Selective Labeling and Growth Inhibition of *Pseudomonas aeruginosa* by Aminoguanidine Carbon Dots

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### Supporting Information

**ABSTRACT:** *Pseudomonas aeruginosa* is a highly virulent bacterium, particularly associated with the spread of multidrug resistance. Here we show that carbon dots (C-dots), synthesized from aminoguanidine and citric acid precursors, can selectively stain and inhibit the growth of *P. aeruginosa* strains. The aminoguanidine-C-dots were shown both to target *P. aeruginosa* bacterial cells and also to inhibit biofilm formation by the bacteria. Mechanistic analysis points to interactions between aminoguanidine residues on the C-dots’ surface and *P. aeruginosa* lipopolysaccharide moieties as the likely determinants for both antibacterial and labeling activities. Indeed, the application of biomimetic membrane assays reveals that LPS-promoted insertion and bilayer permeation constitute the primary factors in the anti-*P. aeruginosa* effect of the aminoguanidine-C-dots. The aminoguanidine C-dots are easy to prepare in large quantities and are inexpensive and biocompatible and thus may be employed as a useful vehicle for selective staining and antibacterial activity against *P. aeruginosa*.

**KEYWORDS:** carbon dots, *Pseudomonas aeruginosa*, antibacterial materials, bacterial labeling, aminoguanidine

*Pseudomonas aeruginosa* is a prominent pathogen responsible for varied nosocomial infections. This bacterial species is associated with pneumonia, urinary tract infections, and surgical wound infections. *P. aeruginosa* infections are particularly hard to treat because the bacteria are capable of developing sophisticated resistance mechanisms against common antibiotics, and in many instances, resistance is even acquired during treatment. *P. aeruginosa*, for example, exhibits significant resistance rates for fluoroquinolones, ciprofloxacin, and levofloxacin (first-line antibiotics), ranging from 20 to 35%. Such multidrug resistance (MDR) characteristics underscore an urgent need for the development of alternative antibacterial substances against *P. aeruginosa*.

Various antibacterial materials including novel antibiotics, antimicrobial peptides (AMPs), metal-based nanoparticles (NPs), and others have been employed as inhibitors and antibacterial substances against *P. aeruginosa*. Metallic NPs, specifically Ag NPs, have been pursued in antibacterial strategies. However, Ag NPs, as well as other inorganic NPs, are toxic and in many cases induce “collateral damage” to host cells and useful human microflora. Carbon dots (C-dots) are a new class of carbon nanomaterials generally exhibiting sizes of <10 nm. C-dots exhibit remarkable physicochemical properties, including tunable fluorescence, low photobleaching, and biocompatibility. C-dots have been employed in diverse applications, including chemical and biological sensing, optics, catalysis, and others. Importantly, because C-dots can be essentially synthesized from any carbonaceous precursors they are generally nontoxic and environmentally friendly, they have attracted significant interest as vehicles for biological and biomedical applications, including bioimaging, drug delivery and targeting, and therapeutics.

Recently, promising microbiological applications of C-dots have been reported. C-dots, for example, have been utilized as fluorescent probes for bacterial detection and labeling, and bacterial viability testing. The synthesis pathways mostly pursued in such systems have focused on conjugating as-prepared C-dots with bacterial targeting agents. These strategies have shown certain limitations, however, such as specifically complex synthesis pathways and often insufficient selectivity. C-dots directed against Gram-positive bacteria have been reported in which bacterial targeting was accomplished through electrostatic interaction between the anionic bacterial membrane and cationic residues on the C-dots’ surface. Another study has shown similar electrostatically based antibacterial activity against *Porphyromonas gingivalis*, an anerobic Gram-negative pathogen related to periodontitis.

Here, we demonstrate construction, for the first time, of C-dots that are selective toward *P. aeruginosa*. The biocompatible...
C-dots were prepared through a simple one-pot synthesis scheme from a mixture of aminoguanidine (AG) and citric acid as the carbon source.\textsuperscript{25} We show that the AG units constitute the primary targeting vehicle of the C-dots toward the \textit{P. aeruginosa} bacterial cells. Notably, an important property of C-dots, which is exploited here, is the retaining of structural and functional characteristics of the C-dot precursors (such as AG employed here) on the C-dots’ surfaces.\textsuperscript{16,18,39,42} Indeed, the experiments presented here demonstrate that AG moieties displayed on the C-dots’ surface play a major role in targeting the C-dots to \textit{P. aeruginosa} cells, affecting cell staining, bacterial growth inhibition, and bacterial biofilm formation disruption. Mechanistic analysis reveals that the AG/CA-C-dots’ selectivity toward \textit{P. aeruginosa} is likely due to specific interactions between the AG residues and LPS moieties displayed on the \textit{P. aeruginosa} cell surface.

