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Letter

Synergy between Microwave Radiation and Silver Ions or Nanoparticles for Inactivating *Legionella pneumophila*

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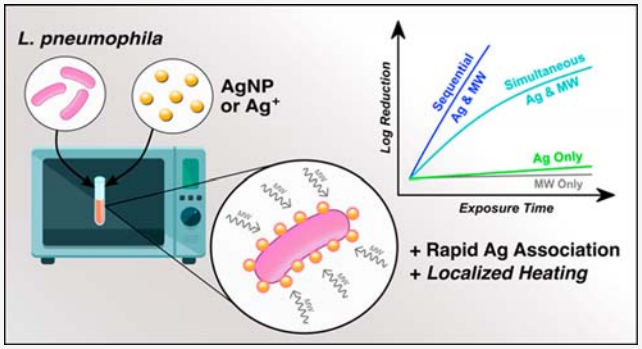
ABSTRACT: The focus on waterborne etiological agents in the developed world has shifted recently from enteric bacteria to opportunistic human pathogens. Of particular interest is the opportunistic pathogen *Legionella pneumophila* (Lp), which can cause legionellosis. Biofilms within premise plumbing systems facilitate the persistence and growth of Lp. Thus, point-of-use treatment approaches that are effective against such opportunistic pathogens are needed. The objective of this study is to evaluate Lp inactivation by utilizing potential synergy between microwave (MW) radiation and silver [Ag^+ and/or silver nanoparticles (AgNPs)]. Lp inactivation can be achieved by the following mechanisms. (i) Initial association of AgNPs with cells, when followed by MW irradiation, causes localized heating. (ii) Pre-exposure to MW radiation weakens the membrane and, when followed by Ag^+ exposure (directly dosed or released from AgNPs), results in increased Ag^+ uptake. (iii) Simultaneous exposure to MW radiation and AgNPs can also lead to localized heating. In these combination treatments, including simultaneous and sequential exposure to silver and MW radiation, Ag^+ is more effective than AgNPs. The synergy between MW radiation and silver could be used to create a point-of-use device that effectively inactivates Lp, thereby overcoming the limitations of chlorine at combating this organism.



INTRODUCTION

The opportunistic human pathogen *Legionella pneumophila* (Lp) poses a unique health hazard because it can persist and proliferate in premise plumbing systems despite upstream drinking water treatment. Inhaling aerosols containing Lp can result in contraction of Legionnaires' disease, a potentially fatal form of pneumonia, or the milder, flu-like Pontiac fever, both of which are known as legionellosis.[1] The number of cases has been increasing for decades, and Lp is the leading cause of waterborne outbreaks throughout the developed world.[2−4] Although outbreaks of Legionnaires' disease garner headlines, a majority of the cases are sporadic (i.e., single cases not associated with other cases); exposure to Lp from premise plumbing in individual residences and large building water systems appears to account for a substantial fraction of these sporadic cases.[5] Alarmingly, the disease burden imposed by Lp might be substantially underestimated.[6]

Legionella is found in multiple locations in the potable-water environment, such as sediments in drinking water storage tanks,[7] hot water heaters,[8] hot tubs,[9] and taps.[10] Lp occurs in biofilms[11,12] in such systems, where its persistence on certain surfaces (e.g., copper) is increased by the presence of free-living amoebae (FLA)[13] that can phagocytose Lp without digesting it.[14] *Legionella* colonization has been observed at potable taps receiving chloraminated water and, more frequently, at taps receiving chlorinated water.[10] Chlorine and chloramines have reduced efficacy against Lp in biofilms as compared to planktonic cells[15] and in biofilms in the presence of FLA as opposed to their absence.[16] Lp, and other opportunistic human pathogens such as *Pseudomonas aeruginosa* and *Mycobacterium avium*, are chlorine-resistant bacteria;[17] for example, a 2-log reduction was obtained at 40 min for Lp and in <1 min for *Escherichia coli* at the same chlorine residual. Lp can persist in the presence of temporary stagnation and low disinfectant residual,[13] which are common conditions in premise plumbing. Lp's ability to persist and grow under drinking water conditions[18] necessitates the development of a point-of-use (POU) treatment approach for its effective inactivation. This approach would be especially useful for protecting immunocompromised people in eldercare facilities.

