Facile production of silver-reduced graphene oxide nanocomposite with highly effective antibacterial performance

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Abstract:

Reduced graphene oxide (rGO) has broad applications based upon its superior electronic properties. In many cases, an additional antibacterial function of these materials requests physical or chemical modification, such as incorporating silver nanoparticles into rGO to produce silver-reduced graphene oxide (Ag/rGO) composite. Most of the previous methods of preparing Ag/rGO are top-down techniques starting from graphite exfoliation into graphene oxide (GO), to the reduction of a mixture of silver nitrate and GO into the Ag/rGO nanocomposite. This method is flawed by its high cost, tedious treatments, and heavy pollution from generated wastes. Herein, we manifest a novel bottom-up strategy of growing Ag/rGO totally from a cellulose acetate and silver nitrate precursor in one step without tedious solution exfoliation and chemical treatment steps. The produced Ag/rGO nanocomposite maintains highly effective antibacterial activity, which is ascribed to the synergy between the effect of rGO size and incorporated bactericidal silver nanoparticles. Our strategy is promising for industrial production from sustainability and cost-effectiveness perspectives.

Key words: reduced graphene oxide; silver nanoparticle; antibacterial performance; oxidative stress; membrane permeability

1. Introduction

Reduced graphene oxide (rGO) is a key derivative of graphene, which exhibits excellent intralaminar electrical conductivity and has been utilized to make various electric and biomedical devices¹⁻⁴. Other emerging applications are the integration of rGO into reverse osmosis membranes for water desalination^{5,6}, and utilization as energy storage materials⁷⁻⁹, etc. Some of these appliances, such as biomedical devices, need to be sterile so that bacterial infections are minimized⁴. As well, reverse osmosis membranes favor an additional antibacterial function to avoid biofouling due to biofilm growth and blockage of membrane pores^{10,11}. Unlike graphene oxide (GO), rGO has relatively fewer functional groups and thereby has a weaker antibacterial capacity than GO¹². Moreover, the traditional method to prepare rGO is considerably more complicated than GO¹²⁻¹⁵. As a result, studies of GO as an antibacterial agent are more abundant than rGO¹⁶⁻¹⁸, although rGO holds greater promise in practical applications.

Silver's antibacterial property has been used to preserve food and reduce wound infections¹⁹. Recently, silver has been incorporated into various nanomaterials, including rGO, for microbial disinfection purposes. Previous researchers prepared a silver-reduced graphene oxide (Ag/rGO) composite through two rather complicated steps²⁰⁻²³. First, they obtained GO from graphite using modified Hummer's method, and then treated a mixture of silver nitrate (AgNO_3) and GO with various reducing agents. This method is limited by high cost and is extremely difficult to upscale.

In this work, we report a relatively easy and sustainable method to prepare Ag/rGO by calcinating an AgNO_3 and cellulose acetate mixture in an H_2 and Ar atmosphere. Our strategy is based on the rationale that 1) carbon atoms of biomass are reported to re-

organize into layered carbon crystals under pyrolysis^{24,25}, and the growth of rGO from biomass under pyrolysis has been reported previously²⁶; 2) cellulose acetate has abundant aldehyde groups, which could reduce Ag⁺ into silver nanoparticle²⁷. The product was identified as highly pure Ag/rGO with Raman spectroscopy, X-ray powder diffraction (XRD), and X-ray photoelectron spectroscopy (XPS). The as-synthesized Ag/rGO was found to maintain a highly effective antibacterial performance using *Escherichia coli* as a model pathogen. Notable advantages of our study include the easiness of material synthesis, abundance and low cost of cellulose acetate precursor, and minimal waste generation. This strategy could be extended to produce a series of other rGO products with desired properties.

2. Material and methods

2.1 Materials

Cellulose acetate (Sigma-Aldrich) with an average molecular weight of 30,000 by GPC (gel permeation chromatography) and silver nitrate (Sigma-Aldrich) was used as precursor for synthesis of Ag/rGO. Acetone purchased from Fisher Scientific was used to uniformly disperse silver ion into cellulose acetate. LB broth and agar for *E. coli* proliferation was bought from BD Difco. MOPS buffer (C₇H₁₅NO₄S) for solution acidity control was obtained from Corning. The Invitrogen LIVE/DEAD BacLight Bacterial Viability Kit was used for visualizing bacteria membrane permeability under a fluorescence microscope (Olympus).