\section*{EXPERIMENTAL SECTION}

\textbf{Materials.} Amino guanidine hydrochloride, citric acid, PBS tablets (pH 7.4), 10,12-tricosadiynoic acid, lipopolysaccharide (LPS) from \textit{P. aeruginosa}, lipopolysaccharide (LPS) from \textit{E. coli}, osmium tetroxide, glutaraldehyde, and ethanol absolute were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hexamethyldisilazane (HMDS) and poly-L-lysine-coated glass coverslips were purchased from Electron Microscopy Sciences (Hatfield, PA, USA). LB broth Lennox (pH range 6.8–7.2) and BHI medium (pH 7.4) were purchased from BD Difco; LB agar was purchased from Pronadisa (Spain), quinine hemisulfate monohydrate 99% was purchased from Alfa Aesar; methanol, ethanol, sulfuric acid 98%, and chloroform were purchased from Bio-Lab Ltd. (Jerusalem, Israel); and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DMPG) were purchased from Avanti Polar Lipids.

\textbf{Carbon Dot Synthesis and Characterization.} Carbon dots were prepared using different weight ratios of the aminoguanidine (AG) and citric acid (CA) precursors. Specifically, AG/CA (2:1) C-dots were prepared by using 50 mg of AG/25 mg of CA in the hydrothermal synthesis, AG/CA (1:1) C-dots were prepared by using 50 mg of AG and 50 mg of CA, and AG/CA (1:2) C-dots were prepared by using 25 mg of AG/50 mg of CA. In all synthesis schemes, aminoguanidine hydrochloride 98% was mixed with citric acid and dissolved in 200 \(\mu\)L of distilled water. The solution was tightly sealed using a Teflon film and then heated in an oven to 150 °C for 2 h. After the reaction was completed, the resultant mixture was allowed to cool to room temperature, yielding a brown precipitate indicating the formation of carbon dots. Then the precipitate was dispersed in 2 mL of methanol through sonication for 2 min and centrifuged at 10 000 rpm for 10 min to remove high-weight precipitate and agglomerated particles. After this, methanol was evaporated under reduced pressure to obtain an orange solid. This was dissolved in distilled water and dialyzed (MWCO 2000) against distilled water for 24 h, and after complete purification, the clear, orange carbon dot solution was sampled for further characterization and use.

The quantum yield (QY) of the AG/CA-C-dots in deionized water was determined by placing the C-dots inside a quartz cuvette and measuring the integrated photoluminescence intensity (in the range of 380–650 nm) upon excitation at 350 nm. The absorbance values of the C-dots at 350 nm were also measured with respect to a standard solution of quinine sulfate dissolved in 0.2 N H\textsubscript{2}SO\textsubscript{4} (\(\Phi = 58\)).

Finally, the quantum yield of each C-dot solution was calculated using eq 1

\[
\Phi_{\text{sm}} = \Phi_0 \times \left(\frac{P_{\text{sm}}}{A_{\text{sm}}}\right) \times \left(\frac{A_{\text{sm}}}{P_{\text{st}}}\right) \times \left(\frac{\eta_{\text{sm}}^2}{\eta_{\text{st}}^2}\right)
\]

\(\Phi\) is the quantum yield, \(P\) is the integrated photoluminescence, \(A\) is the absorbance at \(\lambda_{\text{sm}}, \eta\) is the refractive index of the solvent, \(st\) is the quinine sulfate standard, and \(sm\) is the C-dot sample.

The following techniques were applied to C-dot characterization:

\textbf{Fluorescence spectroscopy.} AG/CA-C-dot solutions in deionized water were placed inside quartz cuvettes, and the fluorescence emission spectra were recorded on an FL920 spectrophotometer (Edinburgh Instruments, U.K.).

High-resolution transmission electron microscopy (HR-TEM). The AG/CA-C-dot solution was placed on a graphene-coated copper grid, and HR-TEM images were recorded on a 200 kV JEOL JEM-2100F microscope (Tokyo, Japan). The sample was dried overnight before the measurement.

\textbf{X-ray photoelectron spectroscopy (XPS).} The AG/CA-C-dot solution was placed on a silicon wafer and dried overnight. Once dried, the samples were measured using an ESCALAB 250 ultrahigh vacuum (1 \(\times\) 10\textsuperscript{-9} bar)-type X-ray photoelectron spectrometer fitted with an Al Ka X-ray source and a monochromator. The beam diameter was 500 \(\mu\)m with a pass energy (PE) of 150 eV for recording survey spectra, while for high-energy-resolution spectra the recorded pass energy (PE) was 20 eV. The AVANTAGE program was used to process the XPS results.