POU devices, such as showerheads equipped with ultraviolet-C light-emitting diodes, have been investigated with respect to Lp inactivation; however, the complex inner geometries of these devices and their efficacy against biofilms

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and protozoan hosts raise concerns.[19] Copper−silver ionization systems commonly are implemented by healthcare facilities to reduce the incidence of nosocomial legionellosis, but they require substantial maintenance and monitoring.[20,21] Thermal treatment is often advised for controlling opportunistic pathogens in premise plumbing, either with thermal shocks up to 80 °C or by maintaining water temperatures of >50 °C.[22] However, logistical concerns in large systems and scalding risk limit its use. Operational limitations and the disinfection resistance of Lp present unique challenges for the development of effective POU devices.

Several novel approaches to hinder or inactivate Lp have been explored. Lactoferrins, which are iron-binding proteins, are bactericidal when unsaturated with respect to iron.[23] Recent research has shown their promise in using a bacterial endosymbiont to reduce Lp proliferation in FLA and its virulence once released.[24] Other Lp inactivation studies have focused on the cell envelope, which has unique components and properties (e.g., very hydrophobic lipopolysaccharide in the cell wall and phosphatidylcholine in the membrane).[25] One study used a natural antimicrobial peptide to increase the membrane permeability of Lp inside FLA.[26] Novel ways to thermally inactivate microorganisms, such as with highly heat-conductive materials, have been investigated. Carbon nanotube polymer composite membranes have demonstrated the effective capture of suspended Lp, where subsequent joule heating can inactivate the captured bacteria.[27] Combining heat-conducting materials with thermal energy delivered via microwave (MW) radiation has potential for Lp inactivation, both because of the global availability and acceptability of MW-generating devices and because localized heating costs much less than bulk heating. Metallic nanomaterials might hold the key to unlocking a MW-enabled treatment approach with the ability to localize MW energy.[28]

The objective of this study is to inactivate Lp by targeting its cell envelope, capitalizing on the potential synergy between MW radiation and metal ions or nanoparticles. Silver is chosen [Ag^+ or silver nanoparticles (AgNPs)] for its high heat conductance (429 W m^{-1} K^{-1})[29] and biocidal capabilities. Inactivation mechanisms are suggested by analyzing the loss of viable Lp under different sequences of silver exposure (Ag^+ or AgNPs) and MW radiation and by assessing the degree of association of silver with cells.

MATERIALS AND METHODS

Materials. AgNPs were synthesized using an established protocol with sodium borohydride as a reducing agent and citrate as a capping agent.[30] The average particle size (13.6 ± 2.5 nm) was determined by transmission electron microscopy (TEM; JEOL 2010F), and colloidal stability was confirmed (hydrodynamic diameter of 24.8 ± 3.2 nm) with dynamic light scattering (DLS; ALV/CGS-3). AgNP characterization results are shown in Figure S-1. Ag^+ stock solutions were prepared from $AgNO_3$ (Alfa Aesar, Tewksbury, MA; ACS grade) and filtered through 0.22 μm membrane filters. Lp (ATCC 33512, American Type Culture Collection, Manassas, VA) was cultured on buffered charcoal yeast extract (BCYE) agar (BD Diagnostics, Sparks, MD), inoculated into buffered yeast extract (BYE) broth, and grown to mid-exponential phase. Additional culturing details are provided in the Supporting Information. A modified phosphate-buffered saline [PBS-25; 1.03 g L^{-1} $NaNO_3$, 0.61 g L^{-1} Na_2HPO_4, and 0.20 g L^{-1} KH_2PO_4 (pH 7.3)] with an ionic strength of 25 mM was used in the Lp assays to minimize AgNP aggregation. $NaNO_3$ was substituted for NaCl (in PBS) to prevent silver chloride precipitation and interparticle bridging.[31]

Exposure Experiments. Bacterial suspensions (from biological duplicates) were pelleted and washed three times with PBS-25 after reaching mid-exponential phase and diluted to ~2.5×10^6 colony-forming units (CFU) mL^{-1}. To a quartz vial were added 0.9 mL of a diluted bacterial suspension and 0.1 mL of a AgNP (2.0 mg L^{-1}) or Ag^+ (0.1 mg L^{-1}) stock solution or PBS-2, and the vials were briefly vortexed. Tolerance experiments (i.e., Lp treatment with MW radiation and/or silver) followed by viable plate counts were conducted according to an established protocol.[32] For MW experiments, a reactor operating at 2.45 GHz with an input power of 70 W, an order of magnitude lower than the typical input power of a household microwave oven, was used. For the maximum MW exposure time of 6 min, the estimated energy consumption is 0.007 kWh. To minimize bulk heating (to avoid confounding MW-specific effects with bulk temperature changes), radiation was delivered in pulses, consisting of irradiation for 1 min followed by a 2 min pause.

