Silver Nanocluster-Embedded Zein Films as Antimicrobial Coating Materials for Food Packaging

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Mei, Lei; Teng, Zi; Zhu, Guizhi; Liu, Yijing; Zhang, Fuwu; Zhang, Jinglin; Li, Ying; Guan, Yongguang; Luo, Yaguang; Chen, Xianggui; and Wang, Qin, "Silver Nanocluster-Embedded Zein Films as Antimicrobial Coating Materials for Food Packaging" (2017). *Publications from USDA-ARS / UNL Faculty*. 1798.

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Silver Nanocluster-Embedded Zein Films as Antimicrobial Coating Materials for Food Packaging

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Supporting Information

ABSTRACT: Highly efficient antimicrobial agents with low toxicity and resistance have been enthusiastically pursued to address public concerns on microbial contamination in food. Silver nanoclusters (AgNCs) are known for their ultrasmall sizes and unique optical and chemical properties. Despite extensive studies of AgNCs for biomedical applications, previous research on their application as antimicrobials for food applications is very limited. Here, for the first time, by incorporating AgNCs (∼2 nm in diameter) into zein films that are widely used as food packaging materials, we developed a novel coating material with potent antimicrobial activity, low toxicity to human cells, and low potential to harm the environment. In addition, we systematically evaluated the antimicrobial activities and cytotoxicity of AgNCs-embedded zein films and compared them to zein films embedded with AgNO₃ or Ag nanoparticles with diameters of 10 and 60 nm (AgNP10 and AgNP60, respectively). At equivalent silver concentrations, AgNCs and AgNO₃ solutions exhibited considerably higher antimicrobial activities than those of AgNP10 and AgNP60 solutions. Moreover, AgNCs exhibited less cytotoxicity to human cells than AgNO₃, with a half maximal inhibitory concentration (IC₅₀) of 34.68 μg/mL for AgNCs, compared to 9.14 μg/mL for AgNO₃. Overall, the novel AgNCs coating developed in this research has great potential for antimicrobial applications in food packaging materials due to its high antimicrobial efficacy, ultrasmall size, and low cytotoxicity.

KEYWORDS: Silver nanoclusters, zein, antimicrobial agent, coating material, food packaging

1. INTRODUCTION

Microbial contamination reduces the shelf life of food products, increases the risk of foodborne illness, and causes huge economic losses to the food industry. One approach to combating microbial contamination in the food supply is to develop antimicrobial food packaging systems and coating materials, which incorporate antimicrobial agents that can interact with food or headspace in the package to extend the shelf life of food products and enhance food safety without affecting food quality. The antimicrobial activities of AgNPs are reported to be highly dependent on the particle size. Decreased particle size contributes to higher antimicrobial activity as a result of increased mobility and surface area to volume ratio, resulting in greater interaction with bacteria. For example, Martinez-Castanon et al. demonstrated that, by reducing the sizes of AgNPs from 89 to 7 nm, the minimum inhibitory concentration (MIC) dropped from 11.79 to 6.26 μg/mL for E. coli and from 33.17 to 7.5 μg/mL for S. aureus. Small AgNPs could attach to and penetrate the cell membrane, altering the permeability and cellular respiration, and causing further damage to intracellular biomolecules such as genomic DNA. Although the development of silver-based antimicrobial pack-
aging materials has advanced greatly in the past decades, many challenges remain to be addressed: (1) creating sustained-release delivery systems of antimicrobials to ensure prolonged antimicrobial efficacy;\textsuperscript{10,11} (2) minimizing antimicrobial agents’ toxicity to human; (3) reducing the residual antimicrobial agents or antimicrobial packaging materials in order to reduce environmental hazard.\textsuperscript{12–14} In this study, we have addressed these challenges by incorporating AgNCs as antimicrobial agents for their broad antimicrobial spectrum, ultrasmall particle size, low cytotoxicity, and highly efficient antimicrobial effects.