\textbf{Bacterial Growth.} Two Gram-positive bacterial strains \{\textit{Staphylococcus aureus} (\textit{S. aureus}) provided by Dr. Marisa Manzano (The National Institute of Health, Italy) and \textit{Bacillus cereus} (\textit{B. cereus})\} and four additional Gram-negative bacterial strains \{\textit{Escherichia coli} K12 (\textit{E. coli} K12), \textit{Escherichia coli} DIHO B (\textit{E. coli} DIHO B), \textit{Salmonella enteritidis} (\textit{S. enteritidis}), and \textit{Salmonella typhimurium} strain ATCC14028 (\textit{S. typhimurium})\} were used in this work, provided by the laboratory of A. Kushmaro (BGU). Two \textit{P. aeruginosa} PAO1 wild type species were kindly provided by the laboratory of M. Meijler (BGU), denoted as \textit{P. aeruginosa} PAO1 A, and \textit{P. aeruginosa} PAO1 B was provided by A. Kushmaro.

\textit{B. cereus} was grown in a brain–heart infusion (BHI) medium containing 7.7 g of calf brains (infusion from 200 g), 9.8 g of beef heart (infusion from 250 g), 10 g of protease peptone, 2 g of dextrose, 5 g of sodium chloride, and 2.5 g of disodium phosphate per 1 L of medium. Some of the bacteria were grown in 30 °C for 12 h, and some of the bacterial species were grown in Lennox medium containing 10 g of tryptone, 5 g of yeast extract, and 5 g of sodium chloride per 1 L of medium. The bacteria were grown for 12 h at 37 °C.

\textbf{Antibacterial Activity of the C-dots.} The antibacterial activity of AG/CA-C-dots was evaluated using a broth dilution assay in which the bacteria were initially grown in medium overnight until full growth was achieved (OD\textsubscript{600} = 1), followed by dilution of the bacteria to OD\textsubscript{600} = 0.05 (CFU = 4 \(\times\) 10\textsuperscript{5})
incubation with AG/CA-C-dots at different concentrations (1, 0.5, 0.25, 0.125, and 0.0625 mg/mL). Growth curves describing the change in OD<sub>600</sub> with time were collected during 24 h of incubation at 37 °C. The growth curves were measured using 96-well plates on a Biotek Synergy H1 plate reader (Biotek, Winooski, VT, USA), and bacterial viability was determined after 18 h of growth.

Minimal Inhibitory Concentration (MIC) of Bacterial Growth. The MIC values were determined using a broth dilution method. Briefly, all bacterial cells were grown under optimum growth conditions with increasing concentrations of C-dots in the medium. OD<sub>600</sub> values were recorded after 24 h of incubation, and the C-dot concentrations in which there is no bacterial growth observed by the unaided eye were determined.

Scanning Electron Microscopy (SEM). Bacterial solutions at OD<sub>600</sub> = 0.05 were incubated together with AG/CA-C-dots for 12 h at 37 °C, following which the bacterial pellet was collected and washed several times with PBS buffer (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4). Subsequently, the bacteria were resuspended in PBS buffer and fixed for the SEM experiments. Bacterial strains were fixed on a poly-L-lysine cover glass, initially using glutaraldehyde 2.5% solution in buffer for 2 h, and then incubated with osmium tetroxide 1% solution, followed by dehydration by rinsing with ethanol/HMDS mixtures. The fixed bacteria were spray coated with a thin gold layer and placed in the microscope for measurements. SEM images were recorded using a JSM-7400 SEM (JEOL LTD, Tokyo, Japan).

Biofilm Analysis. P. aeruginosa PAO 1 A biofilm was grown in a clear-glass-bottomed 96-well plate, typically through placing 300 μL of the medium in each well, followed by the addition of 5 μL of bacterial solutions at OD<sub>600</sub> = 1 (CFU = 8 × 10<sup>6</sup>). The resulting solution was grown for 24 h at 37 °C to yield P. aeruginosa biofilms. The biofilm was washed several times with PBS buffer and imaged by confocal microscopy. In a similar manner, a P. aeruginosa biofilm was grown in a medium containing 1 mg/mL AG/CA-C-dots. The P. aeruginosa biofilm was imaged using a Plan-Apochromat 20×/0.8 M27 confocal laser scanning microscope (CLSM; Zeiss LSM880, Germany). Image processing to obtain 3-D images and to evaluate the biofilm total volume was obtained using IMARIS software (Bitplane, Zurich, Switzerland).

Bacterial Cell Labeling. Bacterial cell labeling was conducted by initially growing all bacteria until full growth (OD<sub>600</sub> = 1; CFU = 8 × 10<sup>6</sup>), followed by centrifugation of the bacterial solution and separation of the supernatant from the bacterial pellet. The bacterial pellet was washed several times with PBS buffer and then resuspended in a solution of 1 mg/mL amino guanidine C-dots dissolved in PBS buffer. The resulting solution was incubated for 3 h at 37 °C, followed by washing of the bacterial pellet in PBS buffer in order to discard C-dots that were not attached to the bacteria. After the final washing, the C-dot-labeled bacterial pellets were resuspended in PBS buffer and imaged with a Plan-Apochromat 20×/0.8 M27 confocal laser scanning microscope.

