Mechanism of virus inactivation by cold atmospheric-pressure plasma and plasma-activated water

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Abstract
Viruses are serious pathogenic contamination that severely affect the environment and human health. Cold atmospheric-pressure plasma efficiently inactivates pathogenic bacteria, however, the mechanism of virus inactivation by plasma is not fully understood. In this study, surface plasma in argon mixed with 1% air and plasma-activated water were used to treat water containing bacteriophages. Both agents efficiently inactivated bacteriophages T4, Φ174, and MS2 in a time-dependent manner. Prolonged storage had marginal effects on the anti-viral activity of plasma-activated water. DNA and protein analysis revealed that the reactive species generated by plasma damaged both nucleic acid and proteins, in consistent with the morphological examination showing that plasma treatment caused the aggregation of bacteriophages. The inactivation of bacteriophages was alleviated by the singlet oxygen scavengers, demonstrating that singlet oxygen played a primary role in this process. Our findings provide a potentially effective disinfecting strategy to combat the environmental viruses using cold atmospheric-pressure plasma and plasma-activated water.

Importance
Contamination with pathogenic and infectious viruses severely threaten human health and animal husbandry. Current methods for disinfection have different disadvantages, such as inconvenience and contamination of disinfection by-products (e.g. chlorine disinfection). In this study, atmospheric surface plasma in argon mixed with air and plasma-activated water were found to efficiently inactivate bacteriophages, and...
plasma-activated water still had strong anti-viral activity after prolonged storage. Furthermore, it was shown that bacteriophage inactivation was associated with the damage to nucleic acid and proteins by singlet oxygen. The understanding of the biological effects of plasma-based treatment is useful to inform the development of plasma into a novel disinfecting strategy with convenience and no by-product.

**Keywords**: virus, cold atmospheric-pressure plasma, plasma-activated water, bacteriophage, reactive oxygen species
Introduction

Contamination with microorganisms, especially pathogenic and infectious viruses, such as poliovirus and foot-and-mouth disease virus, is a threat to public health and animal husbandry. Current disinfection methods include ultraviolet irradiation and chemical disinfectants, the former requiring long processing time and the latter leaving the by-product contamination (1, 2). Thus, development of efficient and safe disinfection strategies of these pathogenic microorganisms is of great significance for human health (3).

Cold atmospheric-pressure plasma (“plasma” for short hereafter) generates at or near room temperature numerous reactive oxygen and nitrogen species (ROS and RNS), such as hydrogen peroxide (H$_2$O$_2$), singlet oxygen (^1O$_2$), ozone (O$_3$), nitric oxide (NO), and hydroxyl radical (OH) as well as electrons, ions and photons. These make plasma attractive for biomedical and environmental applications (4-9).

Currently, plasma has been widely studied for bacterial inactivation and as therapy of infectious diseases (10, 11). Previous studies have reported that a form of plasma, dielectric barrier discharge (DBD), efficiently inactivated very small volumes (20 or 50 μL) of dry and wet Φ174 and λ bacteriophages in Tris-ethylenediaminetetraacetic acid (EDTA) buffer by damaging of protein and DNA of bacteriophages (12-14).

Further, MS2 and feline calicivirus could also be inactivated by direct treatment of a plasma jet, the disadvantage of which was the limitation of the treatment area (15, 16).

Recently, Su et al. used plasma-activated water, saline, and 0.3% H$_2$O$_2$ that were pretreated with a plasma jet to treat Newcastle disease virus and decreased the...
infectivity (17). Therefore, plasma and plasma-activated solutions have become potential alternative disinfectants.

Given the technical challenges and potential safety risk of working with pathogenic viruses, surrogate viruses—bacteriophages—are used to evaluate the anti-viral activities of the plasma in this study. Three bacteriophages with different types of nucleic acids, T4 (double-stranded DNA), Φ174 (single-stranded DNA), and MS2 (RNA) were selected and a surface discharge plasma was used in this study. A surface plasma or water activated by the surface plasma were used to treat water with bacteriophages, respectively. Our aim is to demonstrate anti-viral activity of the surface plasma and surface-plasma-activated water as well as unravel underlying disinfection mechanisms of the surface plasma and plasma-activated water.