Samples were treated individually (exposed to MW radiation or silver), simultaneously (exposed to MW radiation and silver), or sequentially (exposed to MW radiation and then silver or vice versa). Samples treated with only silver had an 18 min exposure to reflect the maximum exposure time for simultaneous treatment. Following silver treatment in the individual or simultaneous scenarios, 10 μL of 0.1 N sodium thiosulfate was added to quench silver, and vials were placed in a 20 °C water bath. Each vial was sacrificial; therefore, after an aliquot was removed for serial dilution, spot-plated onto BCYE agar in triplicate, and incubated at 37 °C for 48−72 h prior to enumeration, the vial was discarded. Moreover, silver (18 min) and MW (6x pulses) treatments were applied sequentially (in both orders). Under silver pre-exposure, sodium thiosulfate was added prior to MW irradiation. The bulk temperature was uncontrolled in the MW pre-exposure experiments; i.e., silver was added immediately following MW pre-exposure (after the last MW exposure), and the heat dissipated naturally. Inductively coupled plasma mass spectrometry (ICP-MS; model 7500ce, Agilent, Santa Clara, CA) was used to quantify aqueous silver released from AgNPs before and after MW irradiation and for association of silver with cells (i.e., uptake or adhesion of AgNPs and Ag^+ to cells); further details are provided in the Supporting Information.

RESULTS AND DISCUSSION

Individual or Simultaneous Treatment. Individual treatments (3x or 6x pulses of MW radiation, 2 mg L^{-1} AgNPs, or 0.1 mg L^{-1} Ag^+) resulted in minor Lp reduction, i.e., <0.4 log (Figure 1a). The toxicity of MW radiation to living organisms is predominantly attributed to thermal stress.[33] While the bulk temperature of PBS-25 increases rapidly upon irradiation (to 40 °C after 1x MW exposure) and plateaus at approximately 50 °C after 6x pulses (Figure 1b), rapid Lp inactivation typically does not occur until the bulk temperature reaches 60 °C (e.g., 4-log reduction after a 2 min exposure to 60 °C).[34] Additionally, previous studies showed Ag^+-only treatment requires substantial time for effective Lp inactivation (e.g., 2.4-log reduction after a 3 h exposure to 0.1 mg L^{-1} Ag^+).[35,36] Thus, low Lp reductions due to individual treatments in the study presented here were expected.

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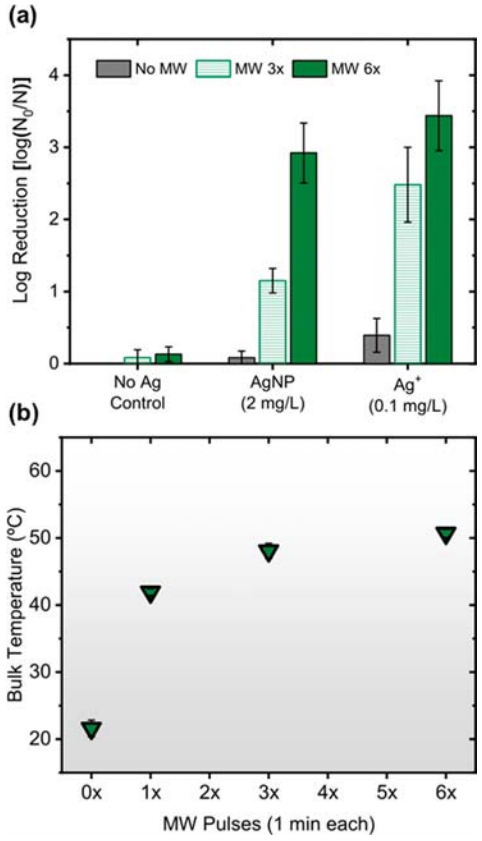

Figure 1. (a) Log reduction in viable Lp after individual or simultaneous exposure to silver (AgNP or Ag^+) and/or pulses of MW radiation (3x or 6x at 2.45 GHz; 70 W) in PBS-25. (b) Bulk temperature of the PBS-25 solution after pulses of MW radiation. Each pulse had a duration of 1 min with 2 min between consecutive pulses. Therefore, 6x pulses of MW radiation, for example, correspond to 6 min of total MW exposure but an 18 min overall duration including the pauses. Error bars represent the standard deviation.