AgNCs, consisting of dozens of atoms, have a diameter about 2 nm, which is close to the Fermi wavelength (~0.5 nm for Ag). With such small sizes, the band structures of AgNCs are discontinuous and break down into discrete energy levels, and AgNCs thus endow unique physical and chemical properties (such as tunable fluorescence with great photostability and quantized charging property), which are different from AgNPs with larger particle sizes.\textsuperscript{15,16} Conventionally, AgNCs are synthesized by reducing Ag\textsuperscript{+} using chemical reductants or templates (e.g., DNA, polymers et al.) are critical for the stability of AgNCs.\textsuperscript{17} AgNCs enabled various applications. For example, Wang et al. reported the use of core–shell structured nanoparticles with hydrophilic surfaces and hydrophobic cores as templates to synthesize AgNCs, in which the templates greatly enhanced the stability and fluorescence intensity of AgNCs.\textsuperscript{18} Previously, AgNCs have been extensively investigated for applications of biosensing\textsuperscript{19} bioimaging,\textsuperscript{20} and disease diagnosis;\textsuperscript{21} however, the exploration of the antimicrobial application of AgNCs, though highly promising, has been limited, especially for food packaging applications. Compared to AgNPs larger than 10 nm, the ultrasmall size of AgNCs impart unique advantages such as a large surface to volume ratio, high local surface Ag concentration, and high mobility. These advantages enhance the antimicrobial potency of AgNCs, enabling the achievement of superior antimicrobial capacity using much smaller amounts of AgNCs than is possible with AgNPs.\textsuperscript{22–24} However, the antimicrobial activity of AgNCs remains to be systematically studied and compared with AgNPs and AgNO\textsubscript{3} solution.

Zein, a group of prolamins from corn, is a Generally Recognized As Safe (GRAS) food-grade ingredient.\textsuperscript{25} Zein films have low water vapor permeability compared to many other biobased films, because three-quarters of the amino acid residues in zein are hydrophobic.\textsuperscript{26} Zein films have previously been developed as antimicrobial food packaging materials by incorporating antimicrobial lysozyme and thymol.\textsuperscript{27} Compared with these biological based antimicrobials, zein films embedded with inorganic and highly potent AgNCs may offer unique advantages such as high efficacy and low volatility.

In this work, we aim to develop a novel antimicrobial coating material for food packaging. We optimized the synthesis of ultrasmall AgNCs in water using polymethacrylic acid (PMAA) as a stabilizer and characterized the AgNC-embedded zein films. Further, we systematically evaluated the antimicrobial activities and cytotoxicity of the resulting AgNC-embedded zein films by comparing them with zein films that were incorporated with AgNO\textsubscript{3} and AgNPs. The developed films showed potent antimicrobial activity and low toxicity to human cells. We envision that, by a simple dry-cast process, this material can be coated and combined with other packaging materials to further enhance antimicrobial potency and broaden the spectra of antimicrobial activity.

2. MATERIALS AND METHODS

2.1. Materials. Silver nitrate was purchased from VWR International (Radnor, Pennsylvania, USA). PMAA was obtained from Polysciences, Inc. (Warrington, Florida, USA). Silver nanoparticles of 10 and 60 nm in diameter were purchased from Alfa Aesar (Haverhill, Massachusetts, USA). Zein was purchased from MP Biomedicals (Santa Ana, California, USA), and ethyl alcohol (ACS grade) was purchased from Pharma-Aaper (Shelbyville, Kentucky, USA).

2.2. Synthesis of Fluorescent AgNCs. AgNCs were synthesized using modifications to a previously reported method.\textsuperscript{28} Briefly, a mixture of AgNO\textsubscript{3} and PMAA in deionized water was reduced by ultraviolet-A irradiation at wavelengths ranging from 315 to 400 nm (UVA Lamp, Sankyo Denki, Japan). To optimize the synthesis conditions to achieve maximum fluorescence emission in minimum time, AgNO\textsubscript{3} and PMAA were dissolved in deionized water with concentration ratios varying from 2:1 to 20:1, followed by exposure to UVA for a series of times. The fluorescence emission of the reducing product was measured every 15 min with excitation at 512 nm for a maximum of 7.5-h UVA exposure.