Cell Culture and Cytotoxicity of AG/CA-C-dots. HeLa, the human cervical adenocarcinoma cell line, and HEK293T, the human embryonic kidney cell line, were grown in DMEM supplemented with 10% FBS and maintained at 37 °C, 95% relative humidity, and 5% CO<sub>2</sub> in a CO<sub>2</sub> incubator under sterile conditions. In a 96-well U-bottomed microtiter plate, 2.5 × 10<sup>5</sup> cells in 1 mL of media/well (n = 3 for each sample) were seeded and left in the CO<sub>2</sub> incubator for 2 h until the cells became recumbent. Cells are incubated for 18 and 48 h separately with increasing titrated concentrations of AG/CA-C-dots (0, 25, 50, 100, 150, and 200 μg/mL).

Measurement of Cell Death by Flow Cytometry. Cell death was measured in a Galliios flow cytometer from Beckman Coulter Inc., with side-scattered light (SSC) analysis combined with 7-AAD uptake recorded in a multwell plate using an excitation 488 nm/emission 695 nm channel on a logarithmic scale. The flow cytometry experiments utilized 2.5 × 10<sup>6</sup> cells for both the HeLa and HEK293T cell lines that were incubated with varying titrated concentrations of AG/CA-C-dots (0, 25, 50, 100, 150, and 200 μg/mL). After 18 and 48 h of incubation separately, all cells were collected for each sample to check for cell death using flow cytometry. Cells were washed with PBS1X, treated with 7-AAD, and incubated on ice for 30 min in the dark for the assessment of apoptotic cells. A flow cytometry measurement was recorded in a density plot of the intensities of side-scattered light (SSC) that is proportional to cell granularity or internal complexity vs 7-AAD, which has a high DNA binding constant and is useful for dead cell discrimination during flow cytometry while being efficiently excluded by intact cells. In an SSC vs 7-AAD dot plot, 1 × 10<sup>4</sup> events were recorded, the data were analyzed with Kaluza Analysis version 2.1 software from Beckman Coulter Inc., and the percentage of dead cells was plotted.

Zeta Potential. C-dot solutions in PBS buffer were placed inside a Malvern DTS 1070 disposable capillary cuvette, and the zeta potential was measured with a Zetasizer Nano ZS (Malvern, Worcestershire).

Elemental Analysis. Powders of AG/CA-C-dots were analyzed for carbon, hydrogen, nitrogen, and sulfur (CHNS) contents. Data were collected using an OEA 2000 Thermo Scientific Flash Smart elemental analyzer.

Lipid/Polydiacetylene Vesicle Assay. Two types of vesicles were prepared: DMPC/DMPC/PDA in a 1:1:3 mole ratio and LPS/DMPC/PDA in a 0.2:2:3 mole ratio. The vesicles were prepared according to established protocols. Briefly, lipid stocks were dissolved in chloroform/ethanol 1:1, mixed together in the appropriate ratios, and dried in vacuo at a constant pressure of 40 mbar for 6 h until a dry powder was obtained. (P. aeruginosa LPS was prepared in doubly distilled water and added to the lipid mixture after drying.) Next, 2 mL of deionized water was added to the lipids to a final lipid concentration of 1 mM. The solution was then probe-sonicated at 70 °C for 3 min to produce vesicular particles, and the vesicles were cooled at room temperature for few hours and placed in a 4 °C environment overnight. The vesicles were then polymerized using UV irradiation at 254 nm (energy flow = 0.3 J/s) for 10–20 s, with the resulting solutions exhibiting an intense blue appearance. For the preparation of the LPS/DMPC/PDA vesicles in which the LPS source was E. coli, the same experimental steps were carried out, with the difference that the E. coli LPS stock was dissolved in chloroform/ethanol 1:1 and mixed with the phospholipids and diacetylene monomers prior to drying.

The fluorescence response assay was used to evaluate the interaction of C-dots with the PDA-containing vesicles, using excitation at 485 nm, yielding emission at 555 nm. Accordingly, the percentage fluorescent chromatic response (FCR%) in 555 nm was calculated using eq 2.
Data were collected on a Fluoroskan Ascent Thermo Scientific fluorescence microplate reader.

**RESULTS AND DISCUSSION**

**Carbon Dot Synthesis and Characterization.** Figure 1 presents the simple synthesis scheme and characterization of the aminoguanidine C-dots (AG/CA-C-dots). As shown in Figure 1A, the AG-dots were prepared via hydrothermal treatment of aminoguanidine and citric acid at a 2:1 mol ratio (150 °C, 2 h). Importantly, the chemical features of the molecular building blocks (in the case here, AG and citric acid) are retained on the C-dot surface (Figure 1A). The AG/CA-C-dots exhibited the typical excitation-dependent emission spectra (Figure 1B) with the maximal emission (upon excitation at 390 nm) at 480 nm (i.e., green-yellow appearance). A representative high-resolution transmitting electron microscopy (HR-TEM) image of the AG/CA-C-dots in Figure 1C underscores the crystalline nature of the C-dots' carbon core, showing a lattice spacing of 0.32 nm corresponding to graphitic carbon. The size distribution of the AG/CA-C-dots, determined by the HR-TEM analysis, was relatively narrow (4.3 ± 0.5 nm, Figure S1). The quantum yield of the AG/CA-C-dots was approximately 3%.

