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Bactericidal Mechanisms Revealed for Rapid Water Disinfection by Superabsorbent Cryogels Decorated with Silver Nanoparticles

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Abstract

The authors have recently reported the fabrication of superabsorbent cryogels decorated with silver nanoparticles (PSA/AgNP cryogels) that demonstrate rapid water disinfection. This paper provides a systematic elucidation of the bactericidal mechanisms of AgNPs (silver nanoparticles), both generally and in the specific context of cryogels. Direct contact between the PSA/AgNP cryogel interface and the bacterial cells is required to effect disinfection. Specifically, the disinfection efficacy is closely correlated to the cell-bound Ag concentration, which constitutes >90% of the Ag released. Cells exposed to PSA/AgNP cryogels show a significant depletion of intracellular adenosine triphosphate (ATP) content and cell-membrane lesions. A positive ROS (reactive oxygen species) scavenging test confirms the involvement of ROS ($\cdot\text{O}_2^-$, H_2O_2 , and $\cdot\text{OH}$) in the bactericidal mechanism. Furthermore, exposed bacterial cells show an

enhanced level of thiobarbituric acid reactive substances indicating the occurrence of cell-membrane peroxidation mediated by ROS. In addition, this study reveals that both Ag^+ and Ag^0 are involved in the bactericidal mechanism of AgNPs via tests conducted using PSA cryogels with bound Ag^+ ions (or PSA/ Ag^+ cryogels without reducing Ag^+ to Ag^0). Significantly, bacterial cells exposed to PSA/ Ag^+ cryogels did not show any cell-membrane damage even though the former had a higher cell-bound Ag concentration than that of the PSA/AgNP cryogels, thus indicating the differential action of Ag^+ and Ag^0 .

Keywords: Silver; nanoparticles; antibacterial mechanism; bio-nano interface; nanohybrids; porous hydrogels;

Introduction

Lack of access to safe, reliable water sources remains a critical problem for millions of people worldwide where waterborne diseases such as diarrhea cause up to 1.8 million deaths annually.¹ This calls for novel water technologies that are robust, low-cost, and energy-efficient.² Silver in the form of a nanoscale material is gaining renewed interest as an alternative water disinfectant due to its unique physicochemical properties and excellent antimicrobial action.²⁻⁷ The application of AgNPs (silver nanoparticles) is currently limited by issues related to dispersion and dissolution that can be circumvented by incorporating AgNPs into various support matrices that are generally being applied in the form of suspensions or filter materials.⁸⁻¹³

The aforementioned composites generally have good disinfection efficacies in which over 3 logs bacterial reduction can be achieved although at varying contact times (usually >0.5 h). However, their applications may be limited due to some operational drawbacks. For example,

composites being applied in the form of suspensions require an additional energy-demanding separation step such as filtration and magnetization. On the other hand, filtration-based systems (e.g., papers or membranes) usually have low water throughputs because of the long treatment and percolation times.

The authors have recently reported a new strategy for water disinfection using superabsorbent poly(sodium/acrylate) cryogels decorated with silver nanoparticles (PSA/AgNP cryogels) that can disinfect up to 3 logs of bacteria within 15 s with a low-level release of dissolved Ag.¹⁴ Unlike the aforementioned materials, PSA/AgNP cryogels developed Loo et al.¹⁴ offer a simpler alternative to point-of-use water disinfection whereby the disinfected water can be recovered by squeezing the swollen cryogels. This provides a new approach for potable-water production in difficult circumstances such as in the aftermath of a disaster where energy and water supplies are disrupted.¹⁵ The surprisingly fast disinfection ability of the cryogels motivated this study of their bactericidal mode of action.