**Materials and methods**

**Plasma device and water treatments**

The surface discharge device consisted of a plane high-voltage electrode, a liquid-facing grounded mesh electrode, and a dielectric layer (made of polytetrafluoroethylene) sandwiched between the two electrodes (Fig. 1A). The surface plasma is generated in the mesh elements of the grounded electrode when a sinusoidal high-voltage is applied, and the discharge power density was maintained at 0.2 W/cm² for this study. As shown in Fig. 1A, each mesh element has a hexagonal shape, and the plasma has a good mesh-to-mesh homogeneity. The petri dish had a dimension of 11 cm × 7 cm (length × width), which was much smaller than that of the surface plasma (12 cm x 8 cm). The bacteriophage suspensions or water (8 mL) in the
petri dish with the depth \((L_w)\) of about 1 mm was placed underneath the plasma, while the air gap \((L_g)\) between the plasma and the liquid surface was 8 mm. The surface air plasma and the bacteriophage suspension were well sealed in an organic glass box. A gas mixture of argon and artificial air \((79\% \text{ N}_2 + 21\% \text{ O}_2)\) was allowed to flow through the box at a constant rate of 4 L/min, and the volume ratio of artificial air was controlled at 1%. Compared to the surface discharge in air, the addition of argon enhanced the production efficiency of the reactive species and their fluxes on underneath the bacteriophage suspension or water by diffusion. For more detail of the surface discharge reactor, please refer to our previous reports (18, 19).

**Measurement of aqueous ROS and RNS generated by plasma**

The concentrations of \(\text{H}_2\text{O}_2\) and \(\text{NO}_2^-/\text{NO}_3^-\) in the water were measured using a hydrogen peroxide/peroxidase assay kit (Thermo Fisher Scientific) and a nitrite/nitrate colorimetric assay kit (Cayman), respectively. \({\cdot}\text{OH}, \cdot\text{O}_2, \cdot\text{NO}, \text{O}_2^-\), \(\cdot\text{NO}_2,\) and \(\cdot\text{ONOO}^-\) were measured using an electron spin resonance (ESR) spectroscopy (Bruker) with relevant spin traps (20). The spin traps were 100 mM 5,5-Dimethyl-1-pyrroline N-oxide (DMPO, Dojindo) for trapping \({\cdot}\text{OH}, 5 \text{ mM N-(Dithiocarbamoyl)-N-methyl-D-glucamine (MGD, Dojindo) for } \cdot\text{NO}, 10 \text{ mM 2,2,6,6-Tetramethylpiperidine (TEMP, TCI) for } ^1\text{O}_2 \text{ and 10 mM 1-hydroxy-2,2,6,6-tetramethylpiperidine (TEMPONE-H, Enzo) for } \text{O}_2^-\), \(\cdot\text{NO}_2,\) and \(\cdot\text{ONOO}^-\).

**Bacteriophages propagation and inactivation assay**

Bacteriophages T4 (provided by Dr. Xiaoqin Lai, Institute of Microbiology, Chinese Academy of Sciences) were propagated and inactivated under plasma.
Academy of Sciences, Φ174 (provided by Dr. Yigang Tong, Beijing Institute of Microbiology and Epidemiology) and MS2 (ATCC 15597-B1) and their host strain *Escherichia coli* JM109 (ATCC53323) were used. The phages (1 mL) was used to infect an early stationary phase culture of the host strain (100 mL), and infected cultures were then propagated at 37°C for 3-5 h. Phage suspensions were then prepared with the infected cultures by centrifugation at 3000 × g for 10 min to remove the bacterial cells and debris and by filtration with 0.22 μm syringe filters (Millipore). The filtered supernatants were dialyzed with water using a concentrator (10 kDa molecular cutoff, Millipore), yielding stocks of phage suspension with a titer of 10^{11-12}. The stocks were stored at 4°C and investigated in 2 weeks.