Simultaneous exposure to MW radiation and silver produces a synergistic effect on Lp inactivation. When 3x or 6x MW pulses are combined with AgNPs, the log reduction is 1.15 ± 0.17 or 2.92 ± 0.42, respectively. Furthermore, the combination of 3x or 6x MW pulses with Ag^+ yields an even greater log reduction of 2.48 ± 0.49 or 3.44 ± 0.48, respectively. We theorize that the synergy observed for the simultaneous treatments is likely due to (i) increased silver uptake by cells, possibly facilitated by membrane damage from MW radiation, and/or (ii) enhanced distributed heating from Ag^+ or localized heating from cell-associated AgNPs. Temperature increases have been indicated to similarly enhance the effectiveness of chlorine against Lp.[37]

Sequential Treatments. As shown in Figure 2a ("Ag^+/MW"), an 18 min pre-exposure to Ag^+ followed by pulsed MW radiation causes substantial Lp inactivation (i.e., >4-log reduction with 3x pulses of MW radiation). An 18 min pre-exposure to AgNPs followed by MW radiation yields 1.74 ± 0.50- and 3.36 ± 0.17-log reduction for 3x and 6x pulses, respectively (Figure 2b, "AgNP/MW"). Thus, sequential exposure to silver and MW radiation yields greater inactivation than does simultaneous exposure to these agents. Additionally, sequential exposure yields greater inactivation than that produced by summing the results of the individual treatments.

Interestingly, when thiosulfate is dosed simultaneously with silver (prior to irradiation in sequential treatment), Lp inactivation is similar to that obtained for individual treatment with MW radiation (Figure S-2); thus, when Ag^+ is complexed with thiosulfate at the outset of treatment, no improvements in Lp inactivation due to MW radiation are observed. However, because enhancement is observed when Lp is pre-exposed to silver, it is possible that Ag^+ associates with cells during the pre-exposure period and has increased uptake during MW radiation and that AgNPs associate with cells during the pre-exposure period and deliver localized heat during irradiation.

The association between silver and cells was investigated via ICP-MS (Figure 3a). Consistent with prior reports,[35] this analysis shows that 20.4 ± 0.8% of the dosed Ag^+ is associated with cells in the absence of thiosulfate. When thiosulfate is added to Lp samples after an 18 min exposure to Ag^+, akin to the pre-exposure period for silver in the sequential exposure experiment, 6.8 ± 0.2% of the dosed Ag^+ is associated with cells. When thiosulfate and Ag^+ are added simultaneously, no detectable silver is associated with cells. The decrease in the level of association of silver with cells in the presence of thiosulfate indicates that silver complexes do not associate with cells to the same degree as free silver. Likewise, 49.0 ± 5.3% of dosed AgNPs associate with cells in the absence of thiosulfate, but 31.8% and 36.5% of AgNPs are cell-associated when thiosulfate is dosed after an 18 min pre-exposure to AgNPs and simultaneously with AgNPs, respectively. This analysis does not distinguish between aqueous and particulate silver or between internalized and surface-associated silver. However, these data confirm that a substantial fraction of dosed silver associates with cells (e.g., in the simultaneous or sequential treatments), which likely contributes to the synergistic action of combined treatment.

In the reverse sequential exposure scenario, where 6x pulses of MW radiation were followed by silver addition, a similar enhancement in Lp inactivation (as compared to the summation of inactivation due to individual treatments or simultaneous treatment) was observed in the case of Ag^+ (Figure 2a, MW/Ag^+). Addition of Ag^+ after 6x pulses of MW radiation achieved 4.08 ± 0.43-log reduction within 10 min of silver exposure (Figure 2b). However, addition of AgNPs after pre-exposure to MW radiation does not increase the inactivation efficacy; here, a 1.31 ± 0.28-log reduction is achieved after AgNP exposure for 15 min, as compared to a 2.92 ± 0.42-log reduction when the treatments are applied simultaneously (Figure 2b). Thus, MW pre-exposure induces nonlethal stress to Lp and facilitates improved inactivation during subsequent Ag^+ (but not AgNP) exposure.