Synthesis of AgNCs for the silver release profile, toxicity, and antimicrobial studies proceeded by reducing 60 mg/mL AgNO\textsubscript{3} and 10 mg/mL PMAA with 60 min UVA exposure, according to the optimal conditions determined in preliminary experiments. After 60 min exposure to UVA light, the solution acquired a pink color, which indicated the formation of AgNCs. The synthesized AgNCs solution was then filtered with dialysis bags (MWCO 1KDa, Spectrum Laboratories, INC, US) to remove unreacted silver ions and stored in a refrigerator for future use. The yield of AgNCs was 10%, which was estimated from Inductively Coupled Plasma (ICP) measurements (ICP–9000, Shimadzu, Kanagawa, Japan). Briefly, a 1 mL stock solution of AgNCs was diluted 10 times with 5% (v/v) aqueous nitrous acid, and AgNO\textsubscript{3} was dissolved by 5% (v/v) aqueous nitrous acid at concentrations of 0.01, 0.1, 1, 10, and 100 mg/mL with a final volume of 10 mL. AgNO\textsubscript{3} solution samples were first tested by ICP to generate the standard curve, and then AgNCs samples were tested.

Synthesis of AgNC-embedded zein film was achieved by dissolving 100 mg zein protein in 1 mL of 70% (v/v) aqueous ethanol and adding AgNC solution to the ethanol in a 3:7 (v/v) ratio. The zein mixture, AgNP60 and zein mixture, AgNP10 (National Institutes of Health, USA). The zeta potential was measured using 1 mL each of AgNC solution, AgNC and zein mixture, AgNP10 (ICPE-9000, Shimadzu, Kanagawa, Japan). Brie

2.3. Characterization of AgNCs. After synthesis, AgNCs were characterized for their fluorescence, morphology, and surface charges. The fluorescence of AgNCs was measured using a microplate reader (SpectraMax, Molecular Devices, LLC, California, USA) with the emission range 560–700 nm with excitation at 510 nm. The morphology of AgNCs was observed via scanning transmission electron microscopy (STEM). Samples were prepared for microscopy as follows. The purified stock solution of AgNCs was diluted 50 times with deionized water, and 5 μL of the diluted solution were dry on a microscopy grid (400 mesh ultrathin carbon film on lacey carbon support film, Ted Pella, INC, US) under the hood for 1 h. The sample AgNC and zein mixture was prepared for STEM by mixing 0.5 μL of AgNCs stock solution with 4.5 μL of 0.01% zein in 70% ethanol, and then dropping 5 μL of the mixture on a carbon grid and drying under the hood for 1 h. These grids were later imaged using STEM (JEM 2100 FEG TEM/STEM, JEOL, Tokyo, Japan), and the size distribution was measured and analyzed using the software ImageJ (National Institutes of Health, USA). The zeta potential was measured on a Zetasizer Nano (Malvern Instruments Ltd., Worcestershire, UK), using 1 mL each of AgNC solution, AgNC and zein mixture, AgNP10 and zein mixture, AgNP60 and zein mixture, and zein mixture in 70% ethanol aqueous solution.

