



Different antibacterial mechanisms of titania nanotube arrays at various growth phases of *E. coli*

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Abstract: To clarify the antibacterial behavior at early adhesion, two titania nanotube (TNT) arrays were fabricated on polished commercially pure titanium (Ti), and the interaction mechanisms between TNT arrays and the model bacteria (*Escherichia coli*, *E. coli*) were investigated. The results show that TNT arrays exhibit a significant early antibacterial effect, which is highly related to the surface free energy and nano-topography. The underlying antibacterial mechanisms include: (1) the anti-initial-attachment effect at the lag phase (0–4 h); (2) the anti-proliferation and physical bactericidal effects at the logarithmic phase (4–12 h); (3) the reduced antimicrobial properties probably due to the overgrowth of bacteria on TNT arrays at the stationary phase (12 h and then).

Key words: titania nanotube array; *Escherichia coli*; antibacterial mechanism; bacterial adhesion; bacterial proliferation

1 Introduction

Titanium-based materials have been widely used as dental and orthopedic implant materials because of their high mechanical strength, corrosion resistance, and biocompatibility [1–3]. Unfortunately, bacteria-related infection (BRI) in implantation of titanium-based materials often occurs clinically and can lead to local infection, system infection, and sometimes, even death [4]. BRI of implants is mainly originated from the bacteria adhered to the surface of the material, which usually leads to the formation of a mature biofilm [5]. Once a mature biofilm has formed

on the implant surface, it becomes extremely difficult to eradicate with conventional therapy techniques [6,7]. Therefore, one of the most promising antibacterial methods is to inhibit the early adhesion of bacteria before the formation of a mature biofilm [8]. By considering the increasing antibiotic resistance of bacteria, the fabrication of a physically antibacterial surface on Ti-based implants is of strong interest for biomaterial researchers [9].

Electrochemical anodization, which can fabricate highly regular self-assembled titania nanotube (TNT) arrays, provides a cost-efficient way to obtain a well-controlled physical surface on Ti-based implants [10,11]. For example, TNT with different sizes was fabricated on Ti–Nb substrates

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by controlling the voltage of anodization [12]. TNT arrays not only exhibit good biocompatibility but also show potential antibacterial properties [13]. For instance, PENG et al [14] found that the TNT arrays on Ti substrates significantly reduce the initial adhesion of bacteria compared with the polished and acid-etched Ti surfaces.

However, the investigations on anti-adhesion behavior of TNT arrays show controversial statements. For example, LEWANDOWSKA et al [15] claimed that the TNT arrays with diameters ranging from 20 to 30 nm are better bacterial-aggregates-inhibitors than the ones with larger diameters. However, a more robust antimicrobial effect is observed on the TNT array with increasing average nanotubular diameter (from 20 to 80 nm) in another work [16]. These inconsistent conclusions should be probably due to the lack of understanding of the interaction mechanisms between bacteria and TNT arrays during the early bacterial adhesion.

Among the BRIs in implants, 65% of the isolates are gram-negative bacteria [17]. Moreover, the gram-negative bacteria show more significant inhibition effects on titania nanostructures, compared with Gram-positive bacteria [18,19]. Since *Escherichia coli* (*E. coli*) is the most common infecting bacteria among gram-negative bacteria, it was selected as the model bacteria to investigate the early antibacterial behavior of TNT arrays with different geometry parameters and further discuss the possible interaction mechanisms between TNT arrays and different growth phases of *E. coli* in this work.

2 Experimental

2.1 Sample preparation

TNT arrays with different diameters were fabricated by electrochemical anodization on Ti discs (99.7%, 10 mm × 10 mm × 2.5 mm, Guanyue Metal Materials Co., Ltd., China) following the similar procedure as reported before [10]. Briefly, Ti discs were mechanically polished with SiC sandpaper, and then cleaned with acetone, ethanol, and deionized water for 10 min and dried at 60 °C. Anodization was performed in a direct-current (DC) powered two-electrode electrochemical cell, by using platinum sheets as cathode and titanium discs as the anode in the electrolytes containing 0.5 wt.%

NH₄F, 97 vol.% ethylene glycol and 3 vol.% water. After electrolysis at voltages of 30 and 50 V for 50 min, respectively, different anodized samples were obtained. After anodization, the samples were immediately rinsed with deionized water and ultrasonically in ethylene glycol for 10 min, dried at 60 °C, and they are referred to as AD30 and AD50, respectively. The mechanically polished pure Ti is used as a control and referred to as CP-Ti. All samples were sterilized by autoclaving at 120 °C for 30 min before bacterial experiments.