2.2 Synthesis and characterizations of Ag/rGO

10 g cellulose acetate fine powder was first dispersed in acetone, then 5 g AgNO_3 was added into the dispersion and mixed with cellulose acetate dispersion uniformly with a magnetic stirrer. The mixture was then dried inside a vacuum desiccator overnight. After that, the mixed powder was put inside a quartz boat, and transferred into a thermal chemical vapor deposition (CVD) system. The furnace temperature was raised to 1040 °C in 30 minutes and maintained at 1040 °C for 1 hour before cooling down to room temperature quickly. The chamber pressure was kept at atmospheric pressure under a continuous flow of 50 sccm H_2 and 350 sccm Ar through the whole CVD process. The obtained product was extensively washed with acetone to remove organic residuals. Afterwards, the sample was oven-dried at 80 °C for 12 h, and then grinded into fine powders for characterization and antibacterial application. Pristine rGO was synthesized from cellulose acetate via the same operation without addition of silver nitrate. rGO was used as a control in our study.

The Raman spectra of Ag/rGO was collected with a Nicolet Almega XR Dispersive Raman. The laser wavelength was 532 nm. A Bruker D2 Phaser was employed to acquire the X-ray diffraction spectra of as-grown Ag/rGO powders. A Kratos AXIS Ultra DLD spectrometer equipped with an Al $\text{K}\alpha$ x-ray source was used to obtain the X-ray photoelectron spectroscopy data on Ag/rGO powders. Ag/rGO nanocomposites were dispersed in ethanol and sonicated for 5 mins, before drop-casted onto a 300 nm SiO_2/Si wafer for atomic force microscopy (AFM) measurements. The AFM images of Ag/rGO in the supporting information were acquired with a Dimension Icon SPM. The scanning electron microscope (SEM) images of Ag/rGO were collected with a Teneo VS SEM. To

prepare the samples for high-resolution transmission electron microscopy (HRTEM) measurements by Titan 80-300, Ag/rGO powders were sonicated inside ethanol for 5 minutes and then drop casted onto a 400 mesh Cu TEM grid. Titan 60-300 (FEI) was used to obtain scanning transmission electron microscopy (STEM) and Energy-dispersive X-ray spectroscopy (EDX) images.

2.3 *E. coli* inactivation assays

Exponential growth phase *E. coli* cells were used for inactivation assays. This is because cells at this stage are sensitive to external stimuli, and the antibacterial performance of the reagent could be more easily visualized. Similar approaches have been used in other studies.^{28,29} At first, a single colony from LB-agar plate was dropped into 5 mL liquid LB medium, and incubated at 37 °C with a shaking speed of 250 rpm for 12 h. Then 50 µL saturated bacterial solution was added into 5 mL fresh LB medium, and incubated at 37 °C with a shaking speed of 250 rpm for another 2 h until the OD₆₀₀ reached 0.4-0.6. The obtained bacterial solution was then collected via centrifugation and further extensively washed with 10 mM MOPS buffer (pH 7). Bacteria suspended in MOPS buffer were stored at 4 °C for antibacterial study within the same day.

For antibacterial tests, typically 100 µg/mL Ag/rGO was prepared into 5 mL bacterial solution. Concentration of Ag/rGO and solution pH are subjected changes as indicated. The mixture was incubated at 37 °C with a shaking speed of 250 rpm. At desired time interval, the solution was withdrawn for bacterial survival quantification. Briefly, 100 µL sample was withdrawn in the first row of a 96-well plate, and then 20 µL liquid was put

into the next row containing 180 μL MOPS buffer, so as to achieve a 10-fold dilution. Afterwards, 5 μL liquid from each well was dropped onto the LB-agar plate with a multi-channel pipette. The colony forming units (CFU) on the plate after incubation at 37 °C for overnight were counted. Bacteria survival rate is expressed as N/N_0 , where N is the CFU of bacteria determined at specific time, and N_0 is the initial bacteria amount.

