Superior Antibacterial Activity of Photochemical Synthesized Ag-CNT Composites and their Synergistic Effects in Combination with other Antimicrobial Agents

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Abstract
Silver nanoparticle-modified multwalled carbon nanotubes (MWCTNs) nanocomposites (AgCNTs) were synthesized by photochemical reduction method, their antimicrobial effect on both Gram negative and Gram positive bacteria, using E. coli and Bacillus anthracis as model bacteria, respectively, were investigated. The results indicated that AgCNTs exhibited more potent antibacterial effects against both Gram negative and Gram positive bacterium compared to Ag nanoparticles (Ag NPs). The minimal inhibitory concentrations (MICs) of AgCNTs against E. coli cells (0.5 µg/mL) and B. anthracis cells (0.8 µg/mL) was 1/20 times and 1/17.5 of the MICs of Ag NPs against E. coli and B. anthracis, respectively. Further study on the antibacterial effect of combination treatment of AgCNTs with oxidizing antimicrobial agents (NaOCl or H₂O₂) to E. coli cells indicated a partial synergistic or a synergistic effect using the fractional inhibitory concentration (FIC) index test or isololograms. The combination treatment of AgCNTs with a natural peptide, nisin, also exhibited enhanced inhibitory effect on E. coli growth, as significant delays in growth of E. coli cells treated by the combination of 0.2 µg/mL AgCNTs and 4 or 8 µg/mL nisin was observed compared to AgCNTs alone nisin alone treatment. The synergistic or enhanced effect of the combination of AgCNTs with other antimicrobial relied on the combination of different action mechanisms in which AgCNTs played a role to damage cell membrane which allowed easier access for other small antimicrobial molecules to penetrate into cells. Such combination strategy could be broadly applicable to the improvement of existing antimicrobial methods or design/discover new effective antimicrobial agents/methods.

Keywords: Bacterial inhibition; Silver nanoparticle modified carbon nanotubes (AgCNTs); Synergistic effect; Combination treatment; NaOCl; H₂O₂; Nisin; E. coli; Bacillus anthracis

Introduction
Inactivation of pathogenic bacteria has been receiving great attention in the fields of environmental safety and public health. Traditional antimicrobial agents may be broadly classified into organic and inorganic materials. Organic antibacterial agents are often complex toxic bactericides that have strong inhibitory effects on bacterial growth, such as organic acids, essential oils, and bacteriocins. However, their low thermal stability, high toxicity, and short life expectancy have limited their applications in practice [1]. Inorganic agents, such as hypochlorite, alcohol, chlorine dioxide, hydrogen peroxide, triclosan, triclocarban, and some metal ions, are often less toxic and have received more and more recognition in the antibacterial product market in recent years [2,3]. Common inorganic antibacterial agents have been widely used in health-care and cleaning products. Besides these traditional antimicrobial agents, with the rapid growing field of nanotechnology in the past several years, a number of natural and engineered nanomaterials have been discovered for their strong antimicrobial properties for promising applications [4]. These nanomaterials include, but not limit to, metal nanoparticles, metal oxide nanoparticles, carbon-based nanoparticles and nanotubes. For example, silver nanoparticles (Ag NPs) are one of the metal nanoparticles that have been intensively studied as a bactericidal agent and their utilization is increasing owing to their strong antimicrobial properties and minimal toxicity [5]. Carbon nanotubes (CNTs), especially single-walled CNTs (SWCNTs), are one of the recently discovered antimicrobial agents against a variety of bacterial species [6-10], whereas multiwalled CNTs (MWCTNs) are found little or non-antimicrobial [11], but a good component in antimicrobial nanocomposite materials [12,13].

In use of any of these traditional and newly emerged nanomaterial-based antimicrobial agents, leaching to the environment, soil, the groundwater and transported to surface waters, chemical residues as well as possible aggravated harmful disinfactant byproducts (DBPs) [4], often raise safety and health concerns. In some cases, direct contact with high concentration of antimicrobial agents may cause extensive damage to biological systems (like human tissues) [14]. One way to minimize such risk is to use low concentration of the antimicrobial agents, but in many cases, low concentration definitely compromises the antimicrobial effect. The use of combinations of different antimicrobial agents is a possible way to achieve high antimicrobial effect with low concentrations of individual agents, because the combination of appropriate agents can generate new effective antimicrobial molecules on-site or synergistically combine two different mechanisms to achieve