2.4. Assessment of Antimicrobial Activity. The agar diffusion test and growth curve measurement were applied to a pathogenic
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A strain of *E. coli* O157:H7 was then measured every 0.5 h with a UV/vis spectrophotometer. AgNO3, AgNP10, and AgNP60 were dissolved in deionized water (DI water) with concentrations of 2 and 0.4 mg/mL Ag equivalents, respectively. Then, 5 μL of each sample were spread and allowed to dry on diffusion disks (VWR International, Radnor, Pennsylvania, USA) with diameters of 7 mm; thus, each disk contained 10 μg or 2 μg Ag equivalents. The diffusion disks were further dried under the hood, followed by loading an additional 5 μL of 10% zein solution and drying. One colony of *E. coli* O157:H7 was dispersed and incubated in tryptic soy broth (TSB, VWR International, Radnor, Pennsylvania, USA) at 37 °C for 16 h, and 100 μL of bacteria suspension (absorbance at OD<sub>600</sub> 1) were spread evenly on tryptic soy agar (25 mL tryptic soy agar per dish, Sigma-Aldrich, St. Louis, Missouri, USA). The dried disks were then placed on the plates. Diffusion disks loaded with 5 μL of TSB, 5 μL of 10% zein solution, and 3.4 μL of 0.05% PMAA solution (equivalent to the amount of PMAA in AgNCs with 10 μg silver equivalents) were used in the control group. After incubating the plates at 37 °C for 24 or 72 h, the width of the inhibition rings surrounding the disks were measured with a ruler. The antimicrobial activity of bare AgNCs, AgNO3, AgNP10, and AgNP60 (without zein coating) was also studied by measuring the growth curves of *E. coli* O157:H7 exposed to the different treatments. *E. coli* was cultured in TSB for 16 h and diluted with TSB to an optical density (OD) at 600 nm of 0.05. The diluted bacteria were then incubated with AgNCs, AgNO3, AgNP10, and AgNP60 with final concentrations of 1, 5, or 10 μg/mL Ag equivalents, respectively, for 9 h in a 37 °C incubator. The absorbance or OD of each sample at 600 nm was then measured every 0.5 h with a UV/vis spectrophotometer ( Beckman Coulter, Brea, CA, USA). To test the MIC of AgNCs, AgNO3, AgNP10, and AgNP60. Mueller-Hinton agar plates were coated by AgNCs, AgNO3, AgNP10, and AgNP60 embedded films with silver concentrations of 0.525, 1.05, 2.1, 4.2, 8.4, 16.8, 33.6, 67.2, 134.4, 168, 201.6, and 235.2 μg/cm<sup>2</sup>. Agars coated with plain zein film were used as the control. Then, 100 μL of bacteria suspension (OD<sub>600</sub> = 1.0) were evenly spread on the pretreated agar and incubated at 37 °C for 24 h. The lowest silver concentrations that resulted in no visible growth of microorganisms were determined as the MIC.

2.5. Cell Viability. The cytotoxicity test of AgNCs, AgNP10, AgNP60, and AgNO3 was performed on human cell line HCT116 (ATCC, Manassas, VA). Briefly, the cells were seeded on 96-well plates and incubated overnight for adhesion, followed by adding 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2, or 102.4 μg/mL of AgNCs, AgNP10, AgNP60, or AgNO3 dissolved in Dulbecco’s Modified Eagle Medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA), respectively. After incubating for 48 h, the cell viability was measured by the cell counting kit-8 (Dojindo Molecular Technologies, Maryland, USA). Specifically, 10 μL of cck-8 solution were added to each well of cells and incubated for around 2 h. The absorbance at 450 nm was recorded by a plater reader (SpectraMax, Molecular Devices, Sunnyvale, California, USA), and cell viability was calculated according to the manufacturer’s guidance. Results were analyzed in GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, California, USA).

2.6. Statistics. The experimental results were analyzed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, California, USA) with significance level p < 0.05. Two-way ANOVA and Tukey’s multiple comparisons test were conducted for the agar diffusion test data. One-way ANOVA and Bonferroni post-test were conducted for the cell viability data.

3. RESULTS AND DISCUSSION

3.1. Synthesis, Optimization, and Characterization of AgNCs. AgNCs were synthesized by reducing silver ions via UVA irradiation with PMAA as a stabilizer. The effects of irradiation time, concentration of AgNO3 and PMAA, ratio of AgNO3 to PMAA, and light sources (e.g., UVA lamp, linear light bulb, and sun light) on the formation and fluorescence intensity of AgNCs were systematically investigated. The fluorescence emission results for different UVA irradiation times are shown in Figure 1A. The fluorescence intensity increased rapidly at the beginning of the reduction due to continuous formation of AgNCs, and after 60 min of UVA exposure, the fluorescence intensity reached a plateau, which indicated the saturation of the AgNCs formation. Further UVA irradiation did not cause a decrease in fluorescence, which verified the great photosensitivity of the AgNCs. The wavelength of maximum emission underwent a red shift at the beginning of the AgNCs formation and then became stable, which corresponded to the formation and saturation of AgNCs. The synthesis of AgNCs was further achieved using other light sources, such as an 18 W linear light bulb and sunlight. The results indicated that the light bulb and sunlight could also...
reduce the Ag\(^+\) and form AgNCs, but they were less effective than UVA irradiation (Figure S1).