2.2 Sample characterization

Scanning electron microscopy (FE-SEM, JEM-6700F, JEOL, Japan) was used to analyze the surface morphology. The contact angles of different solvents on samples were evaluated by the contact angle analyzer (SDC-200S, Sindin, China). Two solvents, i.e., Milli-Q water and bromonaphthalene (Sangon Biotech, China), were used. The tests were repeated three times for each solvent on each sample. Besides, based on the average contact angles by using the two solvents, the surface free energy of each sample was calculated by the software SDS-200S. Atomic force microscope (AFM, MFP-3D™ Stand Alone, Asylum Research, USA) was also used to characterize the surface roughness of specimens.

2.3 Bacteria culture and growth curve

E. coli strain (ATCC 25922) was used to evaluate the antibacterial properties of the samples. A single colony of *E. coli* strain was inoculated into 5 mL Luria-Bertani (LB) medium and incubated overnight at 37 °C, with shaking at 200 r/min. The suspension was subsequently diluted to 1 × 10⁸ CFU/mL with fresh LB medium. Then, 50 μL *E. coli* suspension was added to the Erlenmeyer flask with a pipette in the ultra-clean workbench, and incubated for 24 h at 37 °C, with shaking at 200 r/min. OD 600 nm of the culture medium in the Erlenmeyer flask was measured on a microplate reader (BioPhotometer, Eppendorf, Germany) every 1 h with LB medium as a blank. The bacterial growth curve was performed with at least three replicates.

2.4 Bacterial viability assay

The viability of *E. coli* was assessed by using a counting assay kit-8 (CCK-8, BBoneBot, China)

following the manufacturer's instructions. The diluted suspension of *E.coli* was cultured on CP-Ti, AD30, and AD50, respectively. After incubation for 4, 8, and 12 h, the specimens were washed three times with PBS. Then, 600 μ L mixture solutions of culture medium and CCK-8 (10:1, volume ratio) were added to each well with continuous culture for 2 h in the incubator. Finally, the incubation solution was measured at 450 nm with a microplate reader (EnSpire 2300, Singapore). Each specimen was technically replicated three times.

2.5 Bacterial adhesion

Each sample was incubated with bacterial suspension. After being cultured for 4 and 8 h, the bacterial solution was removed and the samples were rinsed gently with PBS to remove the non-adherent bacteria. After rinsing, the samples were fixed with 4% paraformaldehyde for 30 min and then dehydrated in a gradient concentration (20%, 50%, 70%, 80%, 90%, 95% and 100%) in an ethanol solution. The specimens were then dried overnight, and the surface was sputtered with gold particles for 120 s. Finally, SEM (FE-SEM, JEM-6700F, JEOL, Japan) was applied for bacterial morphology analyses.

2.6 Live/dead bacteria imaging

The live/dead staining (LIVE/DEAD BacLight bacterial viability kits, Molecular Probes, L7012, USA) was performed for the antibacterial activity assessments. Each sample was incubated with bacterial suspension for 8 h. Then, the CP-Ti, AD30, and AD50 samples were rinsed with 0.9% NaCl followed by adding 50 μ L of the combination dye solution (1.5 μ L SYTO 9 + 1.5 μ L propidium iodide + 1 mL 0.9% NaCl). After keeping in the dark for 15 min, the dye solution was removed. Confocal laser scanning microscopy (CLSM, Olympus FV1200, Japan) was used for the characterization of the stained samples.

2.7 Gene expression analysis by RT-qPCR

To further investigate the underlying mechanism of the interaction of the bacteria-TNT array, the expression levels of *E.coli* related genes (*ompA*, *ompF*, *csgA*, and *csgG*) were quantified by real-time quantitative-polymerase chain reaction (RT-qPCR). The suspension of *E.coli* was cultured on different Ti substrates for 8 h. Then, the total

RNA of *E.coli* was extracted with TRIzol reagent (Yeasen, China). The concentration of RNA was quantified using a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA) and then reversely for cDNA with a cDNA reverse transcription Kit (TransGen, China). The obtained cDNA was further amplified using SYBR Green PCR Master Mix (Yeasen, China) under the amplification conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. All primer sequences were presented in Table 1 according to the literature [20], and the targeted gene expression was normalized to *rpoA*. Each sample was technically replicated three times.