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greater antimicrobial effect at relative low concentrations of individual agents. Such strategy has been demonstrated in some antimicrobial agents. For example, Fenton reagent has been reported as an effective sporidical reagent to Bacillus spores [15], which combined hydrogen peroxide or ascorbic acid with various metal ions together in order to generate highly reactive hydroxyl radicals in the immediate vicinity of their targets [16-18]. The combination of NaOCl and vitamin B1 treatment on total aerobic bacteria and E. coli contaminating head lettuce Lactuca sativa showed a significant synergistic effect [19]. Combination of hydrogen peroxide and formic acid showed synergistic antimicrobial effect on six bacterium strains which were representative of contamination bacteria in the food industry [20]. Besides these chemical agents, such combination also works for nanomaterial-based antimicrobial agents. For example, Ag NPs attached to CNTs showed enhanced antimicrobial activity against E. coli [13], because the presence of CNTs in nanoparticles composites can act as a host to increase the long term stability of Ag nanoparticles. They also found that the antibacterial activity of Ag NP-CNTs was dependent on the size of Ag NPs. Jung et al. [12] synthesized Ag-coated CNTs by the use of a simple aerosol technique and prepared AgCNTs-deposited filters. These filters exhibited higher antimicrobial activity than CNTs- and Ag NPs-deposited filters. Additionally, AgCNTs have received attention due to their potential applications as catalysts [21,22], reinforcing agents [23], and advanced materials [24,25].

In this study, photochemically synthesized AgCNTs was evaluated for their antibacterial effects on both Gram-positive bacteria and Gram-negative bacteria using B. anthracis and E. coli as model bacteria, respectively. Further, the combination of AgCNTs with oxidizing antibacterial agents or natural antibacterial agents was studied to determine whether synergistic antimicrobial effect would present on antibacterial agents or natural antibacterial agents. For example, the combination of AgCNTs with sodium hypochlorite (NaOCl) and hydrogen peroxide (H2O2), and the tested natural antimicrobial peptide was- nisin. NaOCl is the effective component of many commercial disinfectants. When NaOCl is used at high concentration, it causes mucous membranes irritation, intraclinal damage on intact skin [26], deterioration in organoleptic quality, an unpleasant odor, residual chlorine, and production of byproducts including the carcinogenic substance trihalomethane [27,28]. Hydrogen peroxide is a powerful oxidizer and an efficient disinfectant. It degrades completely into water and oxygen without producing salt residues. Its safety depends on the applied concentration. For disinfection purpose, high concentrations are required, which raise safety issues. For example, exposure of eyes to 5% of higher H2O2 leads to permanent eye damage. In addition, H2O2 irritates lung by inhalation and causes blisters, burns, and whitening on skin after direct contact. Laboratory tests on bacteria show that H2O2 is mutagenic, causing DNA damage. Because of the potential risk raised by the use of high concentrations of such oxidizing agents, the use of lower concentrations of these agents are advised, but the fact is lower concentrations are less effective [29]. Nisin is a polycyclic antibacterial peptide with 34 amino acid residues and is produced by bacterium pellet was rinsed with deionized water repeatedly. Synthesized AgCNTs were then dried at 80°C under vacuum overnight.

Methods

Characterization of AgCNTs by scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX)

The surface morphology and size of AgCNT nanocomposites were examined by field emission-scanning electron microscope (FE-SEM) (Hitachi 8000). FE-SEM imaging was operated at 10 kV. EDX data were obtained with a Bruker AXS (XFlash detector 5030) coupled with the FE-SEM. Three typical regions of each sample were analyzed, and the average wt.% of each element in AgCNTs composites was determined.

Evaluation of concentration-dependent antibacterial activity of AgCNTs on E. coli K12 cells and Bacillus anthracis cells by optical density (OD) measurement

The experiments were performed in 96-well plates. Bacterial cells were prepared as follows. Bacterial cells were grown in Nutrient broth overnight and were diluted in fresh Nutrient broth to desired concentrations for each experiment. Aliquots of 100 µL diluted cells in nutrient broth at approximately 6.5×10^5 cells/mL were dispersed into the wells of a 96-well plate. Desired volumes of AgCNTs solution were added into the wells to reach the concentrations of 0.2, 0.4, 0.5, 0.6, 0.7, 0.8, and 1 µg/mL, in a total volume of 150 µL in each well by adjusting with deionized (DI) H2O. The plates were incubated at 37°C for 24 h. OD measurements were performed using the Spectra Max M5 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA). The changes in optical density (OD) at 595 nm of the samples after incubation were compared as an indicator of the growths of treated cells, and thus for evaluating the inhibition effect of AgCNTs to bacterial cells. For comparison, the antibacterial effects of Ag nanoparticles (NPs) without CNTs were also tested using the same method.

