and, therefore, the greatest mass impacting onto the bacterial films. Both S3 and S13 caused bacterial inactivation after impaction onto the bacterial films, even though the mass of nanoproduct particles per mm² of agar surface area impacting onto the bacterial films during these tests did not exceed the MIC values for nanoproduct mass per agar surface area. The ability of S3 and S13 to inactivate bacteria upon impaction onto the agar surfaces may be a result of physical damage (e.g., physical bombardment) caused by the nanoparticles upon impaction⁶¹. However, if so, then this effect is specific to the types of nanoparticles present in these product matrices as none of the controls, including 1 µm and 20 nm PSL particles, caused inactivation by physical bombardment. The 1 µm PSL control had a mass concentration similar to the S3 and S13 suspensions and similar mass of PSL particles landed on the agar plates during testing, yet no inactivation was observed.

Thus, the ability of S3 and S13 to inactivate bacteria upon impaction onto the bacterial films may be explained by a combination of the form of the nanosilver in these products and the physical interaction upon impaction which may increase the exposure of bacteria to the nanosilver. Lok, *et al.* (2007) suggested that the antibacterial properties of nanosilver are dependent on multiple physical and chemical properties of the nanoparticles: higher antibacterial activity is associated with increasing exposure of bacteria to oxidized surfaces of nanosilver. Therefore, smaller nanosilver particles and less nanosilver aggregation will increase a suspension's antibacterial activity⁶². Perhaps, the lower mass required from nanoproducts S3 and S13 to inactivate bacteria during the chamber sampling experiments compared to the MIC tests was due to exposure of bacteria to smaller primary nanosilver particles (Figure 7) as aggregates broke up during their aerosolization by Collision nebulizer and, possibly, upon impact onto the agar. The inactivation of bacteria could also have been caused or contributed to by other metals in those products. S3 and S13 had relatively high concentrations of Zn and S13 had a high concentration of Mg. Both zinc oxide and magnesium oxide nanoparticles have been shown to exhibit antibacterial activity^{63, 64}. On the other hand, S1 and S8 also had high concentrations of Zn with more Zn compared to S13, but did not cause inactivation of bacteria. However, S1 had no silver in particulate form and the silver nanoparticles detected in S8 were larger than those measured in S3 and S13.

Compared to other products, S3 and S13 had the smallest mean nanosilver particle diameters (~22 to 30 nm). The size of nanosilver particles has largely been agreed upon as a factor that influences the antibacterial activity of nanosilver, with smaller particles being more effective due to greater exposure of bacteria to these particles and greater surface area for oxidation^{60, 62, 65–67}. Thus, size could be another factor contributing to the ability of these two products to inactivate bacteria.

Aerosolization of nanoproducts S3 and S13 resulted in the highest mass among the investigated nanoproducts impacting onto the surface of the agar and bacterial films. Given the small size of their primary particles, this resulted in the exposure of bacteria to agglomerates comprised of particles of small size. Also, aerosolization of ethanol control showed that ethanol droplets evaporated to sizes that were less than that of the BioStage cut-off size and did not affect the bacterial film. This result suggests that volatile components in the carrier fluid of S3 and S13 should have evaporated during the experiments and should not have affected the bacteria. At the same time, it is possible that semi-volatile components of the carrier fluid adsorbed to the nanoparticle agglomerates and interacted with bacterial film upon contact with those agglomerates. However, without a manufacturer's listing of every ingredient present in each product, it is difficult to determine what semi-volatile components could have contributed to the inactivation of bacteria.

While the antimicrobial properties of nanosilver have been a topic of increasing study, this research calls to attention the need for more studies investigating consumer nanoproducts in their final form. Nanosilver, even if present in relatively high concentrations, may not inactivate bacteria as was found for nanoproducts S7 and S8. Consumer nanoproducts advertised as being nanosilver may actually have high concentrations of other types of nanoparticulate metals, and the roles of these other nano-metals in bacterial inactivation have been given less attention in the literature. The effect of carrier fluid on the inactivating properties of microorganisms should also be considered.

