Research Article

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Electric-field assisted growth and mechanical bactericidal performance of ZnO nanoarrays with gradient morphologies

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Abstract: In response to the widespread bacterial threat, mechanical bactericidal nanostructures with various morphologies have been reported for years. However, the relationship between morphology and bactericidal properties is still yet to be elucidated due to the lack of a fair comparison under similar density of the nanostructures. For this purpose, an electrical-field assisted hydrothermal growth method were utilized to prepare the ZnO nanoarrays with similar array density ($1.9 \times 10^9 \text{ rod}\cdot\text{cm}^{-2}$ – $2.4 \times 10^9 \text{ rod}\cdot\text{cm}^{-2}$) but gradient morphologies from hexagonal prism, hexagonal prismoid to hexagonal pyramid on stainless steel sheets. Moreover, in dark condition, a bactericidal activity was observed on the ZnO nanoarray surfaces within 30 min contact for both *E. coli* and *S. aureus* cells. The bactericidal rate was rapidly enhanced as the average tip width of the nanorods decreasing from 137 to 38 nm. These results suggest that the hexagonal pyramid ZnO nanoarrays have a rapid, efficient and broad-spectrum bactericidal activity, which could contribute to the next-generation aquatic pathogens control strategies.

Keywords: ZnO nanoarrays, mechanical bactericidal activity, surface topography, electric field assisted

Abbreviations

NAs	nanoarrays
SSS	stainless steel sheet

1 Introduction

Microorganisms contaminate the human living and working environments, especially the surfaces of metals, ceramics and polymers [1–3]. Such bacterial settlement can trigger biofilm formation, also known as fouling, which will lead to persistent infection and the release of harmful toxic metabolites [4–6]. In order to circumventing the fouling issue, extensive research has been conducted to develop effective antimicrobial surfaces [7–10]. A variety of chemical bactericidal agents (organic [11–13], inorganic [14–16], photocatalytic [17, 18]) were utilized on the surface. However, these bactericidal agents are either physically or chemically unstable, and moreover, they may lead to potential environmental pollution and bacterial drug resistance [19–21]. Therefore, it is urgent to find alternative bactericidal methods for practical applications.

Studies have shown the promising results of the engineered surfaces with specific micro topography, with the restriction of bacterial cell reproduction activities and the destruction of essential cell structures via a mechanical mechanism [9, 10, 22–24]. Ivanova *et al.* reported that *Psaltoda claripennis* cicada wing surfaces displayed bactericidal behavior against Gram-negative bacteria due to their nanopillar topography, in which the bactericidal mechanism is mechanical and independent of chemical composition [25]. Further they reported a wax nanopillar array on *Diplacodes bipunctata* dragonfly wings that imparted an even stronger bactericidal effect against both Gram-negative and Gram-positive bacteria [26]. The non-chemical bactericidal behavior of natural nanopillar topography has drawn strong attention to the development of contact-based bactericidal surfaces. Thus, a number

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of biomimetic nanopillar topographies for bactericidal applications have been fabricated on a variety of substrates, such as silicon [26] and poly (methyl methacrylate) (PMMA) nanopillars [27]. The densely spaced nanopillars with smaller tip sizes were found to be more effective bactericidal effects [27]. However, there are another paper reporting that loosely spaced nanopillars are more likely to bind strongly to cell membranes, causing them to tear as the cells move [28]. Unfortunately, the discrepancy above is due to the lack of focus on the single morphological factor (density, height and tip-width of nanoarrays (NAs)), while maintaining the other morphological factors relatively similar. Therefore, the relationship between the single morphological factor and the bactericidal performance is yet to be elucidated [29–31].

In this work, we studied the influence of the single morphological factor of the NAs tip-width upon the bactericidal activities. To this end, an electric field assisted hydrothermal method were adopted, so as to maintain the density and height of NAs to be relatively similar. Three types of ZnO NAs, namely hexagonal prism NAs, hexagonal prismoid NAs and hexagonal pyramid NAs with similar density and gradient morphology distribution were grown on the stainless-steel sheet. A correlation between the shape/tip-size of the NAs and the bactericidal activities against both *E. coli* and *S. aureus* cells in dark condition were then established, which together with the synthetic method, could guide the preparation of the next-generation bactericidal surfaces in large area.