Treatment with AgCNTs and Ag NPs and determination of viable cell reduction

A freshly grown overnight E. coli K12 culture was washed 3 times and resuspended in PBS. The washed cells were then treated with Ag NPs or AgCNTs. The treatment was performed in 1.5 ml centrifuge tubes. Each tube included 900 µL bacterial cells, 0.3 µg/mL AgCNTs or 5 µg/mL Ag NPs, and adjusted to the final volume of 1 mL by addition of DI-H2O. Controls contained 900 µL bacterial cells and 100 µL DI water. The tubes were rotated at 15 rpm at room temperature for 0, 15, 30, or 60 min on a Dynal rotator (Dynal biotech Inc., Lake Success, NY). After the treatment, viable cell numbers of all the samples were determined by the traditional plating method. For each sample, serial 1:10 dilutions were made, and aliquots of 100 µL of appropriate dilutions were surface plated on Luria-Bertani (LB) agar plates. Colony numbers were counted after 18 h incubation at 37°C, and colony forming units (CFU) per mL were calculated.

Fractional inhibitory concentration (FIC) index test for the combination of AgCNTs and NaOCl or H2O2

To investigate whether there were synergistic effect in the combination of AgCNTs with NaOCl or H2O2, FIC index test was performed and isobolograms were generated. To do this, a broth microdilution checkerboard method was used based on the recommendations of NCCLS [30-33]. Aliquots of 100 µL of E. coli cells in Nutrient broth were distributed into each well of 96-well plates. AgCNTs solution and NaOCl or H2O2 were added to the wells at various volumes to achieve 2-fold serial dilutions along the ordinate and the abscissa of the plates, respectively. The final volume in each well was adjusted to 150 µL by adding desired volume of DI H2O. The resulting checkerboard contained each combination of AgCNTs and NaOCl or H2O2 at different concentrations. The plates were incubated at 37°C for 48 h. OD values at 595 nm of all samples were read before and after the incubation. Increase in OD indicated bacterial growth, while no change on OD indicated no bacterial growth. Based on the OD measurement
of bacterial growth, the minimal inhibitory concentrations (MICs) of each agent or combined agents was determined, which was the lowest concentration of the agent(s) causing complete inhibition of bacterial growth. Then, the FIC index ($\Sigma$FIC) was calculated as follows: $\Sigma$FIC = FIC of agent A + FIC of agent B, where FIC of agent A = MIC of agent A in combination/MIC of agent A alone, and FIC of agent B = MIC of agent B in combination/MIC of agent B alone. The combination effect was determined according to the resulting FIC index as follows: FIC index $\leq$ 0.5 indicates synergy; FIC index 0.5-0.75 indicates partial synergy; FIC index 0.75-1.0 indicates additive effect; FIC index 1.0-4.0 indicates indifference, and FIC index $>$ 4 indicates antagonism, respectively [34].

**Isobologram**

The FIC values were plotted on an isobologram of AgCNTs versus NaOCl or H$_2$O$_2$ to graphically visualize any interactions between the two reagents. In an isobologram, the concentrations of the two reagents are plotted on the x- and y-axis. Then the MIC of each reagent is plotted on the graph and the two data points are joined by a line. The MICs of mixed reagents are plotted and joined by another line and compared with the previous line. If the line of the mixed MICs lies below the line of individual MICs, the interaction between the two agents is synergistic; If it is above the previous line, the interaction is antagonistic; and if it is on the previous line, there is no interaction between the agents [35].