The cellular response to thermal treatment is generally physiological in nature, such as membrane rupture and protein denaturing.[38] Exposure to sublethal temperatures (i.e., 40 °C due to MW radiation) can cause the formation of temporary pores in *E. coli*.[33] While such poration can be reversible, silver exposure can lead to irreversible cell damage; for example, Ag^+ stress is attributed to its interaction with surface moieties, including thiols, proteins, or nucleic acids.[39] Enhanced Lp inactivation from sequential exposure to MW radiation and Ag^+ is believed to result from exacerbated membrane damage[40,41] or facilitated ion transport.[42] In an ionic solution, microwave-induced heat is generated from dielectric heating.[42] The polar molecular oscillation of water and collisions (with neighboring molecules) due to dipolar polarization combines with ionic oscillation of Ag^+, resulting in electrical resistance

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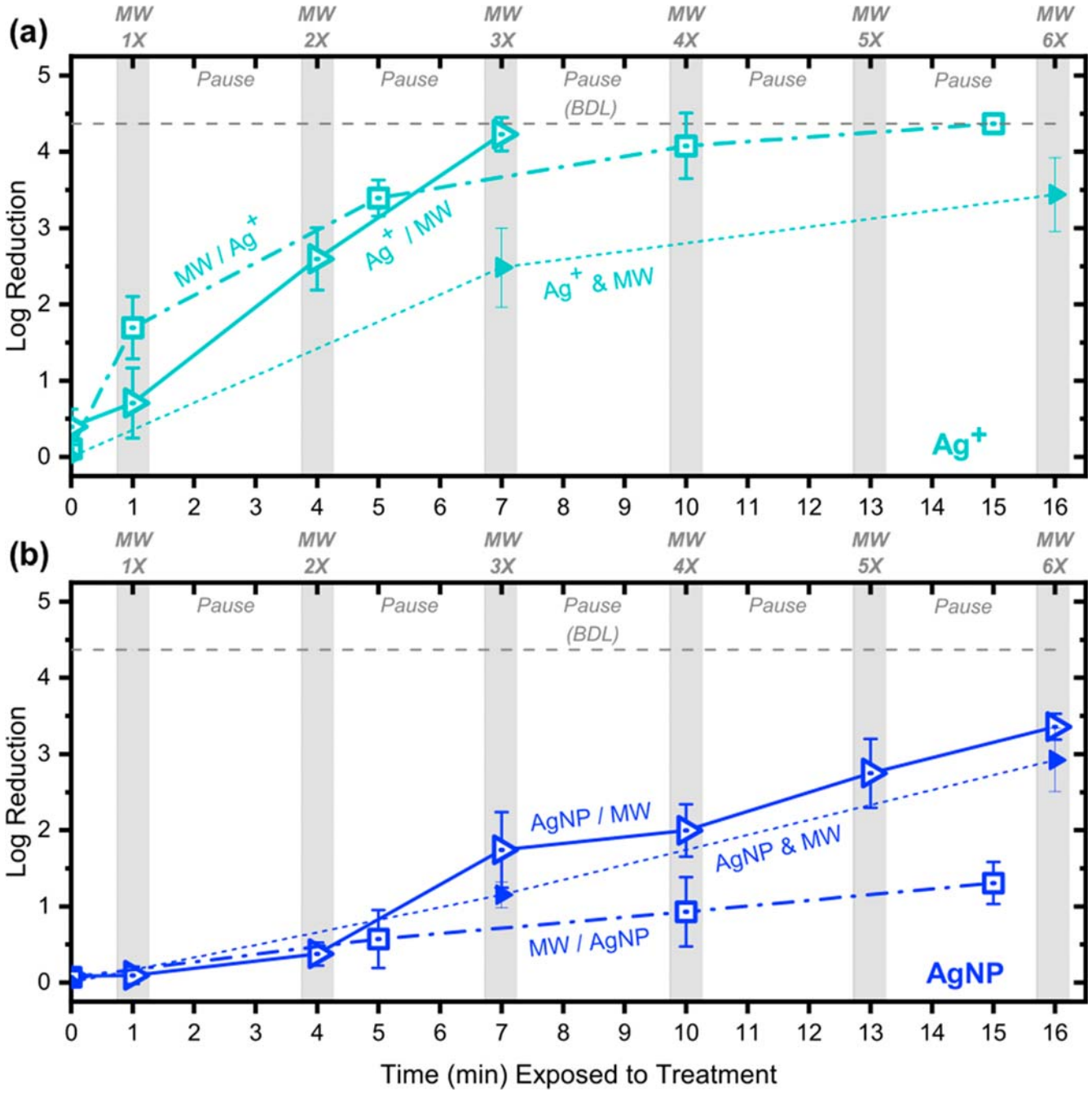