2 Experimental

2.1 Materials

Stainless steel sheet (SSS, 304 austenitic), pure ethanol, monoethanolamine, and dimethylamine borane were purchased from Chang Zheng (Chengdu). Zinc acetate dihydrate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$), zinc nitrate hexahydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) and hexamethylenetetramine (HMTA, $(\text{CH}_2)_6\text{N}_4$) were purchased from Aladdin (Shanghai). Polyethyleneimine (PEI, Mw 600) was purchased from Sigma-Aldrich. All the chemicals used in this work were analytical grade. Deionized water was used as the hydrothermal growth solution.

2.2 Electrical-field assisted hydrothermal growth of ZnO nanoarrays

Zinc acetate dihydrate was dissolved in pure ethanol at 60°C to form the 5 mM precursor solution. The resultant precursor solution was then coated onto a piece of SSS substrate (3 cm × 10 cm) by dip-coating at a speed of 5 cm/min. The as-coated substrate was annealed at 300°C for 10 min. To construct a series of ZnO NAs on SSS, the two-step electrical-field assisted chemical bath deposition (CBD) method was used. First, a ZnO seed layer were formed on SSS substrate by dip-coating-annealing method [32–34]. After repeating the coating and annealing step four times, the SSS substrate were covered by a uniform 30 nm ZnO seed layer.

The electrical-field assisted fabrication process of the ZnO NAs were shown in Figure 1a. An electrode-pair was introduced into the hydrothermal growth system. The SSS substrates with seed layer was horizontally down placed in solution of electrical-field hydrothermal growth system which contained equimolar $\text{Zn}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ and HMTA (20 mM, 400 mL) in water in the presence of PEI solution (4 mM) at 80°C. A direct current voltage (50 V) was applied to the electrodes by cataphoresis apparatus during the crystal growth. After growth, the substrates were thoroughly cleaned with deionized water several times and annealed in air at 400°C to remove any organic residue.

2.3 Fabrication of ZnO film

The ZnO film was prepared as a comparison group. The sol-gel dip-coating method was used to prepare zinc oxide thin films [35]. Zinc acetate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$), 30 mmol) was added into 100 mL 2-methoxyethanol (MEA) at 60°C and stirred for 15 min to yield a clear and homogeneous solution. The solution was cooled to room temperature and aged for 24 h. The ZnO films were deposited on SSS substrates (1 cm × 1 cm) by dip-coating at a speed of 5 cm/min. The resultant film samples were dried at 90°C for 10 min. After repeating the dip-coating-drying step ten times, the final ZnO film samples were annealed at 500°C for 1 h and cooled to room temperature.

2.4 Characterization

To characterize the ZnO NA surface topographies, field emission scanning electron microscopy (FE-SEM, JSM-7001F, JEOL) was used at a voltage of 20 kV. X-ray diffraction patterns were recorded by an X-ray diffractometer

Figure 1: The experimental setup a) and changes in ZnO NAs topography under the influence of setting position of samples. The gradient red shows the gradient change of $\text{Zn(OH)}_2 \cdot 4$ concentration. SEM images of b) anode region; c) middle region and d) cathode region; topographic parameters: e) tip-width, f) density and g) height, as determined by SEM.

(XRD, Philips X' Pert PRO), with Cu $K\alpha$ radiation ($\lambda = 1.5418$ Å).

2.5 Bactericidal properties of ZnO nanoarrays

The bactericidal properties of the ZnO NAs against *E. coli* and *S. aureus* cells were evaluated using the sticking membrane method referring to the GB T 21510-2008 standard and Japanese Industrial Standard (JIS Z 2801). Using this

method, the selected bacterial suspension (*E. coli* stain [ATCC 25922] and *S. aureus* stain [ATCC 6538], purchased from Guang Dong Detection Center of Microbiology, China) was activated on an agar culture medium (AOBOX Co., Ltd. Beijing) at 37°C overnight and proliferated in a nutrient broth medium at 37°C for 4 h. The bacterial cell suspension was then diluted by broth medium to proper concentration (*E. coli*, 1.0×10^7 CFU·mL⁻¹~ 2.2×10^7 CFU·mL⁻¹ and *S. aureus*, 1.0×10^6 CFU·mL⁻¹~ 2.1×10^6 CFU·mL⁻¹) to afford a standardized suspension. All samples (stainless steel, ZnO