**Results and Discussion**

**Characterization of AgCNTs**

The EDX elemental microanalysis results showed that the average content of element C, O, and Ag were 74.63%, 15.00%, and 10.37%, respectively, which further confirmed the presence of Ag NPs on CNTs. In this hybrid nano-composite, the role of acid-treated MWNTs was to stabilize Ag NPs [36]. The stabilizing mechanism was proposed as follows: the oxygen elements on the walls of the acid-treated MWNTs provided the nucleation centers for Ag ion and then stabilized the Ag nanoparticles after they were formed. The formed Ag nanoparticles were able to line up on the MWNTs’ surfaces, producing a stable assembly due to the special structures [36,37]. To completely inhibit the growth of Gram negative bacteria _E. coli_ K12 cells, the minimal concentrations of Ag NPs and AgCNTs were 10 µg/mL and 0.5 µg/mL, respectively (Figures 1A and 1B). To completely inhibit the growth of Gram positive bacteria _B. anthracis_, the minimal concentrations of Ag NPs and AgCNTs were 14 µg/mL and 0.8 µg/mL, respectively (Figures 1C and 1D). The minimal inhibitory concentration (MIC) of AgCNTs to _E. coli_ and _B. anthracis_ cells were 1/20 to 1/17.5 of those of Ag NPs, respectively, indicating that AgCNTs exhibited more potent inhibitory effect against both types of cells than Ag NPs did.

It is noted that AgCNTs exhibited slightly stronger inhibitory effect on Gram negative _E. coli_ cells than Gram positive _B. anthracis_ cells. This was true to Ag NPs too. This observation might be resulted from the distinct structure of cells between Gram-negative and Gram-positive bacteria. In Gram negative bacteria, the cell wall is a 7-8 nm thick layer of peptidoglycan which is sandwiched between an inner cell membrane and an outer membrane, whereas Gram-positive bacteria has a cell wall of 20-80 nm thick layer of peptidoglycan without an outer membrane. The peptidoglycan is a meshlike exoskeleton similar in function to the exoskeleton of an insect, consisting of sugars and amino acids. The Gram-positive cell wall may also include other components such as teichoic and lipoteichoic acids and complex polysaccharides, which can act as chelating agents. The peptidoglycan layer is essential for the structure and for survival in the normally hostile conditions in which bacteria grow. It protects bacteria against antibacterial agents such as antibiotics, toxins, chemicals, and degradative enzymes [38].

It is also noted that the photochemical synthesized AgCNTs in this study showed much stronger antibacterial effect than the AgCNTs reported in a previous study [38], where the reported MIC of AgCNTs to _E. coli_ cells was 130 µg/mL. Yu et al. [39] synthesized Ag NPs/Halloysite nanotubes/graphene nanocomposites with sandwich-like structure, and the MIC to _E. coli_ was 2 µg/mL. It was believed that the synthesis approach thus the resulting actual structure of AgCNTs, the density, and the size of Ag NPs on CNTs are factors that affect the inhibitory effect of the hybrid AgCNTs to bacterial cells. However, the correlation between these factors and the inhibitory effect will need a more systematic study and is not within the scope of this study.

To further investigate the inhibitory effect of AgCNTs during bacterial growth, the dynamic growth curve of _E. coli_ cells in the presence of 0.3 µg/mL AgCNTs was monitored using OD measurement, along with the growth curves in the presence of equal concentration of Ag NPs (0.3 µg/mL), equivalent concentration of Ag NPs (5 µg/mL), and the control (Figure 2A). Figure 2A indicated that 0.3 µg/mL AgCNTs effectively inhibited the _E. coli_ cell growth, whereas AgNPs at the same concentration did not show inhibitory effect. Elevated concentration of Ag NPs to 5 µg/mL showed an inhibitory effect approximately equivalent to 0.3 µg/mL AgCNTs on _E. coli_ cell growth. To reach the OD595 value of 0.2, _E. coli_ cells demonstrated 6.2, 5.85, and 0 h delay after being treated with 0.3 µg/mL AgCNTs, 5 µg/mL Ag NPs, and 0.3 µg/mL Ag NPs, respectively. The growth curve results further confirmed the stronger inhibitory effect of AgCNTs than Ag NPs on bacterial growth.