2.4 Reusability assay of Ag/rGO

After each round of antibacterial test, solution was centrifuged at 10,000 g for 3 min, and the supernatant was discarded. The collected Ag/rGO particles were oven-dried at 80 °C in a sterilized tube to inactivate residual live *E. coli* cells. The Ag/rGO particles were then cooled down to room temperature and added into bacterial solution for another round of antibacterial test. Conditions of antibacterial assay are described above.

2.5 Iron homeostasis measurement

The iron homeostasis was measured with cell lysate with an ultrasensitive Ferene-S assay. Exponential *E. coli* cells in 10 mM MOPS buffer (pH 7) were sonicated on ice to obtain the cell lysate with intact cytoplasm proteins. 20 mM Ferene-S (Sigma-Aldrich) was added into 200 μL cell lysate treated by 100 $\mu\text{g/mL}$ Ag/rGO within a 96-well plate, and the solution was incubated at 37 °C for 1, 2, and 3 h, respectively. Absorbance measured at 593 nm with a UV-Vis spectrometer indicated the complex between iron ion and Ferene-S compound. By comparison with pre-obtained calibration curve (0-30 nM), the free iron ion concentration can be calculated.

2.6 ROS content measurement

Reactive oxygen species (ROS) was measured with a HPF (3'-(p-hydroxyphenyl fluorescein) reporter. Briefly, during antibacterial tests, 5 mM HPF (Thermofisher Scientific) was added into 200 μ L bacteria solutions treated by 100 μ g/mL Ag/rGO. HPF could be oxidized by ROS, in particular HO $\cdot$ into a green fluorescent product. The plate was incubated at 37 $^{\circ}$ C in dark environment, and fluorescence of each well was measured at 0, 1, 2, and 3 h, respectively, with excitation/emission at 490/515 nm.

2.7 NADH content measurement

Cellular NADH content was measured via a cofactor recycling assay³⁰. 5 mL exponential growth phase *E. coli* cells were centrifuged and resuspended into 5 mL ice-cold MOPS buffer. 0.3 mL of 0.2 M NaOH was added into the solution, and heated for 10 min at 55 $^{\circ}$ C. Then, the solution was neutralized with 0.3 mL of 0.1 M HCl. After centrifugation, the supernatant was transferred to a new tube for NADH concentration measurement. Briefly, 50 μ L supernatant collected above was added in a solution containing 50 μ L of commercial yeast ADH of around 25 units activity and 0.9 mL reagents mixture (11% ethanol, 4.4 mM EDTA, 0.47 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, and 3.7 mM phenazine ethosulfate in 100 mM Bicine buffer, pH 8). After reaction, the product will yield blue color, which was measured at 570 nm with a UV-Vis spectrometer.

3. Results and discussion

3.1 Synthesis and characterization of Ag/rGO

Figure 1. (a) Raman spectra, (b) X-ray diffraction pattern, and (c,d) X-ray photoelectron spectra of synthesized Ag/rGO composite. (c) and (d) indicate C 1s and Ag 3d core spectra, respectively.

Pyrolysis of AgNO_3 and cellulose acetate mixture was conducted at 1040 $^{\circ}\text{C}$ under reductive H_2 and inert Ar atmosphere. The purity of synthesized Ag/rGO after extensive washing was analyzed by various spectroscopies. Raman spectroscopy of the Ag/rGO

nanocomposite shows a high, sharp G-peak at 1586 cm^{-1} originating from E_{2g} symmetry, and a slightly broader D-peak at 1346 cm^{-1} due to defects in the conjugated π electrons of graphitic materials^{31,32}. **Figure 1b** shows the XRD spectra of the rGO/Ag nanocomposite. The peaks at 38.1 , 44.3 , 64.5 , 77.3 , and 81.4 are assigned to diffractions from the $(1\ 1\ 1)$, $(2\ 0\ 0)$, $(2\ 2\ 0)$, $(3\ 1\ 1)$, and $(2\ 2\ 2)$ planes of silver nanoparticles, respectively³³. No extra peaks related to Ag^+ were observed in **Figure 1b**, suggesting a complete reduction of Ag^+ to Ag. High resolution XPS survey spectrum of Ag/rGO sample shows that carbon, oxygen and silver elements were detected (**Figure S1a** of supporting information). The fitted C 1s spectrum (**Figure 1c**) shows C=C, C-C, C–O–C/C–OH, C=O, O=C–OH groups and π – π^* shake-up satellite structure located at 284.4 , 285.1 , 286.4 , 287.9 , 289.0 , and 290.6 eV , respectively^{34–37}. The Ag 3d core level (**Figure 1d**) was fitted with two doublets (Ag $3d_{5/2}$ and Ag $3d_{3/2}$) with a fixed area ratio equal to 3:2 and doublet separation of 6.0 eV . The Ag $3d_{5/2}$ component at 371.6 eV was metallic silver satellite signal of 368.1 eV component³⁸. The O 1s core level (**Figure S1b** of supporting information) shows C=O, C-O, and chemisorbed oxygen located at 531.9 eV , 533.1 , and 535.2 eV , respectively³⁹.