Table 1 Primer sequences of *E. coli*

Primer	Nucleotide sequence
<i>ompA-F</i>	5'-GGCGTGCAGACACTAAATCCA-3'
<i>ompA-R</i>	5'-AGCGAAGACCGGAGAAACG-3'
<i>ompF-F</i>	5'-ACACCGATATGCTGCCAGAAT-3'
<i>ompF-R</i>	5'-AACACGACCAACGAAGAAGTCA-3'
<i>csgA-F</i>	5'-TGCAAACCTGATGCCCCGTAAC-3'
<i>csgA-R</i>	5'-CCTGACCAACATCTGCACCAT-3'
<i>csgG-F</i>	5'-TGCCGACACGCAATACCA-3'
<i>csgG-R</i>	5'-ATTGACGACGCGCAGGTT-3'
<i>rpoA-F</i>	5'-AGCTGGTCATCGAAATGGAAAC-3'
<i>rpoA-R</i>	5'-ACGACGAATCGCCTCTTCAG-3'

2.8 Statistical analysis

All experiments were performed in triplicate and all data were presented as means $\pm$ standard deviation (SD). Statistically significant differences (* p < 0.05, ** p < 0.01) were performed with SPSS 19.0 software via the one-way ANOVA.

3 Results

3.1 Surface characterization

Figure 1 shows the surface structures of the CP-Ti, AD30, and AD50 samples. The CP-Ti shows a flat and smooth surface, while vertical and uniform nanotube arrays are formed on the titanium substrate after anodizing. The average diameters of the nanotubes on AD30 and AD50 are approximately 60 and 100 nm, respectively.

The contact angles and surface free energy of all samples are given in Table 2. The CP-Ti presents the highest water contact angle of about 63°. In

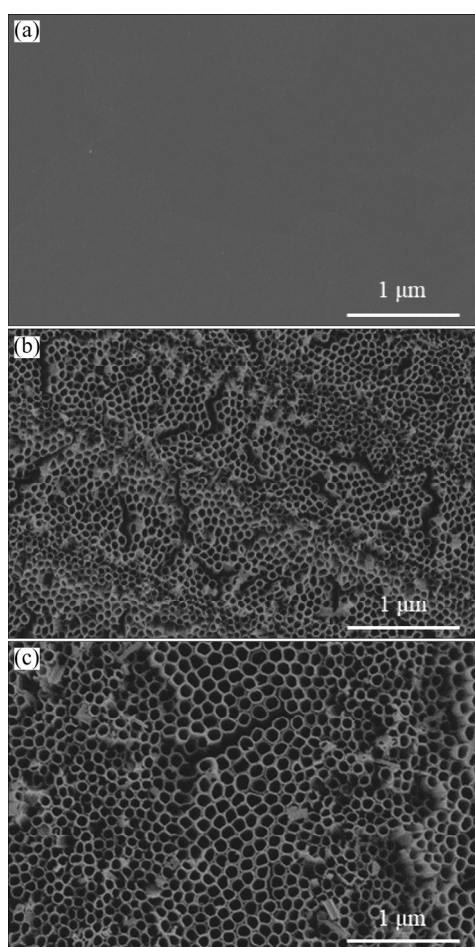


Fig. 1 SEM micrographs of surface morphology of CP-Ti (a), AD30 (b), and AD50 (c)

Table 2 Contact angles and surface free energies of CP-Ti, AD30, and AD50

Sample	Contact angle/(°)		Surface free energy/(mN·m ⁻¹)
	θ_W	θ_B	
CP-Ti	63.19±1.18	13.53±0.34	47.312
AD30	10.59±1.43	2.41±0.65	72.008
AD50	4.95±0.34	1.33±0.21	72.896

θ_W and θ_B are water and bromonaphthalene contact angles; the values of contact angles are mean $\pm$ standard error of the mean ($n=3$).

contrast, the AD30 and AD50 samples show almost superhydrophilic surfaces with water contact angles of around 10° and 5°, respectively. All samples show strong lipophilic behavior when using the non-polar bromonaphthalene. There is a big gap between the surface free energy of the CP-Ti and that of the anodized samples.