Figure 2. Investigation of Lp inactivation with (a) Ag^+ and MW radiation or (b) AgNPs and MW radiation by pre-exposing cells to each treatment, followed by sequential treatment with the other. Ag/MW refers to sequential treatment with silver exposure followed by MW radiation. MW/Ag refers to the reverse sequential treatment scenario, where MW radiation is followed by silver exposure. Simultaneous exposure to silver and MW radiation (AgNP & MW) is shown for comparison. BDL refers to beyond detection limit and is represented by the dashed line. The detection limit in terms of log reduction is dependent on the initial cell density in each experiment and ranged from 4.2 to 4.5. Error bars represent the standard deviation.

within the solution.[43] This interfacial polarization is an effective distributed heating mechanism[44] for such solutions and likely contributes to the observed inactivation efficacy of the simultaneous or sequential Ag^+ and MW treatment. It should be noted that the "free ions" in a solution are known to generate this resistive heating when the solution is irradiated with MW. However, ions that are associated with the surface of a microorganism or internalized within a microorganism have not been proven to possess similar freedom; therefore, it is not known if these cell-associated ions can lead to localized heating.

For AgNPs, particle-specific stresses can involve physical disruption of cellular membranes[39] or periplasmic effects, where particles attach to cell surfaces and deliver Ag^+ into the periplasm with subsequent formation of hydrogen peroxide.[43] With regard to AgNP and MW radiation, the nanometer dimension of AgNPs is much smaller than the penetration depth (on the order of 1 μm for metals) of MW radiation; this allows for an innate suppression of MW reflectance but

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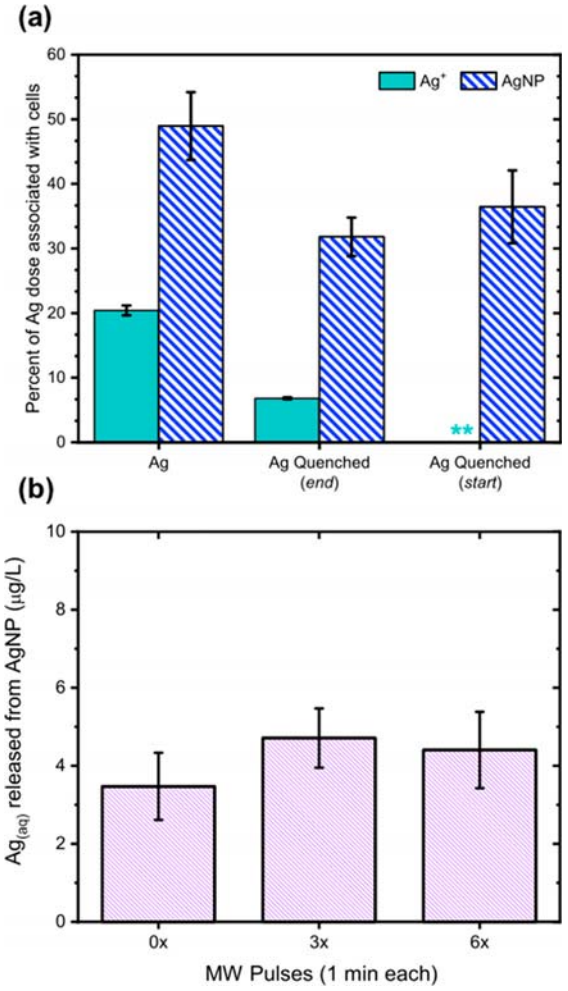

Figure 3. ICP-MS analyses for probing the interactions between silver and Lp. (a) Association of silver (0.1 mg L^{-1} Ag^+ or 2.0 mg L^{-1} AgNP) with Lp cells after an 18 min exposure period under three conditions, i.e., no sodium thiosulfate addition (Ag), sodium thiosulfate added at the end of the 18 min silver exposure period [Ag Quenched (end)], or sodium thiosulfate added at the start of silver exposure [Ag Quenched (start)]. Two asterisks indicate a value below the accurate detection limit (<0.25 μg L^{-1}). (b) Aqueous silver produced in a 2 mg L^{-1} AgNP suspension before and after pulsed MW (3x and 6x) treatment. Each pulse had a duration of 1 min with 2 min between consecutive pulses. Error bars represent the standard deviation.