PMAA plays a critical role in the synthesis of AgNCs. It carried carboxylic acid groups that are capable to coordinate with Ag\(^+\), and the hydrophobic regions in PMAA facilitated the formation of AgNCs. Further, the spatial structure prevented the aggregation of AgNCs.\(^{17,24}\) Thus, different concentration ratios of AgNO\(_3\) to PMAA (AgNO\(_3\)-to-PMAA ratios) were then tested with 60 min of UVA irradiation to optimize the conditions for the synthesis of AgNCs. Holding PMAA at 10 mg/mL and increasing AgNO\(_3\)-to-PMAA ratios initially enhanced the fluorescence intensity of AgNCs. However, a plateau was reached between ratios of 8:1 to 12:1, followed by a slight decrease (Figure 1B). This phenomenon indicated the nonlinear relationship between the AgNO\(_3\) to PMAA ratio and the fluorescence intensity of AgNCs. A similar trend was also observed when the PMAA concentration was fixed at 40 mg/mL; the fluorescence intensity of AgNCs increased and reached a maximum at 240 mg/mL AgNO\(_3\), followed by a slight decrease (Figure S2).

In addition to the changes in AgNO\(_3\)-to-PMAA ratios, changes of absolute concentrations of AgNO\(_3\) and PMAA were also found to affect the fluorescence properties of AgNCs. Thus, AgNCs were synthesized using a series of absolute concentrations of AgNO\(_3\) and PMAA under AgNO\(_3\)-to-PMAA ratios of 2:1, 6:1, 12:1, and 20:1 (Figure 1C). Generally, with the same AgNO\(_3\)-to-PMAA ratio, a higher concentration of these substrates produced a larger amount of AgNCs, resulting in higher fluorescence intensity. However, when the silver and PMAA concentration exceeded a threshold, the fluorescence intensity decreased significantly, indicating that a very high AgNO\(_3\) concentration may inhibit the formation of AgNCs. The concentrations of AgNO\(_3\) for optimal formation of AgNCs varied depending on the different AgNO\(_3\)-to-PMAA ratios.

Figure 2. Characterization of AgNCs. (A) STEM images of AgNCs and (B) AgNCs in 0.1% zein and 70% ethanol solution. (C) Size distribution of AgNCs (n = 100). (D) Zeta potential of AgNCs, zein, and mixtures of zein and AgNCs, zein and AgNP10, and zein and AgNP60.

Figure 3. Agar diffusion test of different silver nanocomposite-embedded zein films. Left: Inhibition zone of E. coli O157:H7 treated by AgNO\(_3\), AgNCs, AgNP10, and AgNP60 with 2 μg and 10 μg Ag equivalents, respectively, for 1 and 3 days. Right: the width of inhibition zone (**p < 0.0001, ns > 0.9999, n = 3).
Furthermore, AgNCs formed by the series of concentrations of AgNO₃ and PMAA, i.e. 600, 50 and 1000, 50 (AgNO₃, PMAA; mg/mL), developed a small amount of pink floccule likely containing AgNCs and PMAA, which was presumably caused by the high ratio of AgNO₃ to PMAA, as well as the gel formation ability of PMAA. At very high concentrations of AgNO₃ and PMAA, i.e. 600, 50 and 1000, 50 (AgNO₃, PMAA; mg/mL), the AgNO₃ was supersaturated and not well dissolved; no dramatic fluorescence emission was observed.

Based on the results of the synthesis method optimization, we determined to synthesize AgNCs by reducing 60 mg/mL AgNO₃ and 10 mg/mL PMAA with 60 min UVA exposure for further study. The characterization of AgNCs was performed by observing their morphology and surface charge. Under scanning transmission electron microscopy (STEM), AgNCs showed a narrow size distribution around 2.2–2.4 nm (Figures 2A, C) and were well dispersed (Figure S3A). After mixing AgNCs with zein in 70% ethanol, no significant changes in the size and dispersion of AgNCs were observed, which indicated that AgNCs were highly stable in the zein-containing 70% ethanol solution (Figures 2B, S3B). The zeta potential of AgNCs was −2.69 and −0.70 mV before and after mixing with zein solution, respectively. The mixture of zein and AgNP10, AgNP60 in 70% ethanol presented zeta potentials of −10.9 and −2.06 mV, respectively.