Figure 2. (a) High-resolution transmission electron microscopy and (b) scanning transmission electron microscopy (STEM) with EDX mapping images.

Microscopic analyses were performed on the Ag/rGO nanocomposite to study its morphology. **Figures S2** and **S3** of supporting information presents SEM and STEM images of Ag/rGO under increasing magnification, respectively. Silver nanocrystals

embedded inside rGO are clearly observable, demonstrating a uniform, dense doping of Ag into rGO. Further, from **Figure 2a**, silver nanocrystals with diameters ranging from 10 - 100 nm are shown. Compositional distribution of elements in Ag/rGO composite was analyzed by energy-dispersive X-ray spectroscopy (EDX), which suggested that C, Ag and O elements were uniformly distributed on Ag/rGO surface (**Figure 2b**). Collectively, these results demonstrated that silver nanoparticles were successfully incorporated into rGO.

3.2 Antibacterial activity of Ag/rGO

Figure 3. Antibacterial performance of Ag/rGO (a) compared with controls, (b) at different Ag/rGO concentration, (c) at different solution pH, and (d) measured Ag⁺ concentration at different solution pH. Typical conditions are 100 µg/mL Ag/rGO, ~ 10⁸ CFU/mL *E. coli*, and 10 mM MOPS buffer (pH 7). Ag/rGO concentration and solution pH vary as indicated.

The antibacterial activity of as-prepared Ag/rGO was then tested. *E. coli* cells of exponential growth phase treated by either a MOPS buffer or rGO without silver were used as negative controls. Results indicated that Ag/rGO exhibited the highest bactericidal performance, i.e., 2.85-log reduction in cell viability after 3 hours; negligible loss of cell viability was detected in the buffer control and 1.03-log cells were inactivated by rGO treatment (**Figure 3a**). The vital role of silver in the antibacterial performance of Ag/rGO is thus suggested. Literature reports that the sharp edges that could penetrate cell membranes and induce the leakage of cellular components mainly accounts for the bactericidal property of rGO¹². Incorporation of bactericidal silver species further enhances the killing efficacy of rGO. In our study, the average height of silver nanoparticles is 15 nm from atomic force microscopy measurement (**Figure S4** of supporting information), that could efficiently diffuse through the outer membrane of *E. coli* and hijack the inherent electron transport chain. It is worth noting that the Ag/rGO synthesized using our method exhibited stronger antibacterial activity than reported Ag/GO^{40,41}, possibly because our bottom-up synthesis strategy could more effectively incorporate silver atoms and expose them to the cell surface.

The antibacterial performance of Ag/rGO at varying concentrations (i.e., 10, 50, and 100 µg/mL) was investigated within a 3 h duration (**Figure 3b**). It was observed that a higher concentration of Ag/rGO mediated greater antibacterial efficiency, because of stronger Ag/rGO-bacteria interactions. The cell viability reductions were 0.61-, 1.47-, and 2.85-log for 10, 50, and 100 µg/mL Ag/rGO, respectively, after treatment for 3 h. Moreover, the bacterial inactivation was also time-dependent, and longer incubation period tended

to increase cell death rate. For example, 100 µg/mL Ag/rGO killed 0.88-log *E. coli* in 1 h, and 2.85-log in 3 h.