**Viable cell reduction in pre-treatment with AgCNTs**

We further investigated the effect of pre-treatment with AgCNTs on the inactivation of bacterial cells, as well as the treatment time dependency. As seen in Figure 2A, AgCNTs and Ag NPs at the concentration of 0.3 µg/mL and 5 µg/mL, respectively, had approximately equivalent inhibition effect on bacterial growth, so we selected to use these concentrations of AgCNTs and Ag NPs to treat _E. coli_ cells for 15 to 60 min, and the reductions in viable cell number after these treatments were determined by the plaque method. Figure 2B shows that the viable cell reduction in logarithmic value after the...
treatments with AgCNTs and Ag NPs for various periods of time. As shown in Figure 2B, upon the immediate mixing with 0.3 µg/mL AgCNTs, the viable cell number reduced 0.82 log (equivalent to 85.75%). As the treatment time prolonged, the viable cell reduced more. Upon 60 min treatment with 0.3 µg/mL AgCNTs, the viable E. coli cell number reduced by 3.64 log (equivalent to 99.98%). Not surprisingly, treatments with 5 µg/ml Ag NPs showed a similar trend and similar magnitudes in viable cell reduction, as 15 min and 60 min treatments produced 0.64 log reduction (equivalent to 77.2%) and 3.02 log reduction (equivalent to 99.91%), respectively. The results here indicated that the inactivation effect of AgCNTs to bacterial cells were effective upon immediate contact and increased with the increasing treatment time. This observation is consistent with observations on the antibacterial activities of Ag NPs or Ag NPs composites reported in other studies, including studies demonstrated that Ag NPs inhibited the bacterial growth rate from the time of first contact [40], and other studies showed that the Ag-impregnated polymers had an improved antibacterial characteristic with increased contact time [41].

We further used SEM microscopy to examine the morphology changes of E. coli cells before and after being treated with Ag NPs or AgCNTs. Figure 3A shows that the untreated E. coli cells, which were intact and rod - shaped with well-defined membranes. After being treated with Ag NPs 15 µg/mL for 30 min, some cells were ruptured or fragmented, and some appeared irregular in shape while others were intact and not much change in morphology compared to the untreated cells (Figure 3B). After 30 min treatment with 1 µg/mL AgCNTs, almost all the cells were ruptured and turned flat on AgCNTs (Figure 3C), such obvious cell damages by AgCNTs proved the effectiveness of their much stronger antibacterial activity compared to Ag NPs.

Looking into the mechanisms of AgCNTs for inhibiting/inactivating bacterial cells, it is accepted that the stronger antibacterial activity of AgCNTs comes from the synergistic effect of Ag NPs and MWCNTs. Several mechanisms on how AgCNTs inactivate bacterial cells have been proposed. Some studies indicated that in an aquatic environment, Ag NPs attached on CNTs partially dissolve in aquatic solution and generate low concentration silver ions (Ag+), and the released Ag+ ions bind to the thiol group (SH) of enzymes and proteins on bacterial surfaces and interrupt the adenosine triphosphate (ATP) pathway, leading to destabilization of bacterial membranes and walls [13]. Other studies proposed that AgCNTs interact with bacterial membrane and generate free radicals reactive oxygen species (ROS) on site, which induce membrane damage [38]. Other proposed mechanism suggested that AgCNTs physically pierce bacterial membrane and wall, which allow the penetration of Ag+ as well as Ag NPs into the internal of bacteria cells and inactivate them [42]. In Fact, Ag NPs accumulations on bacterial cell membrane, the lipopolysaccharide layer in the cell wall, and deep inside bacterial cells were observed by SEM, TEM, and STEM (scanning tunneling electron microscope) tests [43]. Significant morphological changes, including pits and cracks on cell membrane, as well as fragmentary membrane, were also observed on Ag NPs-treated cells [44]. Some other studies suggested that the main role of MWCNTs in the AgCNTs hybrids was to effectively stabilizing Ag NPs because the formation of Ag NPs and CNTs hybrids significantly reduced the reunion of Ag NPs and prevented them from aggregation, which was a factor that greatly affect the effectiveness of Ag NPs’ antibacterial activities [45]. While each of these proposed mechanisms is reasonable from a certain point of view and each has supportive evidence, they
do not exclude each other; and most likely, these mechanisms work together. But much work is still necessary to fully understand and elucidate the detailed mechanisms of AgCNTs for inactivation of bacterial cells.