X-ray photoelectron spectroscopy (XPS) data in Figure 1D illuminate the functional units on the AG/CA-C-dots' surface, confirming the retention of the aminoguanidine and carboxylate acid residues. Specifically, the C 1s spectrum in Figure 1D features deconvoluted peaks at 284, 285.2, 286.5, and 288.5 eV that correspond to C–C, C–N, C–O/C=N, and C≡O bonds, respectively. The N 1s XPS peak in Figure 1D similarly displays deconvoluted signals at 399.7 and 400.7 eV ascribed to the C–N and C≡N bonds, respectively. The XPS results are further corroborated by Fourier transform infrared (FTIR) spectroscopy, showing peaks in areas attributed to C–N, C= N, C≡O, and O–H bonds (Figure S1B). The absorbance spectrum of the AG/CA-C-dots was also measured, displaying peaks corresponding to the carbonyl residues on the C-dots (Figure S1C). The crystallinity of the carbon dots was also estimated by X-ray diffraction (XRD), and the d spacing of the carbon core was found to be 0.366 nm, corresponding to the (002) plane of graphite (Figure S1D).

**Antibacterial Effects of AG/CA-C-dots.** Figure 2 illustrates the selective antibacterial properties of the AG/CA-C-dots. In the experiments summarized in Figure 2, AG/CA-C-dots were added to the bacterial growth medium, and bacterial proliferation was monitored. The concentration-dependent bactericidal effects of the AG/CA-C-dots against *P. aeruginosa* PAO 1 A are depicted in Figure 2A, demonstrating that the proliferation of *P. aeruginosa* PAO 1 A was inhibited with increasing C-dot concentration. Indeed, no bacterial growth was apparent at a C-dot concentration of 0.5 mg/mL (Figure 2A). A concentration-dependent antibacterial effect against *P. aeruginosa* PAO 1 A was also apparent in the agar dilution method, in which the AG/CA-C-dots were incorporated within the agar matrix (Figure S3). Growth curves of all other bacterial strains are presented in Figures S4 and S5.

The bar diagram in Figure 2B demonstrates that the antibacterial effect of AG/CA-C-dots is selective toward *P. aeruginosa*. Indeed, Figure 2B demonstrates that the AG/CA-C-dots had a significant inhibitory effect on *P. aeruginosa* PAO 1 A and *P. aeruginosa* PAO1 B, while the C-dots appeared to exhibit no bactericidal effects in the cases of *Salmonella enteritidis*, *Staphylococcus aureus*, *E. coli* K12, and *Bacillus cereus* (Figure 2B). A small antibacterial effect at a high C-dot concentration was apparent in the case of the *E. coli* DHIO B strain. Table 1 summarizes the minimum concentrations inhibiting cell growth (MIC), further demonstrating the bactericidal selectivity of the AG/CA-C-dots toward *P. aeruginosa* bacterial strains.

**Figure 3** presents scanning electron microscopy (SEM) images showing the effect of the AG/CA-C-dots upon different bacterial cells. Notably, Figure 3 reveals that significant morphology alteration occurred when *P. aeruginosa* PAO 1 A cells were incubated with the AG/CA-C-dots, specifically, the “flattening” and elimination of cell surface smoothness (Figure 3A). This observation likely indicates the leakage of intracellular fluid due to bacterial cell wall permeability induced by the AG/CA-C-dots, similar to membrane perforation induced by other bactericidal compounds. In contrast to the severe morphology transformation of the *P. aeruginosa* PAO 1 A cells following incubation with the AG/CA-C-dots, the SEM images in Figure 3B show no discernible effect of the AG/CA-C-dots upon *S. aureus* cells, consistent with the data in Figure
We further investigated the impact of the AG/CA-C-dots upon biofilm formation (Figure 4). Bacterial biofilms are largely impermeable saccharide/proteinaceous matrixes enclosing bacterial cells, constituting a prominent pathogenic factor.51−53 The microscopy images in Figure 4 show a significant decrease in biofilm abundance when P. aeruginosa PAO 1 A bacteria were incubated with the AG/CA-C-dots for 24 h. Biofilm volume determination, carried out by the analysis of the three-dimension images, revealed an almost 50% decrease when AG/CA-C-dots were added to the bacterial growth medium (Figure 4B).