The bactericidal effectiveness of AgNPs has been ascribed to the toxicity of the dissolved Ag⁺ ions¹⁶⁻²⁰ and the nanoparticle-specific^{13,21-25} physicochemical effects that impair biological action of functional biomolecules such as enzymes, DNA, and membrane proteins.^{3,5,26-29} Although the bactericidal mechanism of AgNPs has been a subject of intense research during the past decade, controversy still exists concerning the exact mechanism. The involvement of multiple pathways and Ag domains in the multifaceted bactericidal action of AgNPs poses significant challenges in gaining an unequivocal understanding of the mechanism. Besides their excellent disinfection properties, the newly developed macroporous cryogels can provide a suitable platform to systematically investigate the involvement of various possible pathways in

the bactericidal mechanism of AgNPs, both generally and in the specific context of PSA/AgNP cryogels.

Experimental

Fabrication and characterization of cryogel hybrids

PSA/AgNP cryogels were prepared as previously described.^{14,30,31} Further description of the protocol can be found in the Supporting Information. Unless otherwise stated, all the tests were conducted using AgNC-170 (Ag content: $166.7 \pm 15.0 \text{ mg g}^{-1}$). The Ag content in the PSA/Ag⁺ cryogels was determined to be $116.0 \pm 7.9 \text{ mg g}^{-1}$.

The specimens were coated with Pt for 30 s (20 mA) using an auto-fine coater (JEOL JFC-1600) prior to imaging using field-emission scanning microscopy (FESEM, JEOL JSM-7600F). To visualize the three-dimensional (3D) pore structure of the cryogels, a high-resolution X-ray tomography scanner (SkyScanner 1172) was used to obtain raw data for the cryogels. The two-dimensional (2D) images were then reconstructed into 3D images using the software Avizo Fire; a detailed description for the quantification of the pore parameters via image analysis of the 2D optical slices can be found in the Supporting Information. The specific pore volume of the cryogels was determined by measuring the mass increase of the cryogels after a 3-h immersion in cyclohexane (0.779 g cm^{-3}) relative to their initial dry mass.³² The mechanical properties of 10-mm thick swollen cryogels were characterized using a computer-controlled mechanical testing system (Instron 5567) at room temperature; the samples were compressed up to 95% strain using a 5 kN load cell at a ramp rate of 10 mm min^{-1} .

Protocol for microbiological assays

Determination of disinfection efficacies and re-growth of exposed cells

The antibacterial activity of the PSA/AgNP cryogels was tested against 4 types of bacteria: Gram-negative, *Escherichia coli* (ATCC25922) and *Salmonella Typhimurium* (NCTC 13348), and Gram-positive, *Bacillus subtilis* (ATCC6633) and *Staphylococcus aureus* (ATCC6538). All experiments were conducted using mid-exponential-phase cultures suspended in phosphate saline buffer (PBS, 0.01M, pH 7.45) forming a suspension with an initial cell density $\sim 10^8$ cfu mL⁻¹.

The spread-plate method was used for quantifying the number of live cells. To determine the death rate of the bacteria, Universal Quenching Agent (UQA, 0.1% peptone + 0.1% Na₂S₂O₃ + 0.5% Tween 80 + 0.07% lecithin)^{33,34} was used to suppress the disinfection reaction. Aliquots of the suspension squeezed out from the gel were diluted 10 times in UQA and left to react for 60 min before the mixture was plated. Various ROS (reactive oxygen species) scavengers such as *N*-acetyl-*L*-cysteine (0.1 mM), superoxide dismutase (10 U mL⁻¹), catalase (10 U mL⁻¹), and DMSO (0.1 mM) were added to the bacterial suspension (15 min before the antibacterial tests) to reveal the role of the ROS in disinfection. Control samples spiked with the ROS scavengers were conducted; all the aforementioned ROS scavengers added at the indicated concentrations did not result in any significant reduction of viable bacteria in the suspension. At least 6 replicates were conducted for all experiments.

In order to separate the excess silver leachate in the squeezed water, 2 mL of the treated bacterial suspensions were centrifuged. The pellets were re-suspended in 50 mL of nutrient-rich medium (tryptic soy broth), and then incubated at 37 °C for 5 h to examine the possibility of cell repair under favorable conditions. Sample aliquots were taken every 0.5 h to determine the OD₆₀₀ using a spectrophotometer (Shimadzu UV-1700).