facilitates coupling with the radiation for uniform and rapid heating. Localized heating from MW-absorbing nanomaterials has garnered a significant amount of interest in terms of catalytic degradation[44,45] and thermal therapy[46,47] applications. Gold nanoparticles in a magnetic field have been shown to produce significantly higher temperatures relative to that of the bulk solution within 2 nm of the particle surface.[48] Lp inactivation due to the association of AgNPs and MW-stressed membranes (sequential treatment MW/AgNP) is less effective than simultaneous treatment or the reverse sequential treatment (AgNP/MW). Thus, we hypothesize that inactivation caused by AgNP exposure in the last two scenarios predominantly occurs by AgNP-rendered localized heating and/or Ag^+-specific effects (where AgNPs serve as a constant source of Ag^+, which can be directly released into the periplasm or transported into the cytoplasm to cause damage).

The release of aqueous silver from AgNPs was quantified with and without MW radiation (Figure 3b); MW irradiation shows a negligible effect on silver release, as indicated by the relatively unchanged $Ag_{(aq)}$ concentration of 5 μg L^{-1}, despite up to 6x MW pulses. This $Ag_{(aq)}$ concentration is substantially lower than that in the Ag^+ experiments (0.1 mg L^{-1}); however, localized delivery of Ag^+ from AgNPs to the cell is possible, given the substantial association of AgNPs with cells (Figure 3a) and could have contributed to the observed Lp inactivation. Preliminary analyses assessing the production of reactive oxygen species were performed as previously described,[28] but no substantial H_2O_2 was detected under any experimental condition.

Several recent studies have evaluated synergistic treatments, aiming to achieve the target disinfection efficacy while reducing disinfectant dosages, energy demand, or contact time. For example, ozone is widely known to damage cell membranes; when combined with UV radiation, it shows effective disinfection of recalcitrant *Bacillus subtilis* spores.[49] Locally enhanced electric field treatment (LEEFT) has been shown to disrupt membrane integrity; when used as a pretreatment for ozonation, it enhances the effectiveness of ozone disinfection and can substantially reduce the required ozone dose.[50] Synergistic effects also have been realized when UV treatment is combined with peracetic acid[51] or ultrasound[52] pretreatment. The synergy between MW and silver demonstrated in this study is a valuable combined treatment technique for Lp inactivation.

Environmental Implications. The number of legionellosis cases is steadily increasing. Biofilm persistence in aging infrastructure, ambient temperature changes, increased likelihood of natural disasters due to climate change, and increases in drinking-water age from prolonged inactivity (e.g., during the current pandemic[53] and after floods[54,55]) exacerbate Lp growth in distribution systems.[56,57] The 2014 Flint, MI, water crisis coincided with an outbreak of Legionnaires' disease that was apparently attributable to low chlorine residual and release of iron, an essential nutrient for Lp, into the system.[58,59] A 2015 outbreak in Quincy, IL, occurred after a change in corrosion control and a decrease in disinfectant residual, among other factors.[60] These incidents highlight the myriad factors contributing to the persistence and proliferation of Lp.

Lp presents a key problem in supplying safe drinking water, the ineffectiveness of residual disinfectants to inactivate Lp in distribution systems. Thus, an alternative disinfection approach is required. MW radiation-based treatment in the presence of aqueous or particulate silver has the potential to achieve effective inactivation of Lp. The *in situ* application modality will need to explore device attachments near the primary sources of Lp exposure,[61] where sequential treatment with MW and silver can render effective Lp inactivation. We demonstrate that silver and MW radiation act synergistically to inactivate this pathogen. The wide availability and acceptability of MW devices can alleviate implementation challenges, while the short duration of exposure (to MW radiation and silver) necessary to achieve a ≤4-log reduction makes this innovative approach a promising treatment option.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.1c00371.

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Materials and Methods, including culturing conditions and media, silver nanoparticle dissolution, and association of silver with cells; characterization of synthesized, citrate-capped AgNPs (Figure S-1); and inactivation of synergistic treatment with thiosulfate quenching (Figure S-2) (PDF)

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Notes

The authors declare no competing financial interest.

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