Fluorescence Staining of P. aeruginosa with Aminoguanidine Carbon Dots. While the experiments presented in Figures 2−4 highlight the selective bactericidal effects of the AG/CA-C-dots against P. aeruginosa, we further examined the feasibility of bacterial cell labeling by the fluorescent C-dots (Figure 5). Indeed, the fluorescence confocal microscopy images in Figure 5 demonstrate that the AG/CA-C-dots can be employed for the selective staining of P. aeruginosa. To accomplish bacterial cell staining, the bacterial suspensions were grown to saturation (OD600 = 1), pelleted, and incubated with the AG/CA-C-dots (1 mg/mL) for 3 h. The fluorescence microscopy images (excitation = 405 nm) dramatically demonstrate that P. aeruginosa cells were labeled by the C-dots, while no fluorescence labeling was apparent in the case of S. aureus. The fluorescence microscopy results in Figure 5 corroborate the selectivity profile of the AG/CA-C-dots, confirming the specific targeting of P. aeruginosa. Furthermore, Figure 5 demonstrates that bacterial staining (rather than bacterial inhibition) can be attained through tailoring the experimental parameters (C-dot concentration, point of C-dot addition to the bacterial suspension, and incubation time).

Toxicity of AG/CA-C Dots toward Mammalian Cells. To evaluate the cytotoxicity of the AG/CA-C-dots, we carried out flow cytometry analysis utilizing HeLa and HEK293T cells (Figure 6; raw data provided in Figure S6). In the experiments, the cells were incubated for 18 and 48 h with increasing titrated concentrations of AG/CA-C-dots, and cell death was measured by flow cytometry. The percentages of dead cells, presented in Figure 6, confirm that for both cell types there is no apparent effect of the AG/CA-C-dots on cell viability, up to a high AG/CA-C-dot concentration of 200 μg/mL.

Table 1. MIC Values for the AG/CA-C Dots Applied to the Growth of Different Bacteria

<table>
<thead>
<tr>
<th>species</th>
<th>gram type</th>
<th>MIC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa PAO 1 A</td>
<td>—</td>
<td>0.5</td>
</tr>
<tr>
<td>P. aeruginosa PAO 1 B</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>S. enteritidis</td>
<td>—</td>
<td>&gt;1</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>—</td>
<td>&gt;1</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>—</td>
<td>&gt;1</td>
</tr>
<tr>
<td>E. coli DIHO B</td>
<td>—</td>
<td>&gt;1</td>
</tr>
<tr>
<td>B. cereus</td>
<td>+</td>
<td>&gt;1</td>
</tr>
<tr>
<td>S. aureus</td>
<td>+</td>
<td>&gt;1</td>
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We further investigated the impact of the AG/CA-C-dots upon biofilm formation (Figure 4). Bacterial biofilms are largely impermeable saccharide/proteinaceous matrixes enclosing bacterial cells, constituting a prominent pathogenic factor.51−53 The microscopy images in Figure 4 show a significant decrease in biofilm abundance when P. aeruginosa PAO 1 A bacteria were incubated with the AG/CA-C-dots for 24 h. Biofilm volume determination, carried out by the analysis of the three-dimension images, revealed an almost 50% decrease when AG/CA-C-dots were added to the bacterial growth medium (Figure 4B).

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Mechanistic Analysis. While the experiments outlined in Figures 2−5 show the selective antibacterial and staining capabilities of the AG/CA-C-dots against *P. aeruginosa*, we further aimed to elucidate the mechanism for *P. aeruginosa* targeting, staining, and bactericidal activity by the AG/CA-C-dots (Figures 7 and 8). To probe the relationship between the C-dot surface properties and bactericidal activity, we synthesized C-dots with different mass ratios between the aminoguanidine and citric acid precursors and assessed their antibacterial activities (Figure 7). Specifically, in addition to the AG/CA-C-dots exhibiting a 2:1 mass ratio between the AG and citric acid (CA) precursors, we also examined C-dots with 1:1 and 1:2 AG/CA mass ratios. [A detailed characterization of the AG/CA-C-dots (1:1) and AG/CA-C-dots (1:2) is provided in Figures S7 and S8.] Importantly, many studies have demonstrated that functional units of the precursor molecules are retained on the surfaces of the C-dots generated from those precursors.54−56 As such, the experiments presented in Figure 7 examine the relationship between the relative abundance of aminoguanidine residues on the C-dots’ surface and the bactericidal activity of those C-dots.

Figure 7A presents zeta potential measurements recorded for the C-dots prepared from the AG and CA precursors at the three different mass ratios. The C-dots were negatively charged because they display abundant carboxylic residues formed through the hydrothermal synthesis process.40 However, Figure 7A demonstrates that the negative charge on the C-dots’ surface, reflected by the zeta potential values, was reciprocal to the relative concentration of AG (which exhibits positive amine residues) in the reaction mixture. Fourier transform infrared (FTIR) spectral analysis of the three C-dot species (Figure S9A) corroborates the zeta potential data, indicating that the AG abundance on the C-dots depended on the AG/CA precursor ratio.