Determination of bacterial cell membrane integrity

The morphological changes of the bacterial cells after exposure to the cryogels were observed using field emission scanning electron microscopy (FESEM; JEOL JSM-7600F). Samples were prepared by fixation with 2% glutaraldehyde for 1 h followed by repeated washing in 0.1 M sodium cacodylate buffer. The samples were subsequently dehydrated in an ethanol series of increasing concentration for 15 min before they were placed in a freeze-dryer for at least 24 h. The dried samples were coated with Pt prior to imaging. The integrity of the bacterial cell membranes was also evaluated by staining the cells with SYTO 9 (6 mM) and propidium iodide (PI, 30 mM) (Live/Dead BacLight kit, Molecular Probes). Stained samples were observed under an epifluorescence microscope (Olympus BX60) using a 100X oil objective. Note that both dyes are excited at 470 nm but emit at different wavelengths, namely 630 nm (PI) and 530 nm (SYTO 9). A nucleic acid double-staining protocol based on energy transfer of SYTO 9 to PI was used to assess the degree of cell-membrane damage.^{35,36} Control and treated samples (about 2×10^6 cfu/mL) were mixed with equal volumes of SYTO 9 (0.6 mM) and PI (3 mM) dye mixture for 15 min in the dark. Singly stained green and red control samples were prepared by incubating live and dead (killed in 70% ethanol for 12 h) cells in SYTO 9 and PI, respectively. Flow-cytometric analyses were conducted using an LSR II flow cytometer (Becton Dickinson).

Intracellular ROS assay

Intracellular ROS generation was assessed by monitoring the enhancement of the fluorescence intensity of the exposed samples relative to that of the control. Bacterial samples were incubated with 2 μ g/mL of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min before they

were exposed to the PSA/AgNP cryogels for 2 h in the dark. The bacterial suspension then was squeezed out and its fluorescence intensity was measured using a microplate reader (Biotek Synergy 2) at excitation and emission wavelengths of 485 and 535 nm, respectively. DCFH-DA is a nonfluorescent dye that is cell-permeable. DCFH-DA hydrolyzes to the nonfluorescent DCFH (2',7'-dichlorodihydrofluorescein) by esterases in the cells. In the presence of ROS, DCFH oxidizes to the fluorescent DCF (2',7'-dichlorofluorescein). Thus, enhanced fluorescence is indicative of an elevated level of ROS in the cell.

Lipid peroxidation assay

The thiobarbituric acid reactive substances (TBARS) assay was employed to determine the occurrence of lipid peroxidation. The TBARS assay measures the formation of malondialdehyde, which is one of the by-products of lipid peroxidation, although there has been some criticism of the specificity of this test. However, the TBARS assay is a well-established method that is widely accepted as a measure of lipid peroxidation. Further details about the assay can be found in review papers.^{37,38} For this assay a bacterial suspension of cell density 10^{10} cfu mL⁻¹ was used. The cells were exposed to the PSA/AgNP cryogels for 15 min before the assay. First 1 mL of the exposed cells was lysed in 0.5% sodium dodecyl sulfate. Then 2 mL of the TBA reagent (that comprises 20% trichloroacetic acid; 0.5% thiobarbituric acid; and 2.5 N HCl) was added to the sample. The resultant mixture was incubated in a water bath at 95 °C for 1 h. After cooling, the mixtures were centrifuged at 10,000 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (Shimadzu UV-1700).

Determination of the leakage of cytoplasmic contents

The bacterial cell-membrane integrity was examined using UV spectrophotometry (Shimadzu UV-1700) at 260 nm. When the bacterial membrane damage is severe, the release of cytoplasmic contents can be detected. The amount of DNA and RNA released from the cytoplasm can be estimated by the detection of the absorbance at 260 nm.^{39,40} After 5 min incubation with the PSA/AgNP and PSA/Ag⁺ cryogels, the bacterial suspension was immediately squeezed out. The bacterial suspension was filtered through a 0.20 µm syringe filter and its absorbance at 260 nm was measured.