AFM was applied to visualizing the surface topography of all samples (see Fig. 2). The surfaces of AD30 and AD50 samples exhibit R_q (root-

mean-square roughness) values of 73.15 and 112.85 nm, respectively, which are significantly higher than the value of 3.56 nm for CP-Ti.

3.2 Bacterial growth curve

The growth curve of *E. coli* is presented in Fig. 3. The whole curve consists of three parts: the lag phase, the logarithmic phase, and the stationary phase. The lag phase ends at around 4 h, followed by the logarithmic phase from 4 to 12 h. From then on, the bacterial growth reaches the stationary phase.

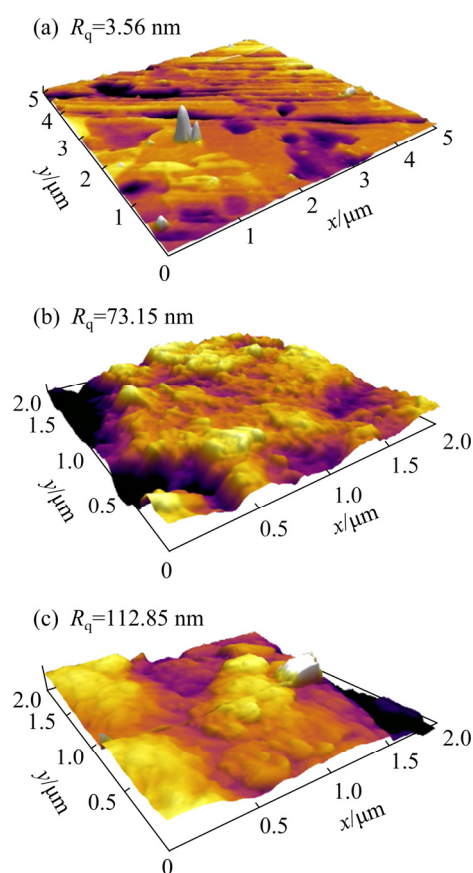


Fig. 2 AFM images of surface roughness: (a) CP-Ti; (b) AD30; (c) AD50

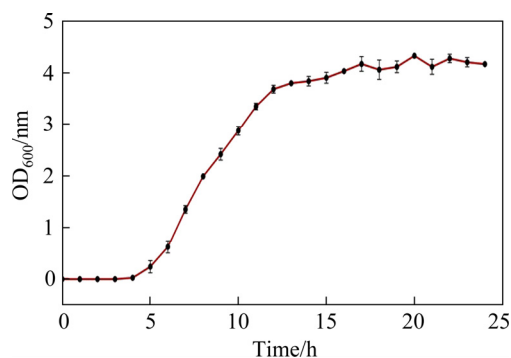


Fig. 3 Growth curve of *E. coli*

3.3 Antibacterial behavior of TNT samples

The viability of *E. coli* cultured for 4, 8, and 12 h on the blank control, CP-Ti, AD30, and AD50 is depicted in Fig. 4. In general, with increasing culture time, the OD values of all groups show an increasing tendency, but the bacteria activity on CP-Ti does not show a significant difference after being cultured for 8 and 12 h. The CP-Ti and blank groups show comparable OD values after incubation for 4 and 8 h, but the bacterial activity seems to be lower on the former than on the latter after being cultured for 12 h. The OD value of the AD30 and AD50 anodized groups is significantly lower than that of the CP-Ti group after culturing *E. coli* for 4 and 8 h ($p < 0.01$), while after being cultured for 12 h, only the AD50 group shows significantly decreased OD values compared with the CP-Ti group ($p < 0.01$). Besides, after being cultured for 8 and 12 h, the growth of *E. coli* is significantly inhibited on the substrate with increasing nanotube diameter.