Figure 2: Characteristics of three locations ZnO nanoarrays on SSS.

film and ZnO NAs) were cut into $1.5\text{ cm} \times 1\text{ cm}$ and immersed into 75% ethanol for 1 min and dried in an ultraclean cabinet prior and placed on the bactericidal experimental device (Figure S1) to form a cavity ($1\text{ cm} \times 1\text{ cm} \times 0.12\text{ cm}$). 100 μL of standardized bacterial cell suspension was added into the cavity and incubated at 37°C in dark for 0.5 h, 2 h and 4 h, respectively. Each sample was thoroughly washed with sterilized physiological saline (10 mL). The bacterial cell suspensions were subsequently diluted in sterile physiological saline for 10 times. Cell counts were obtained by spreading 100 μL of bacterial cell suspension onto agar plates. Colony forming units (CFU) on each agar plate were counted after incubation at 37°C for 18 h. Each test was repeated four times. The bactericidal ratio was calculated by equation (1).

$$\text{Bactericidal ration} = \frac{N_0 - N}{N_0} \times 100\% \quad (1)$$

Where N_0 is the number of living bacteria on the control sample surfaces and N is the number of living bacteria on the test sample surfaces. All the experiments are carried out in dark conditions.

3 Results and discussion

3.1 Fabrication and topography characterization of ZnO nanoarrays

ZnO NAs were fabricated by a two-step electrical-field assisted hydrothermal growth method. First, uniform ZnO seeds were pre-produced on a piece of stainless steel sheet (SSS) by a dip-coating-annealing process [32, 33] (Figure S2) and then hydrothermally grown under electric field

to form ZnO NAs on SSS substrate. In order to obtain ZnO NAs with gradient changing topography, electric field were introduced into the growth system (Figure 1a).

The ZnO NAs fabricated by the electrical-field assisted hydrothermal growth method were shown in Figure 1b-d. By placing the samples at different positions in the electric field, three different types of topographies could be fabricated. Near the anode region, the aligned hexagonal prism ZnO NAs with flat rod tips and an average tip-width of 137.0 nm were obtained (Figure 1b). When the sample moved to the middle region, a hexagonal prismoid NAs were formed (Figure 1c). It is obvious that the tip-width of NAs growth in cathode region was further decreased to 37.7 nm and a hexagonal pyramid like ZnO NAs was fabricated (Figure 1d). These experimental results indicated that there is a gradient change of the unit morphology of the ZnO NAs in the electric field. Moreover, by controlling the density of the seed layer, the density of the three different NAs could be maintained quite similar. Since the height is mainly controlled by the growth time, there is no significant difference observed of the height among the NAs (Figure 1g).

The X-ray diffraction (XRD) patterns of the ZnO NAs show diffraction peaks at 31.8° , 34.4° , and 36.5° , which were indexed to a typical ZnO (JCPDS card, No. 001-1136) wurtzite structure (see Figure 2). The peak at 34.4° , corresponding to the (001) plane, is much higher than those at 31.8° (100) and 36.5° (101), which confirming the preferred growth direction of the ZnO crystal along [001] orientation. The relative peak strength of (101) on the surface of hexagonal prism, hexagonal prismoid to hexagonal pyramid decreased with the peak strength of (001), corresponding to their difference in morphology. On the other hand, the peaks at 42.9° , 49.9° , and 73.4° are characteristic of the stainless steel (JCPDS card, No. 65-4150) austenite structure [32].

To better understand the effect of electric field on ZnO crystal growth, The PEI concentration of hydrothermal solution from different regions were also measured by high performance liquid chromatography (HPLC). The results show that the PEI concentration in cathode region, middle region and anode region are almost the same (Table S1). Besides, the adsorption of PEI on three NA surfaces were also detected by energy-dispersive X-ray spectroscopy (EDX). Before test, all the samples were pre-annealing at 300°C to sublimate the absorbed HMTA (sublimation temperature 280°C). The N element feature count point reveals that there was no significant difference in the amount of PEI absorbed on the surface of each area (Figure S3). It is reported that the PEI can absorbed on the ZnO (100) crystal face and thus increased the rela-