Determination of cellular ATP levels

E. coli cells exposed to PSA/AgNP cryogels for various contact times were assayed for ATP (adenosine triphosphate) by a luciferin/luciferase assay using a BacTiter-Glo microbial cell viability assay kit (Promega). The luminescence signal was measured in a microplate reader (Biotek Synergy 2). Triplicate experiments were conducted.

Note that control studies using abiotic samples exposed to cryogel samples were also conducted for all photometric-based assays (i.e., leakage of cytoplasmic contents, ATP, live/dead, intracellular ROS, and TBARS assay) to account for possible interference due to the optical properties of Ag species.

Analytical methods for quantification of Ag species

The procedure used for the Ag speciation study is summarized in Fig. S4a (Supporting Information). Note that the bacterial cells were separated via centrifugation at 10,000 rpm for 15

min. Also, Ag^0 was separated by filtration through a centrifugal ultrafilter (Amicon, MWCO 3 kDa).⁴¹ The extracellular phase was obtained by filtering (0.2 μm) the supernatant of the bacterial suspension. Either an inductively coupled plasma optical emission spectrophotometer (ICP-OES, Perkin Elmer Optima 2000DV) or an inductively coupled plasma-mass spectrometer (ICP-MS, Elan DRC-e) was used to determine the total Ag concentration in the samples. Note that all samples were digested in concentrated HNO_3 prior to total Ag analyses. The concentration of free Ag^+ was determined using a silver/sulfide solid-state ion-selective electrode (ISE, Orion) connected to an ion meter (Orion Dualstar). The method of standard addition⁴² was employed due to the low concentration of Ag^+ in the samples. Note that all standards and samples were buffered with an ionic strength adjuster (Orion) before ISE measurements. The total organic carbon (TOC) concentration in the filtered samples was determined using a TOC analyzer (TOC-V_{CSH}, Shimadzu). All experiments were conducted in triplicate.

Results

Salient properties and bactericidal effects of PSA/AgNP cryogels

Well-prepared cryogels are highly porous with well-interconnected pores (Supplementary Information, Fig. S1) that can repeatedly sorb and desorb water for over 1000 cycles with little change in their morphology and mechanical properties (Figs. 1a and b; Supplementary Information, Fig. S2). The specific pore volumes of both the cryogels range from 2.8 to 3.1 $\text{cm}^3 \text{g}^{-1}$. Furthermore, most of the Ag incorporated is located on the cryogel surface as evidenced by the EDX line-scan profile and Ag elemental mapping that shows the majority of Ag being distributed on the outer surface of the pore walls (Supporting Information, Fig. S3). It was found

that 20 mg of the cryogel (containing less than 4 mg silver) can inactivate 3×10^6 *Escherichia coli* (*E. coli*) cells/s within the first 30 s of contact (Fig. 1c). Note that this unprecedented rapid water disinfection can be achieved without any external energy input unlike methods such as electroporation.^{43,44} Besides *E. coli*, the cryogels are also effective in disinfecting other types of bacteria including *Salmonella typhimurium*, *Bacillus subtilis*, and *Staphylococcus aureus* (Fig. 1c, inset). The cryogels are so effective that the *E. coli* cells contacted with the gels for 3 minutes or longer cannot be revived even after 5 h of incubation in a nutrient-rich medium (Fig. 1d). In view of the excellent bactericidal activity of the PSA/AgNP cryogels, they serve as an interesting model system to unravel the intricate disinfection mechanism by enabling a precise manifestation of the AgNP bactericidal action and to gain a deeper understanding of factors contributing to rapid disinfection.