3.3 Effect of solution pH

The role of solution pH in Ag/rGO antibacterial performance was assayed. *E. coli* cells were incubated with 100 µg/mL Ag/rGO in buffered solution at a pH range of 5.5-8.5, and cell viability was measured every one hour. It was shown that lower pH mediated higher antibacterial activity of Ag/rGO (**Figure 3c**). For example, at pH 5.5, 7, and 8.5, the losses of *E. coli* viability were 3.39-, 2.85-, and 1.03-log, respectively, after treatment for 3 h. pH values outside this range were not tested, because the extreme pH alone would result in significant bacterial death and cause false positive observations (**Figure S5** of supporting information). Bacterial cell death under extreme pHs is mainly due to denatured protein functions and increased membrane permeability,^{42,43} which cause osmotic shock. The pH-dependent antibacterial activity of Ag/rGO was likely linked to the dissolution of silver ions (Ag⁺) in solution. During the synthesis of Ag/rGO using AgNO₃ and cellulose acetate, Ag⁺ was reduced into zero-valent silver nanoparticles, possibly by the reductive aldehyde groups of cellulose acetate²⁷. However, as the solution turned acidic, silver nanoparticles of Ag/rGO might dissolve, releasing Ag⁺ into solution⁴¹. To test our hypothesis, we used Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to measure the amount of released Ag⁺ in solution. Results indicated that an acidic pH of 5.5 particularly mediated the highest release of Ag⁺ into solution (i.e., 80.3 ppb), compared with pH values of 7 and 8.5 (i.e., 20.3 and 9.5 ppb) (**Figure 3d**). Moreover, the release of Ag⁺ linearly increased with incubation time. The presence of Ag⁺ ought to improve the antibacterial performance

of Ag/rGO, because Ag^+ is more mobile than silver nanoparticles and can directly enter cell membranes to disrupt disulfide bond interactions of proteins. With these coupled results, we reason that an acidic pH increased the antibacterial activity of Ag/rGO mainly by dissolving silver nanoparticles into the solution.

3.4 Reusability assay

Figure 4. (a) Reusability assay of Ag/rGO for *E. coli* inactivation, and (b) released Ag^+ into solution at each round of assay. 100 $\mu\text{g/mL}$ Ag/rGO, $\sim 10^8$ CFU/mL *E. coli*, and 10 mM MOPS buffer (pH 7).

The reusability of Ag/rGO for *E. coli* disinfection was then tested for multiple rounds (**Figure 4**). After each round of reaction, the particles were centrifuged and oven-dried to inactivate residual bacteria. It was found that, during the consecutive five rounds of experiments, Ag/rGO inactivated 99.86%, 95.43%, 94.31%, 92.3%, and 85.3% *E. coli* respectively after 3 h treatment each. The slightly decayed antibacterial potency of Ag/rGO was likely because of dissolution of silver nanoparticles from Ag/rGO. To confirm this hypothesis, we measured the dissolved Ag⁺ concentration in each round of experiment, which was 20.3, 15.4, 17.5, 12.9, and 10.4 ppb respectively. The decreased Ag⁺ concentration detected in the solution indicated weakened silver nanoparticle content of Ag/rGO. Nonetheless, results suggested that our synthesized Ag/rGO could be reused for multiple rounds with steady antibacterial performance.

3.5 Antibacterial mechanism

Figure 5. Antibacterial mechanism study. (a) Measurement of free iron ion concentration with an ultrasensitive Ferrocene-S assay, (b) quantification of ROS generation with a fluorescent HPF probe, (c) detection of depleted NADH with a cofactor recycling assay, (d) and fluorescent images of *E. coli* cells after treatment with live/dead fluorescent dyes to indicate cell membrane permeability.

We finally studied the antibacterial mechanism of Ag/rGO using a molecular biology approach. It has been verified that Ag/rGO kills bacteria via the combination of size effect of the Ag/rGO that cuts open the cell membrane and the released Ag^+ that enters bacterial cytoplasm to further disrupt protein functions. The cell response to such stimuli has