Figure 3: The bactericidal ratios of three ZnO NAs in darkness with the incubation time varying from 30-120 min a) against *E. coli* and b) against *S. aureus*.

tive c-axis growth rate to form nanopyramid arrays with smaller tip-widths [36]. However, the PEI concentration in the hydrothermal system is not affected by the electric field. This is due to the high temperature hydrothermal system improves the diffusion property of PEI molecule [37, 38]. On the other hand, by pre-nitrolysis method, the total Zn^{2+} concentration in the three regions was detected by atomic absorption spectrometry, and the results showed that the total Zn^{2+} concentration in different regions is significantly different (Table S2), which cause the pyramid NAs. The difference in ZnO NAs morphology might be attributed to the difference in the relative concentration of PEI and $\text{Zn}(\text{OH})_2 \cdot 4$ [36]. Flocculent aggregates composed of $\text{Zn}(\text{OH})_2 \cdot 4$ are easier to migrate and harder to diffuse in the electric field.

3.2 Tip-size dependent mechanical bactericidal properties of ZnO nanoarrays

To evaluate the bactericidal properties of three ZnO arrays, two types of standard bacterial suspensions (*E. coli* and *S. aureus*) were respectively dropped into the cave of the bactericidal apparatus and incubated for 30 min-120 min. The experiment was carried out in dark conditions to avoid the influence of the reactive oxygen species generated by the photocatalytic effect of ZnO. From the bactericidal experiment results of *E. coli* (Figure 3a). The bactericidal ratio on the hexagonal prism NAs was $93.1 \pm 3.5\%$ after 30 min of bacterial cell contact, where the bacteri-

cidal ratio is defined as the ratio of the number of dead bacteria on the tested sample surfaces to the number of living bacteria on the control sample surfaces. The bactericidal ratio increased to $> 99.9\%$ after 2 h incubation. The number of living bacteria on the hexagonal prismoid NA surface was even lower (Figure 3a) after 30 min incubation, with the bactericidal ratio reaching $96.8 \pm 2.4\%$, a distinctly bactericidal effect. Notably, the hexagonal pyramid NAs exhibited the highest 30 min bactericidal ratio, $99.3 \pm 0.6\%$, and thus exhibited the best bactericidal property (Figure 3a). From these results, it can be inferred that the stronger bactericidal behavior of ZnO nanopyramid arrays against *E. coli* is related to their sharp tip-width. Similar results were also obtained from the bactericidal experiments against *S. aureus* (Figure 3b), which further confirms the nano-topography-based bactericidal behavior has a broad-spectrum effect. Notably, the bactericidal behavior of the surfaces was stronger against *E. coli* than *S. aureus*, which was attributed to the difference between the structures of the cell walls, where the Germ-negative bacteria (*E. coli*) are more vulnerable to mechanical damage due to a thinner peptidoglycan layer [39].

In recent years, two bactericidal mechanisms of ZnO have been recognized [40–44]. In the reactive oxygen species (ROS) bactericidal mechanism, the generation of the superoxide anion radical ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot\text{OH}$) by ZnO was thought to disrupt cell membranes and kill bacteria [40, 41]. The radicals ($\cdot\text{O}_2^-$ and $\cdot\text{OH}$) are only generated under light or exposure to other types of irradiation. Since the bactericidal experiments were all carried out in dark conditions,

the production of $\cdot\text{OH}$ and $\cdot\text{O}_2^-$ was believed to be minimal. Furthermore, by using a hydrogen peroxide kit [42], the absorbance of Fe^{3+} -xylenol orange compounds and formazan compounds at 560 nm and 450 nm indicated that the concentration of H_2O_2 on ZnO prism NAs was higher than on ZnO pyramid NAs (Figure S4, S5), demonstrating that H_2O_2 is not the major factor responsible for the observed bactericidal behavior. Thus, the ROS bactericidal mechanism cannot account for the bactericidal behavior of the ZnO nanorod arrays observed here. In the other proposed mechanism, it is hypothesized that Zn^{2+} ions leach out from the ZnO NAs causing membrane injury [45] and reformation [46]. However, since the concentration of Zn^{2+} leaching out from the ZnO pyramid NAs used in this study was even lower than that of ZnO prism NAs (Table S3), this mechanism cannot be applied to this study either. Therefore, it can be speculated that the hexagonal pyramid NAs with the sharpest tips puncture the cell membranes more easily and then were more efficient in killing the cells than the other two NAs.