As described by Nazarenko, *et al.* (2011), it should be noted that the method of product nebulization (i.e., hand-held sprayer, versus standard nebulizer) will affect the resultant particle number size distribution produced from the product liquid suspension with a tendency towards larger droplets produced by hand-pumped sprayers. However, the use of the Collison aided in the desiccation of the aerosolized droplets which was desired to desiccate the volatile components of the suspensions, and it would not have changed the metals content³⁹. Regardless, the influence of the selected method for nebulizing the products in this study should be considered and future study should consider including nebulization methods to more closely match the size distribution of product particles produced by hand pumps.

Overall, this research illustrates that certain nanosilver sprays have antibacterial potential and they may affect human and environmental bacterial flora. At the same time, the inactivation potential cannot be solely presumed based on the nanosilver presence and concentration in the product. The inactivation of bacteria by nanotechnology-enabled consumer sprays is likely due to multiple factors, and the potential for nanosilver in consumer products to cause bacteria inactivation will depend upon that product's final

manufactured form. Nanosilver is being touted as one of the answers to the dawning era of antimicrobial resistance, and it is suggested to be used in medical dressings, devices, lotions, etc.^{68, 69}. We suggest that caution and more research is needed as these products and nanoparticles become ever present in our environment, and at the moment it is unclear how exactly they may affect us⁵⁷. For all nanoparticulate metals present in product liquid suspensions, future work should include determination of the oxidation state with an understanding of which forms are relevant for aqueous solutions⁷⁰, as well as the size distribution for all nanometals. In the present study, this was performed by spICPMS only for nanosilver. Also, future work should include microscopy techniques to determine if the nanoparticulate metals are actually taken up by the bacteria⁷⁰, or if there is membrane associated damage on the surface of the microbes caused, for example, by physical contact with the nanomaterials⁷¹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental setup for investigating inactivation of bacteria by aerosolized nanoparticle agglomerates.

Figure 2.

Particle mass size distribution of aerosolized nanoparticle products and controls after the product matrix was desiccated in the test chamber. S1 to S13 represent the coded nanoparticle products investigated in this study. The three controls include 1) 1 μm polystyrene latex (PSL) test particles suspended in water to match the peak mass concentration of tested nanoparticle products, 2) ethanol (EtOH, 70%), and 3) 20 nm PSL suspended in ethanol (70%) at a final concentration similar to that of the nanoparticle products (10 ppm). The aerosolized nanoparticle products were sampled from the chamber using either a BioStage impactor (SKC Inc.) or Six-Stage Viable Cascade Impactor (Tisch Environmental); the lines indicate the cut-point diameters of the six cascade impactor stages; the cut-point of the single-stage BioStage is equivalent to that of N6 of the Six-Stage Viable Cascade Impactor.

Figure 3.

Particle number size distribution of aerosolized nanoparticle products and controls after the product matrix was desiccated in the test chamber. S1 to S13 represent the coded nanoparticle products investigated in this study. The three controls include 1) 1 μm polystyrene latex (PSL) test particles suspended in water to match the peak mass concentration of tested nanoparticle products, 2) ethanol (EtOH, 70%), and 3) 20 nm PSL suspended in ethanol (70%) at a final concentration similar to that of the nanoparticle products (10 ppm). The aerosolized nanoparticle products were sampled from the chamber using either a BioStage impactor (SKC Inc.) or Six-Stage Viable Cascade Impactor (Tisch Environmental); the lines indicate the cut-point diameters of the six cascade impactor stages; the cut-point of the single-stage BioStage is equivalent to that of N6 of the Six-Stage Viable Cascade Impactor.

Figure 4.

Spread-plated bacterial films of *E. coli* exposed to substances sampled by a multi-nozzle BioStage impactor: a) dry air (control), b) 1 μm PSL particles (control), c) agglomerated S13 particles and d) 70% ethanol (control). White circles in (c) indicate the absence of bacterial growth – inactivation of bacteria caused by nanoparticle particles observed as holes in the bacterial film. Darker dots in (b) indicate accumulated fluorescent 1 μm PSL particles. Colors in (b) and (c) were enhanced for clarity. The uniform dot pattern is present due to the configuration of impactor nozzles.

Figure 5.