Bacteriophage suspensions were treated with plasma for increasing times, plasma-activated water (the volume ratio 1:1) that were pretreated with plasma for increasing times, 500 μM H_{2}O_{2} + 75 μM NO_{2}^- + 500 μM NO_{3}^- or 1% formaldehyde, then incubated at 22°C for 1 h or indicated times, and the bacteriophage titers in the water were measured using the double agar layer method. The early stationary phase host strain was collected by centrifugation, washed once with 10 mM magnesium sulfate (MgSO_{4}) and suspended in 10 mM MgSO_{4}. After treatment, 100 μL phages were incubated with 200 μl host strain suspension at 37°C for 20 min. Then the incubated solutions were mixed with soft agar (LB broth with 0.7% agar), that was preheated to 45°C, and plated onto the bottom layer of the agar (LB broth with 1.5% agar). The plates were cultured at 37°C overnight, and the plaques were subsequently counted.
Bacteriophages inactivation assay of the plasma-activated water after storage

The water was treated with plasma for 100 s and stored in 1.5-mL Eppendorf tubes made of polypropylene or glass tubes at 22°C in the dark or in the light for different days as indicated. After storage, the plasma-activated water was mixed with T4 bacteriophage suspensions (the volume ratio 1:1) and incubated at 22°C for 1 h. Then the bacteriophage titers in the water were measured using the double agar layer method as described previously.

Analysis of bacteriophage DNA

Phage genomic DNA was extracted from treated or untreated T4 bacteriophage samples using a viral DNA kit (Omega). Then the T4 DNA samples were digested with DraI (Takara) and separated using 0.8% agarose gels in 0.5×Tris/borate/EDTA (TBE) buffer at 50V for 3 to 4 h. The gels were stained with ethidium bromide (EtBr), then examined and photographed using a BioDoc-It Imaging System (UVP).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

T4 bacteriophages treated with plasma, plasma-activated water, or untreated (20 μL) were mixed with Laemmli buffer (Bio-Rad) and analyzed using 6-15% gradient SDS-PAGE. The gels were stained with Coomassie blue R-250 and scanned using a GS-800 calibrated densitometer (Bio-Rad).

Transmission electron microscopy (TEM)

T4 bacteriophages treated with plasma, plasma-activated water, or untreated were dropped onto carbon-coated grids and kept for 10 min at 22°C. Then, the excess liquid
was discarded and the grids were covered with 1% uranyl acetate for 30 s at 22°C. These stained samples were examined using an FEI Talos F200C transmission electron microscope operating at 200 kV at ×28000 magnification.

**Analysis of ROS and RNS scavengers**

Chemical scavengers for ROS and RNS, mannitol (MP Biomedicals), tiron (Sigma-Aldrich), sodium azide (Sigma-Aldrich), L-histidine (Sigma-Aldrich), and ebselen (TCI) were used. For direct plasma treatment, the final concentration of 200 mM mannitol, 20 mM tiron, 10 mM sodium azide, 10 mM L-histidine or 1 mM ebselen was added into the water containing bacteriophages to the indicated concentration. Then, the samples with or without scavengers were treated with plasma for 2 min and incubated at 22°C for 1 h. For the plasma-activated water treatment, the final concentration of 200 mM mannitol, 20 mM tiron, 10 mM sodium azide, 10 mM L-histidine or 1 mM ebselen was added into water to the indicated concentration before or after plasma treatment for 2 min. Then, the water with or without scavengers was incubated with an equal volume of water containing bacteriophages at 22°C for 1 h. The inactivation rates were examined as described above.