### 3.2. Potent Antimicrobial Activity Exhibited by AgNCs-Embedded Zein Film

The antimicrobial activity of AgNCs-embedded zein film was tested on pathogenic *E. coli* O157:H7 using both an agar diffusion test and a growth curve measurement. AgNO₃, AgNP10, and AgNP60 were included as comparisons (Figure 3). In the agar diffusion test, silver composites of each kind (silver equivalents: 2 μg and 10 μg, respectively) and zein (2.5 mg) were loaded on diffusion disks to test their antimicrobial activities. Diffusion disks loaded with 5 μL TSB, 1.7 μg PMAA, and 2.5 mg zein were used as the control.

After 1- and 3-day treatments, no inhibition rings were observed in the control group, and some bacterial colonies were present on the agar plate on day 3. After treating bacteria with 2 μg silver equivalents of AgNCs or AgNO₃ for 1 day, clear inhibition zones of 0.93 mm and 1.26 mm widths were observed around the AgNCs and AgNO₃ saturated disks, respectively. When bacteria were treated with 10 μg Ag equivalents of AgNCs and AgNO₃, greater antimicrobial effects were observed, as indicated by inhibition zones of 1.95 and 2.05 mm, respectively. There was no significant difference between the width of inhibition rings after 1-day or 3-day treatments of AgNO₃ and AgNCs with 10 μg Ag equivalents, which verified the comparable antimicrobial activities of AgNO₃ and AgNCs. Moreover, as expected, the inhibition activities of both AgNO₃ and AgNCs were concentration-dependent. However, no clear inhibition rings were observed in bacteria treated by AgNP10 or AgNP60 of 2 μg or 10 μg silver equivalents, and there was no significant difference in inhibition zones between AgNP10 or AgNP60 treated groups and the control group.

These results can be explained by both the high surface to volume ratio and high mobility of AgNCs relative to those of AgNPs. The higher surface to volume ratio of AgNCs results in greater surface contact with bacteria and consequently higher antimicrobial activity. The greater mobility of silver nanocomposites is another key factor that contributes to improved antimicrobial activity. Specifically, the antimicrobial activity can be influenced by the release rate of Ag from different silver nanocomposites that were embedded in zein films. Thus, we studied the release rate of Ag from these films by submerging different silver nanocomposite-embedded zein films in water and testing Ag concentrations in the surrounding water every half day. We observed that AgNP10 and AgNP60 embedded zein films released Ag at the slowest rate, whereas both AgNCs-embedded zein film and AgNO₃ steadily released Ag at a much faster rate (Figure S4). This result corresponded to the weak antimicrobial activity of AgNP10 and AgNP60 and the potent antimicrobial activity of AgNCs and AgNO₃. To determine whether zein films inhibited the mobility of AgNP10 and AgNP60, we performed agar diffusion tests for bare AgNCs, AgNP10, AgNP60, and AgNO₃ (no zein coating) at 10 μg silver equivalents. The antimicrobial efficacies were similar to those with zein coatings, and no clear antimicrobial activity was observed for AgNP10 or AgNP60 (data not shown). We also examined the agar diffusion test for large AgNPs with diameters of 550 nm (AgNP550) (Figure S5). Again, no clear bacteria inhibition zone was observed with 50 μg AgNP550 treated for 1 day.

Figure 4 shows the effect of UVA irradiation time (40 min, AgNC40 and 100 min, AgNC100) during AgNCs synthesis on antimicrobial activity assessed by agar diffusion tests at 2 μg and 10 μg Ag equivalents. After 1-day bacterial treatment, AgNC100 (2 μg Ag equivalents) showed a clear inhibition zone of 1.98 mm width, which was comparable to that observed.
for AgNCs with 10 μg Ag equivalents synthesized using 60 min UVA irradiation (1.95 mm). The widest inhibition zone, measuring 2.62 mm, was observed for AgNC40 with 10 μg Ag equivalents, which was significantly larger than that for AgNC100 (10 μg Ag equivalents). However, no significant difference was observed between the inhibition rings treated by AgNC40 and AgNC100 with 10 μg Ag equivalents after 5 days (Figure S6). Nor was there any significant difference observed between the inhibition rings for AgNC40 and AgNC100 at 2 μg Ag equivalents for either 1-day or 5-day treatments. These results indicate that the reducing time is not an important factor contributing to the antimicrobial effect of AgNCs.