To further assess the relationship between the AG display on the C-dots and their antibacterial effects against *P. aeruginosa*, we quantitatively correlated the percentage of nitrogen (which solely originates from the AG precursor) and the bacterial
viability (Figure 7B). The results indicate a dramatic reciprocal relationship between the percentage of nitrogen in the C-dots and the \textit{P. aeruginosa} viability: lower nitrogen concentration (i.e., lower AG display on the C-dots’ surface) yielded greater bacterial viability and vice versa. The full data set regarding the C, N, and H atomic percentages in all of the C-dots tested is presented in Figure S9B.

The correlation between AG concentration and antibacterial properties of the C-dots is similarly clearly manifested in the \textit{P. aeruginosa} growth curves, recorded in the presence of identical concentrations (1 mg/mL) of the three types of C-dots, each prepared from the two precursors at different mass ratios (Figure 7C). For example, the total inhibition of \textit{P. aeruginosa PAO 1 A} growth was achieved upon addition of the C-dots exhibiting a 2:1 mass ratio between AG and CA (Figure 7C, red curve). In comparison, C-dots prepared from a lower AG concentration (1:1 mass ratio between AG and CA) had a smaller antibacterial effect (blue curve in Figure 7C), while C-dots synthesized from a 1:2 AG/CA mixture did not inhibit the growth of \textit{P. aeruginosa PAO 1 A} (Figure 7C, green curve).

Crucially, the \textit{P. aeruginosa PAO 1 A} growth analysis in Figure 7C reveals that the addition of aminoguanidine alone...
did not have a discernible antibacterial effect (Figure 7C, light-purple curve). This important observation indicates that the display of the AG units on the C-dots’ surface makes a critical contribution to the antibacterial properties of the particles. The enhanced bactericidal functionality of the C-dot-displayed AG may be ascribed to both the presentation of a large number of the AG units on the C-dots as well as the surface immobilization of biologically active AG conformations. Previous studies have similarly demonstrated enhanced activities of therapeutic molecules immobilized on the surfaces of nanoparticles.40,41,57

While the data in Figure 7 confirm the correlation between the bactericidal properties and the display of aminoguanidine residues on the C-dots’ surfaces, we further elucidated the likely molecular targets of the AG/CA-C-dots on the bacterial cell surfaces (Figure 8). Figure 8 presents fluorescence emissions of lipid/polyacetylene (PDA) vesicles following the addition of AG/CA-C-dots. Lipid/PDA vesicles constitute a useful biomimetic membrane model which has been widely employed for studying bilayer interactions of membrane-active molecules.45,46,48,58–60

In general, lipid/PDA vesicles mimic bilayer membranes, and the conjugated PDA units constitute sensitive fluorescent reporters for the binding of soluble species onto the bilayer surface.45,46,48 In the experiments depicted in Figure 8A,B, we recorded the AG/CA-C-dot-induced fluorescence emitted by lipid/PDA vesicles comprising two distinct compositions: DMPG/DMPC/PDA vesicles (1:1:3 mol ratio) designed to mimic the outer membranes of Gram-positive bacteria that are relatively rich in phosphatidylglycerol (PG)61–63 and P. aeruginosa-extracted lipopolysaccharide (LPS)/DMPC/PDA vesicles (0.2:2.3 mol ratio) mimicking the outer membranes of P. aeruginosa.64–67 Importantly, LPS molecules are not present in the membranes of Gram-positive bacteria, which are encased by a thick peptidoglycan layer.62,63

The data in Figure 8A,B shows significant differences between the fluorescence response of the LPS/DMPC/PDA vesicles induced upon addition of AG/CA-C-dots compared to DMPG/DMPC/PDA vesicles. Specifically, the kinetic analysis in Figure 8A demonstrates that the fluorescence emitted by DMPG/DMPC/PDA vesicles on AG/CA-C-dots addition increased much more rapidly and to a higher fluorescence value than for the LPS/DMPC/PDA vesicles. Previous studies have linked enhanced fluorescence emission in lipid/PDA vesicles to bilayer surface interactions of membrane-active molecules.69–71 Accordingly, the results in Figure 8A are indicative of more pronounced surface binding of the AG/CA-C-dots in the DMPG/DMPC/PDA vesicles, while deeper bilayer penetration of the AG/CA-C-dots likely occurred in the case of the LPS/DMPC/PDA vesicles.35,70