Contact-killing action of PSA/AgNP cryogels via targeted Ag^+ delivery

To understand the mode of action of the PSA/AgNP cryogels, the disinfection efficacy of the absorbed water that was squeezed from the PSA/AgNP cryogels (that contains dissolved Ag^+) was also studied (Supplementary Information, Fig. S4). The water squeezed from the cryogels has virtually no bactericidal activity (Fig. 2a) indicating that the bacterial cells need to interact with the Ag species on the cryogels for disinfection to take place. One possible mechanism advanced in the literature attributes cell death to injury inflicted from physical interaction with sharp surfaces of the AgNPs.^{21,22,45} However, this contradicts recent reports that found fully reduced AgNPs have no antibacterial effect when the experiments were conducted under anaerobic conditions (that preclude the formation of Ag^+) underscoring the importance of Ag^+ ions.^{16,18,19}

In fact, this study has shown for the first time that there is a close correlation between disinfection efficacy and the cell-bound Ag concentration (Fig. 2a, inset). However, no such correlation was observed for the dissolved Ag concentration (Supplementary Information, Fig. S5). The Ag speciation study revealed that most of the Ag species released from the AgNPs (> 90%) was taken up by the bacteria (Fig. S5b). Only a small fraction ends up in the dissolved phase mainly in the form of dissolved complexes ($< 100 \mu\text{g L}^{-1}$) bound to the phosphate ligands or bacterial exudates.^{46,47} Note that the extracellular TOC concentration was determined to be $6.2 \pm 0.6 \text{ mg L}^{-1}$. The concentration of extracellular Ag^0 was negligible ($< 10 \mu\text{g L}^{-1}$) while no free Ag^+ was detected (Fig. S5b). This explains the lack of antibacterial activity using the indirect contact approach since the majority of the Ag^+ ions in the solution are bound to ligands that reduce their bioavailability to the bacterial cells (Fig. 2a).

PSA/AgNP cryogels destroy cell membranes and the differential disinfection mode of AgNPs and Ag^+ ions

Silver delivered to the cell in the form of Ag^+ ions, which upon uptake, can cause a myriad of adverse effects on the cellular metabolic functions of the bacteria. For example, a rapid drop in the cellular adenosine triphosphate (ATP) content (by 69-86%) was observed following the exposure to PSA/AgNP cryogels (Supplementary Information, Fig. S6). In order to distinguish the specific functions of the Ag domains, comparative tests were performed using PSA cryogels impregnated with bound Ag^+ ions (or PSA/ Ag^+ cryogels without reducing Ag^+ to Ag^0). Although the PSA/AgNP cryogels led to a substantially lower concentration of cell-bound Ag and poorer ATP inhibition than did the PSA/ Ag^+ cryogels, the PSA/AgNP cryogels surprisingly caused severe cell-membrane lesions to the majority of the cells (Fig. 2b-d; Supplementary Information,

Figs. S7). In contrast, bacteria contacted with the PSA/Ag⁺ cryogels did not show any cell-membrane damage; however, the presence of a few elongated cells could indicate stress and disturbed cell division (Fig. 2c; Supporting Information, Fig. S8). This puzzling result implies that the mechanism may be more complex than just the toxicity caused by intracellular Ag⁺ ions alone.

Role of ROS in mediating cell lesions

The time-dependent nature of the cell-membrane damage as shown in Figs. 3a and b (with more details given in the Supplementary Information, Fig. S9) suggested that the lesions on the cells exposed to PSA/AgNP cryogels are caused by chemical reaction rather than physical interaction. Furthermore, if the membrane damage were due to physical puncturing of the cell walls by Ag⁰, random pits would be present all over the cells. The chemical reaction is probably a self-propagating one since sustained exposure of the cells to PSA/AgNP cryogels caused the indentations on the cells to develop into holes that are localized mainly in the mid-point of most injured cells (Fig. 3a). It is hypothesized that such damage is likely mediated by ROS whose presence could initiate a chain reaction. Indeed, the results from a ROS scavenging test confirm the involvement of ROS ($\cdot\text{O}_2^-$, H_2O_2 , and $\cdot\text{OH}$) in the bactericidal action of PSA/Ag cryogels (Supplementary Information, Fig. S10); it is known that $\cdot\text{O}_2^-$ and H_2O_2 are precursors to the more reactive $\cdot\text{OH}$ species. In addition, it was found that both PSA/AgNP and PSA/Ag⁺ cryogels could induce the formation of intracellular ROS albeit to different extents (Fig. 3c). This observation was again perplexing because, even though the PSA/Ag⁺ cryogels had a higher Ag content and delivered more Ag⁺ into the cells than the PSA/AgNP cryogels (AgNC-90 and