**Statistical analysis**

All experiments were performed independently at least three times. Statistical analysis was performed using Graph-Pad Prism, version 5. Error bars in all graphs show the standard deviation (± SD). Statistical analyses were performed in SPSS 13.0 (IBM, Armonk, NY, U.S.A.) using the t-test and Kruskal–Wallis test. Statistical significance of data was established at P < 0.05.
Results and discussion

ROS and RNS generated by plasma

Numerous types of gaseous ROS and RNS are generated by surface discharge, and some diffuse across the air gap ($L_g$) and then dissolved into the liquids. The plasma-induced aqueous ROS and RNS levels in the water were measured after plasma treatment for 1 and 2 min. We found that the long-lived species $\text{H}_2\text{O}_2$, $\text{NO}_2^-$, and $\text{NO}_3^-$ as well as the short-lived species $'\text{OH}$, $'\text{O}_2$, $'\text{NO}$, $'\text{O}_2^-$, $'\text{NO}_2$, and ONOO$^-$ diffused into the water. The concentrations of aqueous $\text{H}_2\text{O}_2$, $\text{NO}_2^-$, and $\text{NO}_3^-$ after 2 min plasma treatment were 221, 8 and 216 $\mu$M, respectively (Fig. 1B).

Short-lived species of ROS and RNS were identified and quantitated by ESR spectroscopy by using four spin traps DMPO for trapping $'\text{OH}$, MGD for $'\text{NO}$, TEMP for $'\text{O}_2$ and TEMPONE-H for $'\text{O}_2^-$, $'\text{NO}_2$, and ONOO$^-$. The results were the concentrations of spin adducts, which only reflected the relative concentrations of the specific ROS and RNS. The concentrations of the spin adducts, DMPO-$'\text{OH}$, TEMPO, nitrocyl-Fe, and TEMPONE after a 2-min plasma treatment were 0.5, 145, 30, and 188 $\mu$M, respectively (Fig. 1B). These results indicated that the concentration of aqueous $'\text{OH}$ should be very low, while that of aqueous $'\text{O}_2$ should be much higher.

ROS and RNS generated by plasma are not a simple system but chaotic system involved in numerous chemical reactions (19). ROS and RNS are widely believed to play a crucial role in the plasma-induced biological effects (21).

Inactivation of bacteriophages by plasma or plasma-activated water

To evaluate the abilities of plasma and plasma-activated water to inactivate
bacteriophages, different bacteriophages suspensions were exposed to plasma or incubated with the plasma-activated water that were pretreated with plasma for increasing times, and the infectivity of treated bacteriophages was assayed by determination of their residual PFU. Direct treatment of T4 bacteriophage with plasma for 40 s reduced the PFU from $8.7 \times 10^{10}$ to $3.7 \times 10^5$ PFU/mL, and 80 s treatment gave a residual infectivity of approximately 400 PFU/mL, indicating more than 99.99% phages lost their infectivity. Furthermore, treatment with plasma for 100 s completely abolished the infectivity of the T4 bacteriophage suspension (Fig. 2A). Treatment of T4 bacteriophage with plasma-activated water that was pretreated with plasma for 60 s or 120 s for 1 h reduced the level from $5.8 \times 10^{11}$ to $6.0 \times 10^6$ and to approximately 20 PFU/mL, respectively (Fig. 2B), suggesting that the effective species could be stably maintained in the plasma-treated water. The same experiments with Φ174 and MS2 bacteriophages showed that, after treatment with plasma for 30 s, the infectivity of Φ174 and MS2 bacteriophages was reduced by approximately 4.2 and 4.6 orders of magnitude, respectively, and both phages were almost completely inactivated after 60 s treatment (Fig. 2A). These results indicated that Φ174 and MS2 bacteriophages are more sensitive to ROS and RNS generated by plasma than T4 bacteriophages. Then, the sensitivity of Φ174 and MS2 bacteriophages to plasma-activated water were tested by incubation of bacteriophage suspension and the water pretreated with plasma for 60 s and this revealed that the number of infective phages was reduced close to the detection limit for both phages (Fig. 2B). These results indicated that both plasma and plasma-activated water effectively inactivated
the bacteriophages.