usually pointed to an increase of reactive oxygen species (ROS) content, which then oxidizes functional enzymes and interrupts the electron transport chain. The generation of ROS is ascribed to Fenton chemistry, whereby ferrous iron ions react with hydrogen peroxide (H_2O_2) to produce hydroxyl radicals ($\text{HO}\cdot$)⁴⁴. To probe how ROS was generated, we at first quantified the iron homeostasis. Cell lysate was mixed with the Ag/rGO nanocomposite and free Fe^{2+} was quantified with an ultrasensitive Ferene-S probe. As shown in **Figure 5a**, cell lysate of the control group contained 6.32 nM Fe^{2+} , but after treatment with Ag/rGO the content of Fe^{2+} increased to 10.35, 11.46, and 11.34 nM at 1, 2, and 3 h, respectively. This is because Ag^+ could disrupt Fe-S clusters of many functional proteins, resulting in the release of Fe^{2+} .⁴⁵ In addition, Ag^+ also adversely impairs respiration-associated proteins. During the interruption of an electron transport chain, electrons tend to abnormally accumulate and are accepted by oxygen molecules to form H_2O_2 .⁴⁶ However, molecular probes for H_2O_2 are restricted by their specificity and detection limit. Thus, we alternatively measured the generated ROS with a commercial fluorescent probe (**Figure 5b**). It is observed that after incubation with Ag/rGO for 3 h, cellular ROS content increased by 18%, indicative of significant oxidative stress.

Scheme 1. Illustration of antibacterial mechanism of Ag/rGO.

The physiological responses of *E. coli* to the oxidative stress induced by the Ag/rGO nanocomposite are remarkable. Contents of reduced nicotinamide adenine dinucleotide, i.e., NADH, serve as an indicator of the cellular oxidative stress. Under oxidative stress, NADH is transformed into NAD⁺ after losing an electron. Around 20-100% of NADH could be depleted in response to direct or indirect oxidative stress^{28,46,47}. We thereby measured the cellular NADH contents with a cycled enzymatic assay. As expected, the overall NADH content sharply decreased by 66.2% after Ag/rGO treatment for 3 h, further suggesting the oxidative stress induced by Ag/rGO (**Figure 5c**). The oxidative stress also

affects membrane permeability. It has been reported that Ag^+ induces the aggregation of proteins, and increases membrane permeability, even at low dosage levels of Ag^+ .⁴⁵ To test the permeability of *E. coli* cell membranes, we treated the cells with two fluorescent dyes. When a cell membrane has low permeability, only the green fluorescent dye (SYTO[®] 9 stain, 485/498 ex/em) can enter these cells. However, both green and red fluorescent (propidium iodide stain, 485/636 ex/em) dyes can penetrate *E. coli* outer and inner membranes if a cell exhibits high permeability, showing red fluorescence overall. Both pristine rGO and Ag/rGO at a sub lethal concentration of 10 $\mu\text{g/mL}$ were added to *E. coli* cell suspensions and treated for half an hour. Over 85.3% of these cells should be alive by Ag/rGO treatment, as shown in **Figure 3a**. As a result, cells treated by Ag/rGO showed exclusive red fluorescence indicating high cell membrane permeability, whereas cells treated by rGO exhibited relatively strong selectivity against exogenous molecules (**Figure 5d**). The increase of membrane permeability by Ag/rGO treatment could result in leakage of cellular components and loss of metabolic activity, and eventually lead to cell death after long-term incubation. The overall mechanism of antibacterial action of Ag/rGO is shown in **Scheme 1**.

4. Conclusion

In summary, we demonstrate a rapid way for the mass production of Ag/rGO nanocomposite by directly calcinating the mixture of silver nitrate and cellulose acetate precursors in an H_2 and Ar atmosphere. A possible transformation route is the graphitization of cellulose acetate under pyrolysis, whereby silver ions are reduced into silver nanoparticles through the reductive aldehyde groups of cellulose acetate. The as-

synthesized Ag/rGO uniformly incorporates silver nanoparticles of 10-100 nm, and maintains strong antibacterial activity. Results showed that the bactericidal mechanism of Ag/rGO involves increases in oxidative stress and membrane permeability, which depletes NADH and causes cellular contents leakage, respectively. The applications of Ag/rGO include the fields of water disinfection and electric and biomedical devices with bacterial inactivation functions. For instance, this strategy could be used to mass synthesize large-area Ag/rGO, that could be integrated into the membrane filtration facility. It is anticipated that the synthesized products should exhibit superior performance in various fields. Our strategy of producing rGO-derived products is promising, because it is easy to upscale and generates much less waste than traditional methods.

■ ASSOCIATED CONTENT

Supporting Information of XPS spectra of Ag/rGO, SEM images under increasing magnification, AFM images, and *E. coli* survival rate at different solution pH.

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■ NOTE

The authors declare no competing financial interest.

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