We also measured the fluorescence response following the addition of the AG/CA-C-dots to lipid/PDA vesicles comprising LPS extracted from E. coli compared to lipid/PDA vesicles containing LPS derived from P. aeruginosa (Figure 8C). The chromatic response curves of the two vesicles upon addition of the AG/CA-C-dots (at the same concentration) were significantly different; a much steeper curve (more pronounced color/fluorescence transformation) was observed in the case of the vesicles displaying E. coli LPS, reflecting more pronounced surface localization of the AG/CA-C-dots. Accordingly, the PDA vesicle assay results in Figure 8C further illuminate the probable mechanism accounting for the AG-C-dot selectivity; the LPS moieties displayed on the P. aeruginosa cell surface promote the insertion of the AG-C-dots into the bacterial membrane bilayer, which permeate the cell membrane, thereby exhibiting bactericidal action (akin to the effect of pore-forming antimicrobial peptides). This model may also explain the relatively high MIC values (on the milligram range) presented in Table 1 because sufficiently high concentrations of membrane-internalized C-dots are likely required to achieve a perforation threshold. In the case of E. coli (or other Gram-negative bacteria), the PDA vesicle chromatic data in Figure 8C points to the preferred accumulation of the AG/CA-C-dots at the membrane surface; this translates to lesser bilayer insertion, which explains the significantly lower bactericidal effect.

The role of LPS in promoting the insertion of the AG/CA-C-dots into the biomimetic membrane bilayer is further highlighted in the bar diagram in Figure 8B, depicting the fluorescence responses of the two types of lipid/PDA vesicles, recorded 30 min after the addition of different concentrations of the AG/CA-C-dots. Figure 8B demonstrates that at all AG/CA-C-dots concentrations, the fluorescence signals emitted by the LPS/DMPC/PDA vesicles were significantly lower than the corresponding values recorded for DMPG/DMPC/PDA vesicles. These results confirm that P. aeruginosa LPS induced deeper penetration of the AG/CA-C-dots into the lipid bilayer. Indeed, membrane insertion and permeation are key antibacterial determinants in varied compounds and nanoparticles.10,12,49,72

Figure 9 outlines a mechanism for the cell staining and bactericidal activity of the AG/CA-C-dots toward P. aeruginosa, based upon the data presented in Figures 2–8. The aminoguanidine units on the C-dots’ surface hone onto the LPS residues displayed on the outer membrane of P. aeruginosa bacterial cells, likely through electrostatic attraction. Indeed, guanidinium thiocyanate has been reported to target LPS.37,75 The LPS-bound AG/CA-C-dots penetrate the
bacterial cell membrane, resulting in bilayer permeability and cell death. This interpretation is consistent with the SEM data in Figure 3, showing pronounced leaching of the intracellular contents upon incubation of *P. aeruginosa* cells with the AG/C-dots. Importantly, while LPS is a universal membrane marker of Gram-negative bacteria, *P. aeruginosa* (as well as other Gram-negative bacterial species) exhibits distinct organization and structural features of LPS, likely accounting for the significant selectivity of the AG/C-dots observed here. The small difference in inhibitory activity of the AG/C-dots toward the two *P. aeruginosa* strains (i.e., Figure 3B) likely reflects the known genetic and phenotypic divergence of *P. aeruginosa* PAO-1. Selective binding to the LPS moieties of *P. aeruginosa* may also account for the fluorescence labeling properties of the AG/C-dots (Figure 5) because LPS-mediated attachment to the *P. aeruginosa* cell surface facilitates bacterial staining.

## CONCLUSIONS

New carbon dots were synthesized from aminoguanidine and citric acid molecular precursors, exhibiting remarkable labeling and antibacterial activities toward *P. aeruginosa*. Depending upon the experimental conditions, we demonstrated both specific staining of *P. aeruginosa* bacterial cells and the inhibition of *P. aeruginosa* growth and biofilm formation. Mechanistic analysis reveals that *P. aeruginosa* targeting by the AG-displaying C-dots was likely due to interactions between the AG units and LPS residues on the *P. aeruginosa* cell surface. The aminoguanidine-C-dots are biocompatible and easily prepared from inexpensive reagents and could be employed as a useful diagnostic platform and effective weapon against *P. aeruginosa*.

## ASSOCIATED CONTENT

[2] Supporting Information The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfectdis.8b00270.

Size distribution; FTIR, UV–vis, and XRD spectra; and cytotoxicity of AG/C/C-dots (2:1). UV–vis and fluorescence spectra and FTIR and AFM images of AG/C/C-dots (1:1) and AG/C/C-dots (1:2). Agar dilution of AG/C/C-dots (2:1) and growth curves of *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli K12*, *Escherichia coli DH5α*, *Salmonella enteritis*, *Salmonella typhimurium strain ATCC14028*, and *Pseudomonas aeruginosa* PAO 1 B in the presence of AG/C/C-dots (2:1). (PDF)

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**Notes**

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

The authors are grateful to Nicholas Zerby and Dr. Rina Jeger for help with SEM imaging.

## ABBREVIATIONS

AG/C/C-dots, aminoguanidine carbon dots; CFU, colony-forming unit; LPS, lipopolysaccharide; MIC, minimum inhibitory concentration; MDR, multidrug resistance.

## REFERENCES