T4, Φ174, and MS2 bacteriophages were almost completely inactivated by plasma-activated water that was pretreated with plasma for 120 s (for T4) or 80 s (for Φ174 and MS2) (Fig. 2B). Then, water pretreated with plasma for half of the time, 60 s for T4 or 40 s for Φ174 and MS2, was incubated with bacteriophage suspensions, and the bacteriophages infectivity at different time points was measured (Fig. 3). After incubation for 4 and 8 h with plasma-activated water, the T4 bacteriophage was reduced approximately 7.2 and 8.8 orders of magnitude, respectively (Fig. 3A). The Φ174 and MS2 bacteriophages were reduced to 124 and 21 PFU/mL after incubation with plasma-activated water for 6 h and for 4 h, respectively (Fig. 3B and C). These results exhibited that the inactivation of bacteriophages by plasma-activated water was time-dependent. Compared with the treatment of 1‰ formaldehyde, a reagent for virus inactivation, which only reduced the levels by less than 2 orders of magnitude after incubation for 8 h, the plasma-activated water was more effective (Fig. 3).

Then, the term of validity of the plasma-activated water was measured, and plasma-activated water stored at 22°C for 10 days exhibited a slightly weaker anti-viral activity than that of the freshly prepared plasma-activated water, probably due to the decay of reactive species during the storage (Fig. 3D). The plasma-activated water stored in glass tubes did not exhibit differences in inactivating viruses from that stored in Eppendorf tubes, indicating that plastic ware and glassware were both suitable for storing plasma-activated water (Fig. S1). Moreover, exposure to light did not significantly undermine the anti-viral activity of plasma-activated...
water since there is no significant difference compared with that stored in the dark (Fig. S1). Despite its slightly reduced anti-viral activity, the plasma-activated water was storable and transportable, demonstrating that the plasma-generated ROS and RNS might be used in both gaseous and aqueous form depending on the mode of application.

**Plasma caused aggregation of bacteriophages**

Next, we investigated how the plasma could inactivate the bacteriophages. To this end, the DNA and proteins of T4 bacteriophage treated with plasma or plasma-activated water were analyzed. A large fraction of T4 genomic DNA was retained in the loading wells for T4 bacteriophage treated with plasma or plasma-activated water, while the DNA from untreated T4 bacteriophage exhibited a single band (Fig. 4A). However, after digestion with DraI, the signals in the sample wells were digested, and the DNA from both treated and untreated samples exhibited similar bands (Fig. 4A). The results suggested that T4 genomic DNA could have been cross-linked themselves, or with the coat proteins during plasma-based treatment, forming large DNA-protein complexes that cannot migrate during agarose gel electrophoresis. Compared with the untreated T4 bacteriophage, parts of the proteins from T4 bacteriophage treated with plasma or plasma-activated water slightly decreased, indicated by grey rectangles, which probably due to the degradation of proteins through oxidation (Fig. 4B) (22). The damage induced by plasma-activated water was lesser than that induced by direct plasma treatment, which was likely because plasma-activated water lacked part of the species generated by plasma, such as some short-lived reactive species and UV (23).
These results indicated that reactive species of plasma induced both DNA and protein damages to bacteriophages.

Further, the morphological changes in the T4 bacteriophage induced by plasma and plasma-activated water were investigated using TEM. The untreated T4 bacteriophages exhibited a typical structure with an icosahedron head and tail (Fig. 4C). After plasma or plasma-activated water treatment, the T4 bacteriophages were seriously aggregated and formed large complexes (Fig. 4C). The morphological study suggested that reactive species of plasma caused an interaction between adjacent bacteriophages, leading to the aggregation of T4 bacteriophages.

Singlet oxygen played a primary role in bacteriophage inactivation

Plasma-treated solutions contain numerous active species, such as the long-lived species $\text{H}_2\text{O}_2$, $\text{NO}_2^-$, and $\text{NO}_3^-$ as well as the short-lived species $\cdot\text{OH}$, $^1\text{O}_2$, $\cdot\text{NO}$, $\text{O}_2$•, \NO, and ONOO$^-$ (Fig. 1B). To evaluate the effect of long-lived species, a mixture of $\text{H}_2\text{O}_2$ (500 μM), $\text{NO}_2^-$ (75 μM), and $\text{NO}_3^-$ (500 μM) was used to treat the bacteriophages. However, this only reduced the levels by 1.6–3 orders of magnitude, indicating that the three long-lived species are not the main functional factor (Fig. 3).