The growth curves showing the absorbance at 600 nm for E. coli O157:H7 cultures treated with bare AgNCs, AgNO3, AgNP10, and AgNP60 at 1, 5, and 10 μg/mL Ag equivalents, respectively are shown in Figure 5. At concentrations of 1 and 5 μg/mL Ag equivalents, AgNCs exhibited comparable antimicrobial activity to AgNO3. At the concentration of 10 μg/mL Ag equivalent, a longer lag phase (5.5 h) was observed for AgNO3-treated E. coli than for AgNCs-treated E. coli (4 h). However, after 8 h of growth, both AgNO3-treated and AgNCs-treated E. coli reached the stationary phase, and the maximum cell densities of two treatments were comparable. In addition, a longer generation time and lower maximum cell density were reached for AgNO3-treated cells compared to AgNCs-treated cells. The IC50 for AgNCs was 34.68 μg/mL, in contrast to 9.14 μg/mL for AgNO3. AgNP10 and AgNP60 showed less toxicity than both AgNCs and AgNO3, and this was possibly caused by the same reasons for their lower antimicrobial efficacy, namely, the lower mobility, slower cell membrane penetration, and inefficient silver release of silver nanoparticles.

4. CONCLUSIONS

In summary, we developed a novel antimicrobial coating material by embedding antimicrobial AgNCs into zein films. The fluorescence of AgNCs depended on the UVA irradiation time, light sources, concentration ratio of AgNO3 to PMAA, and absolute concentrations of AgNO3 and PMAA. The antimicrobial efficacy and toxicity of AgNCs were systematically evaluated and compared with those of AgNO3, AgNP10, and AgNP60. AgNCs presented comparable dose-dependent antimicrobial efficacy to AgNO3, but with significantly lower toxicity toward human cells than AgNO3. Further, AgNCs presented much greater antimicrobial capacity than AgNP10 and AgNP60, which indicates that the administration dose of AgNCs for antimicrobial applications could be dramatically reduced compared to that of AgNPs. Overall, the study indicated that the low toxicity, low volatilization, and ultrasmall size of AgNCs enhanced their antimicrobial properties and that
the AgNC-embedded zein film is promising antimicrobial coating material for food packaging.

**Associated Content**

**Supporting Information**
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.7b08152.

Fluorescence intensity of AgNCs reduced by different light sources; effect of concentration ratios of AgNO3 to PMAA on the fluorescent intensity of AgNCs with fixed PMAA concentration of 40 mg/mL; TEM images of AgNCs before and after coating by zein protein; release profile of Ag from zein films embedding AgNCs, AgNO3, AgNP10, and AgNP60; agar diffusion test of zein films embedding AgNCs, AgNO3, and AgNP550 on E. coli; agar diffusion test of zein films embedding AgNCs40 and AgNCs100 on E. coli (PDF).

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**Author Contributions**
L.M. designed this work and performed most experiments, analyzed and interpreted data, and drafted and revised the manuscript. Z.T. designed experiments and revised the manuscript. G.Z. participated in the conception and design of the work, analyzed data, and performed the zeta potential test. Y.L. performed the ICP test for the releasing profile. F.Z. designed experiments and participated in the synthesis of AgNCs. J.Z. designed experiments and participated in the cell viability test. Y.L. designed experiments and participated in the synthesis of AgNCs. Y.G. participated in the TEM imaging and viabilities test. Y.L. designed experiments and participated in the cell viability test. J.Z. designed experiments and participated in the cell viability test. L.M. designed this work and performed most experiments.

**Notes**
The authors declare no competing financial interest.

**Acknowledgments**
This work was supported by the Maryland Agricultural Experiment Station (MAES) under the Project Number MD711 and the USDA National Institute of Food and Agriculture (No: 2014-67021-21585). The authors are grateful for the technical support of the Maryland NanoCenter of the University of Maryland in Transmission electron microscopy.