4 Conclusions

Before biomimic bactericidal nanopillars can be applied for the next-generation antibacterial applications, it is important to understand the relationship between the nano-morphology of NAs and bactericidal performance. In this report, an electric field assisted hydrothermal growth method was adopted to prepare ZnO NAs with gradient morphologies, while maintaining similar height and density of the NAs. We found the NAs with the sharpest tip were most effective against both *E. coli* than *S. aureus* cells. After excluding the ROS and Zn^{2+} effect, the results could be explained by the mechanical bactericidal mechanism that the sharpest rod are most effective in puncturing the cell wall structures. This study offers a facile method for the fabrication of large area bactericidal surfaces with rapid, efficient and broad-spectrum bactericidal activities, which could contribute to the practical fabrication of the next-generation bactericidal surfaces.

Supporting Information: Device diagram and procedures of bactericidal experiment, Topography of ZnO seed layer on SSS, The concentration of PEI in the hydrothermal solution of different regions, The absorption of PEI molecular on ZnO NA surfaces, Total Zn^{2+} concentration of three regions in electric field-hydrothermal system, Difference in $\cdot\text{O}_2^-$ productivity of ZnO films and ZnO NAs in dark, Differ-

ence in H_2O_2 productivity of ZnO films and ZnO NAs in dark, Difference in Zn^{2+} productivity of three ZnO NAs.

Conflict of Interests: The authors declare no competing financial interest regarding the publication of this paper.

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Supporting Information

A: Device diagram and procedures of bactericidal experiment

Figure S1: Device diagram and procedures of bactericidal experiment.

B: Topography of ZnO seedlayer on SSS

Figure S2: Topography of ZnO seed layer on SSS.

C: The concentration of PEI in the hydrothermal solution of different regions

Table S1: The concentration of PEI in the hydrothermal solution of different regions.

regions	PEI concentration (mg/mL)
anode region	2.43
middle region	2.37
cathode region	2.66

D: The absorption of PEI molecular on ZnO NA surfaces

Figure S3: The EDX mapping images of nitrogen residual on different ZnO NA surfaces. (a) pristine ZnO NA. ZnO NAs grown at (b) anode regions, (c) middle regions and (d) cathode regions.

E: Total Zn^{2+} concentration of three regions in electric field-hydrothermal system

Table S2: Total Zn^{2+} concentration of three regions in electric field-hydrothermal system.

	total Zn^{2+} concentration ($\text{mg}\cdot\text{L}^{-1}$)
Anode region	2.825
Middle region	1.129
Cathode region	0.618

F: Difference in $\cdot\text{O}_2^-$ productivity of ZnO films and ZnO NAs in dark

A superoxide assay kit was used to compare the difference productivity of $\cdot\text{O}_2^-$. The absorbance at 450 nm of formazan compounds indicated the concentration of $\cdot\text{O}_2^-$ by the ZnO film is much higher than that of ZnO NAs, indicating $\cdot\text{O}_2^-$ is not the major factor responsible for the as-observed rapid bactericidal behavior.

Figure S4: Production of $\cdot\text{O}_2^-$ of ZnO film and three ZnO NAs as the function of time.

G: Difference in H_2O_2 productivity of ZnO films and ZnO NAs in dark

Figure S5: Production of H_2O_2 of ZnO film and three ZnO NAs as the function of time.

A hydrogen peroxide kit was used to compare the productivity of H_2O_2 of these samples. The absorbance at 560 nm of Fe^{3+} -xylenol orange compounds indicated the concentration of H_2O_2 by the ZnO film is higher than that of ZnO NAs, showing H_2O_2 is not the major factor responsible for the as-observed rapid bactericidal behavior.

H: Difference in Zn²⁺ productivity of three ZnO NAs

Table S3: Atomic absorption spectrometry results of Zn²⁺ leaching out from ZnO film and three ZnO NAs.

	Zn ²⁺ concentration (mg·L ⁻¹)
ZnO film	0.235
ZnO prism NAs	0.204
ZnO prismoid NAs	0.195
ZnO pyramid NAs	0.195