Hence, short-lived species should be considered more important. ROS and RNS scavengers, mannitol for $\cdot\text{OH}$, tiron for $\text{O}_2$•, sodium azide and L-histidine for $^1\text{O}_2$, and ebselen for ONOO$^-$ were used to distinguish the role of the different short-lived species, and the scavengers did not obviously affect the bacteriophage infectivity (Fig. 5A). Sodium azide and L-histidine almost entirely eliminated the inactivation effects of direct plasma treatment, whereas other ROS and RNS scavengers exhibited
non-significant effects (Fig. 5B). The effects of plasma-activated water were also
eliminated by sodium azide and L-histidine, which were added to the water both
before and after plasma treatment, and were not eliminated by other ROS and RNS
scavengers (Fig. 5C). Singlet oxygen was detected in both water treated with plasma
directly and plasma-activated water using a highly selective probe of singlet oxygen
— trans-1-(2’-methoxyvinyl)pyrene (tMVP) (Fig. S2). These data suggested that
singlet oxygen was the main functional species of the plasma and plasma-activated
water in the inactivation of bacteriophages.

Singlet oxygen is highly active and readily react with various biological
molecules including DNA and proteins (24-26). Singlet oxygen rapidly reacts with
cysteine to generate the major product of cystine (R-cys-S-S-cys-R) with disulfides,
which could result in the interaction and aggregation of bacteriophages (22). Singlet
oxygen also selectively reacted with tyrosine, tryptophan, and histidine to produce
hydroperoxides, which could inactivate enzyme activities (22). For DNA, singlet
oxygen could oxidize guanine and induce cross-links between guanine and lysine,
which would be responsible for the large complexes formed by T4 genomic DNA (27).
The bacteriophage inactivation by singlet oxygen generated in UV illuminated fullerol
similarly induced the cross-linking of capsid proteins which were the probable cause
of phage inactivation (28). The inactivation of bacteriophages by plasma was mainly
mediated by the multiple effects of singlet oxygen.

Plasma-based treatment provides an effective strategy for environmental
disinfection in spite of some limitations. Singlet oxygen could also react with a wide
range of organic compounds, such as olefins and phenols (29). The presence of organic compounds would inevitably reduce the inactivation ability of plasma and plasma-activated water. Therefore, plasma-based disinfectants are more applicable to space and water disinfection, which contains less organic matter.

**Conclusions**

Based on these results, a model of bacteriophage inactivation by plasma and plasma-activated water was proposed (Fig. 6). Plasma-generated reactive species, especially singlet oxygen, efficiently inactivated different kinds of bacteriophages in water, including double-stranded DNA, single-stranded DNA, and RNA bacteriophages, by damaging both nucleic acid and proteins, and causing aggregation of the bacteriophages. It is useful for the design and improvement of novel plasma devices with the potential application by understanding the biological and chemical mechanisms of virus inactivation by plasma-based treatment. Compared with ROS derived from inorganic or organic chemicals, ROS generated in plasma was the direct additives, so it would not bring chemical residual contamination after the treatment process. Plasma-based treatment efficiently inactivated different classes of viruses and could be explored as a novel strategy for disinfection to combat environmental problems caused by viruses.

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**Figure legends**

Figure 1. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by surface plasma. **A.** Diagram of bacteriophage suspensions or water treated with surface plasma. **B.** The concentrations of ROS, RNS, and spin trap in the water treated with plasma for 1 and 2 min.

Figure 2. Inactivation of bacteriophages by plasma and plasma-activated water. **A.** Direct plasma treatment. **B.** Plasma-activated water treatment. Bacteriophage suspensions were directly treated with plasma or mixed with water that was treated with plasma, for the indicated times, and the treated samples were incubated for 1 h at 22°C. Surviving infectivity was quantified using serial dilution, plating, and counting of the resulting PFU. Data are representative of three independent experiments. Error bars represent standard deviation (SD).