AgNC-170), the amount of ROS generated by the PSA/Ag⁺ cryogels was significantly lower (Fig. 3c). This result suggested the critical role of AgNPs in ROS formation.

The resultant ROS, especially $\cdot\text{OH}$, can subsequently injure cells by reacting with various biomolecules. One possible route for ROS-mediated cell injury is via lipid peroxidation of the cellular membrane as indicated by the results from the TBARS (thiobarbituric acid reactive substances) assay in this study (Supplementary Information, Fig. S11); an enhanced TBARS level was assumed to be indicative of the presence of lipid peroxidation. As noted earlier, both *E. coli* and *B. subtilis* cells that were exposed to PSA/Ag cryogels show an elevated level of TBARS (Fig. S11). A higher amount of TBARS was detected for cryogels with a higher Ag content that may be attributed to the formation of more ROS (specifically $\cdot\text{OH}$) (Fig. 3c; Supporting Information, Fig. S11). Increased TBARS levels in bacteria have also been observed when exposed to other ROS-generating materials such as Cu-based materials, graphene oxide, and TiO_2 among others.⁴⁸⁻⁵¹ These studies also provide evidence that although the level of polyunsaturated fatty acids (PUFAs) in bacterial membranes is low, ROS-mediated injury can proceed via a lipid peroxidation mechanism; note that PUFA is the preferred substrate for lipid peroxidation because it forms a radical that is stabilized by molecular rearrangement to form a conjugated diene. Furthermore, overexpression of *yqhD* — the gene responsible for aldehyde reductase activity for cell defence against the harmful effects of aldehydes derived from lipid peroxidation — in *E. coli* when exposed to ROS-generating compounds provides further evidence of the occurrence of lipid peroxidation in bacterial membranes.⁵²

Discussion

Based on this evidence, it is proposed that there are two concerted pathways that enable the PSA/AgNP cryogels to achieve a virtually instantaneous bacterial disinfection (Fig. 4). The cryogels provide a conducive physical pathway (which is densely decorated with AgNPs) for cell-AgNP interaction due to their unique micro-architecture and swelling behavior. Rapid imbibition of water by the hydrophilic cryogel network essentially drives an instant flow of bacterial cells into the tortuous microchannels of the cryogels where the cells can interact with the exposed AgNPs (anchored on the cryogel pore surface) within a short diffusional distance (Fig. 4a); note that these interactions with the AgNPs can happen again during the one-second water recovery or expulsion step. While in the microchannels, close proximity between the AgNPs and the cells is assured due to comparable size scales (of the microchannels and cells).

Close proximity between the AgNPs and cells is just one of the prerequisites to trigger a cascade of events. It is known that a narrow region outside the bacterial cell wall is acidified due to proton extrusion under the influence of a proton motive force^{16,53} (Supplementary Information, Fig. S12). Furthermore, the high surface-charge of the PSA/AgNP cryogels (due to the presence of numerous carboxylate groups) could lower the interfacial pH as a cell approaches the surface.^{54,55} Consequently, Ag^0 dissolution at the cell-AgNP interface is substantially accelerated due to rapid oxidation under low-pH conditions (Supplementary Information, Fig. S13). The enhanced Ag^0 dissolution at the cell-AgNP interface translates into a unique "on-demand" Ag^+ -release behaviour (i.e., triggered in the presence of bacteria), which is an interesting new insight on the bactericidal action of AgNPs (Fig. 4b). In fact, the Ag speciation data reveal that only PSA/AgNP but not PSA/ Ag^+ cryogels display the on-demand release behavior as shown by their significantly higher cell partition ratio (Supplementary Information, Fig. S14). For PSA/AgNP cryogels, more than 95% of the Ag consumed is attributed to cell-uptake while for the case of