**References**


Supporting Information

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1. Materials and methods.

Materials

AgNO\textsubscript{3} (VWR International, Radnor, Pennsylvania, USA) and PMAA (PMAA, Polysciences, Inc, Warrington, USA) were used to synthesize AgNCs. AgNP10 and AgNP60 were purchased from Alfa Aesar (Haverhill, Massachusetts, USA). AgNP550 was synthesized through reducing AgNO\textsubscript{3} (VWR International, Radnor, Pennsylvania, USA) by sodium borohydride (VWR International, Radnor, Pennsylvania, USA). Zein protein was purchased from MP Biomedicals (Santa Ana, California, USA), and ethyl alcohol (ACS grade) was produced from Pharmco-Aaper (Shelbyville, Kentucky, USA).

Synthesis of AgNCs by sunlight and lamp light.

AgNCs were synthesized by reducing AgNO\textsubscript{3} under UVA radiation with a stabilizer PMAA. Briefly, 60 mg/mL AgNO\textsubscript{3} and 10 mg/mL PMAA were mixed and exposed to sunlight for 40 hours or to lamp light for 30 hours.

Release profile of silver from zein film

AgNCs was synthesized by reducing 60 mg/mL AgNO\textsubscript{3} and 10 mg/mL PMAA with UVA light for 60 min, and then mixed with ethanol in ratio of 3:7 (v/v). Zein protein was dissolved in 70% ethanol. Zein solution at concentration of 10% were then mixed with 100 µg/mL AgNCs, AgNP10, AgNP60, and AgNO\textsubscript{3}. Later, 1 mL of each mixture were casted and dried into film. The casted films were then immersed in 20 mL water in dark for 3 days, and every half day 1 mL of surrounding solution was collected. The collected solution samples were diluted with 5% nitric acid and measured for Ag concentration by ICP (5000 ICP-OES, Agilent Technologies, Santa Clara, California, USA).
Synthesis of silver nanoparticles.

AgNP550 were synthesized by reducing AgNO$_3$ with sodium borohydride. To be specific, 0.01mM NaBH$_4$ were dropped into 0.25mM AgNO$_3$ followed by vigorous shake. The mixture was then heated up to boiling temperature for 1h to remove unreacted NaBH$_4$. The synthesized AgNP550 were then dialyzed and refrigerated.

2. Fluorescence of AgNCs reduced by different light sources.

![Fluorescence intensity vs Wavelength](image)

**Figure S1.** AgNCs synthesized by 40 h sunlight, 30 h linear light bulb light and 60 min UVA lamp light.

3. Effect of concentration ratios of AgNO$_3$ to PMAA on the fluorescence of AgNCs with fixed PMAA concentration of 40 mg/mL.
**Figure S2.** Fluorescence intensity of AgNCs synthesized with different concentration ratios of AgNO₃ to PMAA with fixed PMAA concentration of 40 mg/mL.

4. TEM of AgNCs before and after mixing with zein.

**Figure S3.** STEM images of AgNCs before (A) and after (B) mixing with zein.

5. Release profile of silver from zein films embedding AgNCs, AgNO₃, AgNP10, and AgNP60.
**Figure S4.** Releasing profile of AgNCs, AgNO₃, AgNP10, and AgNP50 embedded zein films submerged in water.

6. **Agar diffusion test of AgNPs550 embedded zein films on E. coli cells.**

**Figure S5.** Inhibition ring of zein film embedding AgNCs, AgNO₃, and AgNP550. Left: Inhibition zone of E. coli treated by 12.5 μg and 50 μg AgNCs, AgNO₃, and AgNP550 for 1 day. Right: the width of inhibition rings.

7. **Agar diffusion test of AgNCs40 and AgNCs100 embedded zein films on E. coli cells.**
**Figure S6.** Inhibition ring of zein film embedding silver nanoclusters synthesized by 40 minutes (AgNCs40) and 100 minutes (AgNCs100) UVA radiation. Left: Inhibition zone of E. coli treated by 2 μg and 10 μg AgNCs40 and AgNCs100 for 5 days. Right: the width of inhibition rings (ns>0.9999, n=3).