Figure 3. Comparative analysis of bacteriophages inactivated by plasma-activated water. **A.** T4. **B.** Φ174. **C.** MS2. Bacteriophage suspensions were directly treated with water treated with plasma for 60 s or 40 s, 500 μM H₂O₂ + 75 μM NO₂⁻ +500 μM NO₃⁻ or 1% formaldehyde, and the treated samples were incubated at 22°C. Surviving infectivity was quantified at indicated time points using serial dilution, plating, and counting of the resulting PFU. **D.** The storage of plasma-activated water. Plasma-activated water was stored at 22°C for indicated times. Then the plasma-activated water were mixed with bacteriophage suspensions and incubated at 22°C for 1 h. Surviving infectivity was quantified using serial dilution and plating for
PFU. Data are representative of three independent experiments. Error bars represent standard deviation (SD).

Figure 4. Analysis of T4 bacteriophages treated with plasma and plasma-activated water. A. The genomic DNA of T4 bacteriophages. The genomic DNA of T4 bacteriophage treated with plasma, plasma-activated water, and untreated digested with DraI or undigested were separated using 0.8% agarose gels and stained with ethidium bromide (EtBr). B. The proteins of T4 bacteriophages. The proteins of T4 bacteriophage treated with plasma, plasma-activated water, and untreated were analyzed by 6–15% gradient SDS-PAGE and stained with Coomassie blue R-250. C. Transmission electron microscopy (TEM) analysis of T4 bacteriophages treated with plasma and plasma-activated water. The T4 bacteriophage treated with plasma, plasma-activated water, and untreated were negative-stained and examined using TEM.

Figure 5. Singlet oxygen of plasma played a primary role in bacteriophages inactivation. A. The scavengers for reactive oxygen species (ROS) and reactive nitrogen species (RNS) did not evidently affect the infectivity of bacteriophages. ROS and RNS scavengers added to water with bacteriophages and incubated for 1 h at 22°C. B. Direct plasma treatment. The ROS and RNS scavengers added to bacteriophage suspensions, then the bacteriophage suspensions treated with plasma directly and incubated for 1 h at 22°C. C. Plasma-activated water treatment. The ROS and RNS scavengers added into the water before or after the plasma treatment. Then the plasma-activated water in the presence and absence of scavengers incubated with
bacteriophage suspensions and kept for 1 h at 22°C. Surviving infectivity was quantified by serial dilution, plating, and counting of the resulting PFU. Data are representative of three independent experiments. Error bars represent standard deviation (SD).

Figure 6. The inactivation of bacteriophage T4 by reactive oxygen species (ROS) and reactive nitrogen species (RNS) of plasma.
Figure A: Survival infectivity (Lg PFU/ml) of bacteria when scavengers are added to phage suspensions.
- Untreated
- No scavengers
- 200 mM Mannitol
- 20 mM Tiron
- 10 mM NaN₃
- 20 mM L-histidine
- 1 mM Ebselen

Figure B: Survival infectivity (Lg PFU/ml) of bacteria when scavengers are added to phage suspensions before plasma treatment.
- Untreated
- No scavengers
- 200 mM Mannitol
- 20 mM Tiron
- 10 mM NaN₃
- 20 mM L-histidine
- 1 mM Ebselen

Figure C: Survival infectivity (Lg PFU/ml) of bacteria when scavengers are added to water before and after plasma treatment.
- Scavengers added to water before plasma treatment
- Scavengers added to water after plasma treatment
- Treated with plasma-activated water (Water pretreated for 100s)
ROS & RNS generated by gas plasma

- \( \text{NO}_3^- \)
- \( \text{HNO}_3 \)
- \( \text{ONOO}^- \)
- \( \text{ONO}^- \)
- \( \text{H}_2\text{O}_2 \)
- \( \cdot\text{NO} \)
- \( \cdot\text{OH} \)
- \( \cdot\text{O}_2 \)
- \( \text{NO}_2^- \)
- \( \text{HNO}_2 \)

T4 capsid

T4 capsid with DNA and protein damages