PSA/Ag⁺, about 30% of the Ag consumed dissolves into the bulk solution resulting in waste and potential hazards. These new insights on the triggered-Ag⁺-release provide a plausible explanation for the enhanced disinfection with improved AgNP contact and excellent disinfection efficacies despite the observed low Ag leaching for PSA/AgNP cryogels and other Ag nanohybrids that were previously not understood.^{8,10-12,14,56}

Cellular uptake of the Ag⁺ results in a chain of events in the subsequent metabolic pathway (Fig. 4b). First, the cells are almost instantaneously inactivated due to metabolic arrest as a consequence of a drastic drop in the ATP level. Ag⁺ has been found to inhibit ATP formation by either behaving like a protonophore⁵⁷ or by inhibiting enzymes in the respiratory chain^{58,59} to dissipate the proton motive force. In addition, the interaction between Ag⁺ and coupling enzymes (bearing cysteine groups with a strong affinity to Ag⁺ such as NADH and succinate dehydrogenase) impedes the electron-shuttling process causing the increased intracellular production and build-up of $\cdot\text{O}_2^-$ and H_2O_2 .^{58,60-62} The presence of Ag⁺ also enhances the susceptibility of the bacterial cells to ROS by depleting intracellular antioxidants such as glutathione.⁶³ Note that the *E. coli* membrane shows substantial permeability (16 $\mu\text{m/s}$) towards H_2O_2 .⁶⁴ Therefore, when H_2O_2 persists long enough, which is the case when the ROS defence mechanism is compromised, it can efflux from the cell to react with the AgNPs (primarily Ag⁰) forming $\cdot\text{OH}$ (Fig. 4c). In fact, a recent study has shown that AgNPs can form $\cdot\text{OH}$ in the presence of H_2O_2 , but only under acidic conditions.⁶⁵ Although the proposed Fenton-like pathway⁶⁵ for the AgNP-induced formation of $\cdot\text{OH}$ requires further validation, other corroborative studies shed some insight into the reaction mechanisms.^{66,67} Hence, effective decomposition of H_2O_2 to $\cdot\text{OH}$ in the present study is anticipated since the close proximity between the cell and the exposed AgNPs provides the prerequisite acidic conditions. The

resultant $\cdot\text{OH}$ can react with the cell membrane causing severe structural distortion resulting in impaired cell functions that culminate in cell death (Fig. 4c). In the present study the injury was severe as indicated by the leakage of cytoplasmic contents causing irreversible cell damage (Supplementary Information, Fig. S15). Evidently, both Ag^+ and AgNPs work together to effect disinfection; $\cdot\text{OH}$ would not have been formed if either Ag^+ or AgNP were absent.

The mechanism proposed here provides a conceptual framework to better understand the bactericidal action of AgNPs in general and in the specific case of our PSA/AgNP cryogels. The elusive mechanism can be unraveled because the cryogels provide a conducive environment to elicit the intricacies of the bactericidal action mode of AgNPs; they not only provide a high surface-area platform for cell-AgNP interaction in the microchannels, but the rapid swelling of the cryogels also provides the driving force for the bacterial cells to approach the AgNPs. The intimate contact between the AgNP and cells within a confined environment ensures their interaction to be biologically significant. This enables the manifestation of triggered- Ag^+ release that reveals the interaction of the protons in the cell-AgNP interface (generated from cell metabolism and — to a lesser extent — the high surface-charge of the cryogels) with the Ag^0 resulting in efficient uptake of Ag^+ by the contacting bacteria. This new insight can guide future design of environmentally safe Ag-based disinfectants that exhibits excellent disinfection with very low Ag leaching. Furthermore, the use of cryogels impregnated with either Ag^0 or Ag^+ allows uncovering their differential bactericidal action while minimizing confounding effects due to solution chemistry (e.g., aggregation and complexation) when the AgNPs or Ag^+ are directly dispersed in the exposure medium. As a result, the disparity in the cell injury caused by the Ag^+ alone versus that of AgNPs (that show the combined effect of Ag^0 and Ag^+) can be clearly distinguished. Most importantly, it was found that both Ag^+ and Ag^0 are involved in the