**Antibacterial effect of the combination of AgCNTs and oxidizing agents**

Another way to examine whether a synergistic effect exist is to use the isobologram. Figure 4A shows the isobologram of the combination treatment with AgCNTs and H$_2$O$_2$, where the line of MICs in the combination treatment lies below the line linking the MICs of individual AgCNTs and H$_2$O$_2$, indicating a synergistic effect between AgCNTs and H$_2$O$_2$. Similar isobologram was obtained for the combination treatment with AgCNTs and NaOCl (Figure 4B), which indicated a synergistic effect between AgCNTs and H$_2$O$_2$ in inhibition of *E. coli* cells. The synergistic effect roots back from a multiple-damage mechanism in which two different disinfection methods enhance their antibacterial activity by damaging the microorganisms in different ways [46]. When AgCNTs were combined with NaOCl or H$_2$O$_2$, the damages in cell walls and membranes caused by AgCNTs led to more penetration of the active antimicrobial components into the cells and more effectively inactivate to cells.

NaOCl and H$_2$O$_2$ are oxidative antimicrobials and typically work by forming ROS to inactivate bacterial cells. Looking into its reaction in aquatic solution, NaOCl exhibits a dynamic equilibrium as the following equation:

$$\text{NaOCl} + \text{H}_2\text{O} \rightarrow \text{NaOH} + \text{HOCl} \rightarrow \text{Na}^+ + \text{OH}^- + \text{H}^+ + \text{OCI}^-$$

The produced hypochlorous acid (HOCl) is the key microbicidal component owing to its great potency as a nucleophilic non-radical oxidant and its efficacy lies in the fact that neither bacteria nor mammalian cells can counteract its toxic effect since they lack the enzymes required for its catalytic detoxification [47]. HOCl is known to react with primary amines and other n-compounds to rapidly yield chloramines and nitrogen-chlorine derivatives [14,48]. At the molecular level, a study on the genome-wide transcriptome response to NaOCl-induced oxidative stress in *P. aeruginosa* revealed a downregulation of virtually all genes related to oxidative phosphorylation and electron transport and an upregulation of many organic sulfur transport and metabolism genes [49]. The inactivation efficiency of NaOCl is also dependent on other factors including pH value and bacteria species. The high pH of NaOCl interferes in the cytoplasmic membrane integrity [50]. Different bacterial species have different resistance to NaOCl [14]. It was reported that the theoretical NaOCl concentration required for total bactericidal effects is high (≥0.5%), which causes health issue for human beings. The LD50 was 290 mg/kg of an oral dose and 33.3 mg/kg of an intravenous dose, which was obtained by single-dose toxicity studies in rats using 1.1% NaOCl solutions [51].

H$_2$O$_2$ is thermodynamically unstable and produces hydroxyl free radicals, which are highly reactive and attack DNA, RNA, proteins, and lipids in microbes [52]. A 3-6% (v/v) solution is widely used as an antiseptic and general surface disinfectant. H$_2$O$_2$ at the concentration of 16.3 mM was highly inhibitory for glycolysis and mainly bacteriostatic of *E. coli* K12 a model organism. The growth curves of *E. coli* cells in the presence of nisin, AgCNTs, and the combination of AgCNTs and nisin, along with the controls. The presence of 4 or 8 µg/mL nisin, the cell growth curves were similar to the control during 25 h of growth, which indicated that nisin did inhibit the growth of Gram negative bacteria. No effective inhibition was observed even in the presence of 500 µg/mL nisin during 24 h incubation at 37°C (data not shown). Other studies observed similar results on Gram negative *E. coli* O157:H7 (ATCC43895) after being treated with 320 AU/mL partially-purified nisin Z [58]. The ineffectiveness of nisin against Gram negative believed that this combination strategy is broadly applicable to improve the efficiency of existing antimicrobial agents/methods or design/discover new effective antimicrobial agents/methods.

In this study, we examined the possibility of using the combination of AgCNTs with nisin on Gram negative bacteria using *E. coli* K12 as the model organism. The growth curves of *E. coli* cells in the presence of nisin, AgCNTs, and the combination of AgCNTs and nisin, along with the controls. The presence of 4 or 8 µg/mL nisin, the cell growth curves were similar to the control during 25 h of growth, which indicated that nisin did inhibit the growth of Gram negative bacteria. No effective inhibition was observed even in the presence of 500 µg/mL nisin during 24 h incubation at 37°C (data not shown). Other studies observed similar results on Gram negative *E. coli* O157:H7 (ATCC43895) after being treated with 320 AU/mL partially-purified nisin Z [58]. The ineffectiveness of nisin against Gram negative believed that this combination strategy is broadly applicable to improve the efficiency of existing antimicrobial agents/methods or design/discover new effective antimicrobial agents/methods.

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