Nanoparticle mass per impactation surface area of agar plates (mm^2). The nanoparticles tested here are represented by product code numbers with an “S” label. For growth-inhibiting (i.e., inactivating) nanoparticles S3 and S13, the equivalent nanoparticle mass per agar surface area for their MICs are indicated with horizontal reference lines at $36.79 \mu\text{g}/\text{mm}^2$ and $11.66 \mu\text{g}/\text{mm}^2$, respectively. On the x-axis, N1 to N6 represents the six stages of the Six-Stage Cascade Impactor for which nanoparticles S3 and S13 exhibited inactivation of bacterial films as evidenced by holes (“voids”) in the bacterial film growing on the agar surface. S3 and S13 inhibited bacterial growth on impactor stages N5–N6 (cut sizes $0.65 \mu\text{m}$ to $2.10 \mu\text{m}$), and N4–N6 (cut sizes $0.65 \mu\text{m}$ to $3.30 \mu\text{m}$), respectively. The BioStage impactor is equivalent to the cascade impactor stage N6 (cut size $=0.65 \mu\text{m}$) and was used to sample all tested nanoparticles.

Figure 6.

The concentrations (ppb) of the three primary metals detected in nanoparticle suspensions. S1 to S13 represent the coded nanoparticles tested in this study.

Figure 7.

The particle number size distributions in the consumer nanoproduct's liquid suspensions. The text inset represents the mean $\pm 1SD$ of silver nanoparticle size for each nanoproduct. Nanoproduct S1 was found to contain only dissolved silver, i.e., no silver nanoparticles. This was determined based on the particle number size distribution of S1 in comparison to the dissolved silver background as depicted in Figure S1 of the Supplemental Material.

Table 1

Descriptions of Studied Nanospray Products

Nanoproduct Code	Manufacturer Reported Composition	Advertised Intended Use
S1	99.99% pure silver in de-mineralized water	Cosmetic skin spray
S3	0.001% weight silver nanoparticles in ethanol	Shoe deodorant spray
S7	20 ppm silver in deionized water	Antifungal skin spray
S8	10 ppm silver in deionized water	Nasal spray
S11	99.99% pure silver ionizing foam in deionized water	Cosmetic skin spray
S13	Nano-polymers, nonionic surfactants, synergists (this product has since revised its claims to no longer contain silver)	Clean, decalcify and seal bathroom surfaces

Table 2

Controls used when investigating inactivation of bacteria by aerosolized nanoproducts

Control Type	Description and Purpose
Dry air	Used to investigate potential desiccation damage to bacteria films
70% Ethanol	Used to verify that volatile nanoproduct components sufficiently evaporated before reaching bacterial plates
1 µm polystyrene latex (PSL) particles (Thermo Scientific G0100)	1 µm PSL particles were suspended in Milli-Q water to give a peak concentration similar to the largest agglomerated nanoproduct particles; this control investigated potential inactivation caused by kinetic impact of large particles onto bacterial films
20 nm PSL (Thermo Scientific 3020A)	20 nm PSL particles were diluted in 70% ethanol to achieve a final concentration similar to that of the nanoproducts (10 ppm); this control investigated potential physical inactivation caused by agglomerated, nano-sized, inert particles.

Table 3ICPMS Detected Metal Concentrations (unit = ppb, $\mu\text{g/L}$)

Nano-product	Sr	Ag	Ba	Pb	Mg	V	Cr	Mn	Fe	Ni	Cu	Zn
S1	0.58	6888	3.37	1.48	31.40	1.74	0.17	1.08	28.00	2.41	6.24	1515
S3	0.44	12.20	1.98	3.52	18.10	1.26	0.07	0.42	11.90	1.01	2.84	34315
S7	0.81	19332	4.39	0.94	32.60	3.96	1.95	1.13	30.10	2.18	9.49	37.40
S8	11.50	10996	16.20	1.69	226	7.66	7.11	2.03	150	2.95	34.30	1047
S11	2.95	502	3.47	0.38	45.00	16.80	14.30	1.11	20.40	0.31	31.10	286
S13	112	3.61	50.50	4.43	2349	14.20	12.30	7.26	202	30.20	149	409