bactericidal mechanism of AgNPs in the cryogels. We believe that this ultimately resolves the long debate over the disinfection mechanism of AgNPs. Although most mechanistic studies reported in this paper were on *E. coli*, PSA/AgNP cryogels have been shown to work with both Gram-negative and Gram-positive bacteria. It is believed that the mechanistic understanding developed herein could be extended to other bacteria although this requires further study.

Associated content

Method description for PSA/AgNP and PSA/Ag⁺ cryogels fabrication, and quantification of pore parameters via image analysis; and additional results (Figs. S1-15). This material is available free of charge via the Internet at <http://pubs.acs.org>.

Acknowledgments

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- Fig. 2** (a) Comparison of the disinfection efficacies of the cryogels via direct or indirect contact with *E. coli* cells; the inset shows the correlation between bacterial disinfection efficacies with Ag^+ uptake. (b) Comparison of the cell-bound Ag concentration, and the extent of ATP reduction in cells exposed to PSA/ Ag^+ (116.0 ± 7.9 mg/g) and PSA/AgNP cryogels (Ag content of 166.7 ± 15.0 mg/g). FESEM images of *E. coli* cells after a 5-min exposure to (c) PSA/ Ag^+ cryogels and (d) PSA/AgNP cryogels. Note: the scale bars represent $0.5 \mu\text{m}$. The green arrow in (c) points to a cell that appears to be elongated indicative of stress, while the red arrows in (d) point to the holes on the cells.
- Fig. 3** (a) FESEM images, and (b) Fluorescence images (recorded using dual color channels) of *E. coli* cells contacted with the cryogels for various times. The inset of (b) shows the corresponding fluorescence images recorded using the red channel. The scale bars in (a) represent $1 \mu\text{m}$. (c) Fluorescence measurement of intracellular ROS generation using PSA/AgNP cryogels with different Ag content, and PSA/ Ag^+ cryogels (120 mg Ag/g). Significant differences between the fluorescence intensity of the exposed cells are denoted by asterisks: $*p < 0.001$ (Student's t test, $n = 3$). Note that RFU denotes relative fluorescence unit. (d) Intracellular ROS fluorescence images of the control cells and those exposed to the PSA/AgNP cryogels; increased fluorescence indicates enhanced buildup of intracellular ROS.
- Fig. 4** Illustration summarizing the proposed bactericidal mechanism of PSA/AgNP cryogels. (a) Rapid water absorption draws an immediate flow of bacteria into the microchannels of the cryogels allowing frequent encounter with the anchored AgNPs. (b) pH-lowering effect as the cell approaches the AgNPs interface triggers the release of Ag^+ . The Ag^+ is instantaneously taken up by the cell and subsequently inhibits ATP synthesis and the electron transport chain. The latter process results in the indirect formation of ROS ($\cdot\text{O}_2^-$ and H_2O_2). (c) In an acidic environment, AgNPs can form $\cdot\text{OH}$ by causing the decomposition of H_2O_2 . The resultant $\cdot\text{OH}$ can initiate a peroxidative sequence that inevitably results in cell death by destroying the cell membrane.⁶⁸ Note: $\cdot\text{L}$ and $\text{LOO}\cdot$ denote a carbon-centered radical and a peroxy radical, respectively.

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