Figure 5 shows the adhesion and morphology of *E. coli* on the CP-Ti, AD30, and AD50 samples. *E. coli* exhibits a regular rod shape with a smooth surface and aggregates on the CP-Ti as clusters after being cultured for 4 and 8 h (see Figs. 5(a, d)). In contrast, bacterial accumulation on the anodized samples is remarkably inhibited

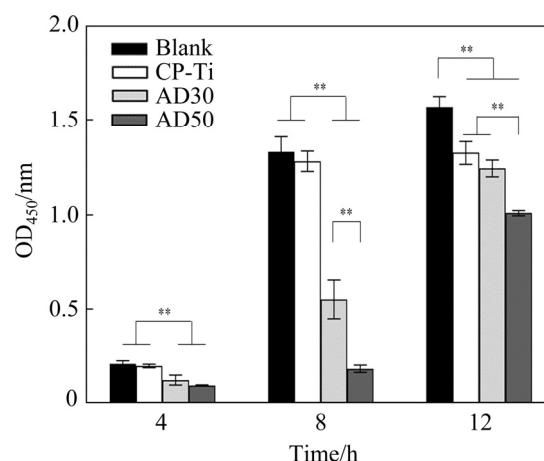


Fig. 4 *E. coli* viability after being cultured for 4, 8, and 12 h on blank, CP-Ti, AD30 and AD50 (** $p < 0.01$)

compared with that on CP-Ti as shown in Figs. 5(b, c, e, f). Besides, the red arrows in Figs. 5(b, c) present that the bacterial surfaces on AD30 and AD50 samples start to corrugate after being cultured for 4 h. However, after incubation for 8 h, the surface morphology of *E. coli* becomes very rough, and a phenomenon of cell membrane disruption is also observed on both anodized groups (see the red arrows in Figs. 5(e, f)).

The fluorescence images of live/dead assay after incubation for 8 h are shown in Fig. 6. The green fluorescence indicates live bacteria, while the

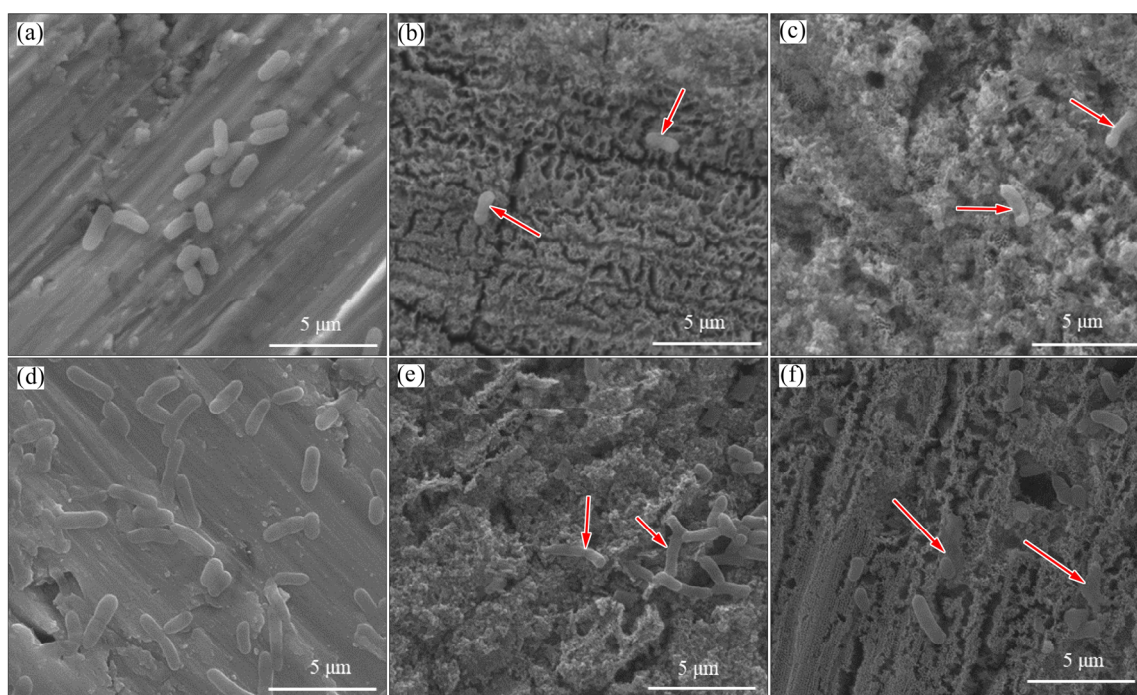


Fig. 5 Morphologies of *E. coli* after incubation for 4 h (a–c) and 8 h (d–f) on CP-Ti (a, d), AD30 (b, e), and AD50 (c, f) (The red arrows show the corrugated (or even dead) cells)

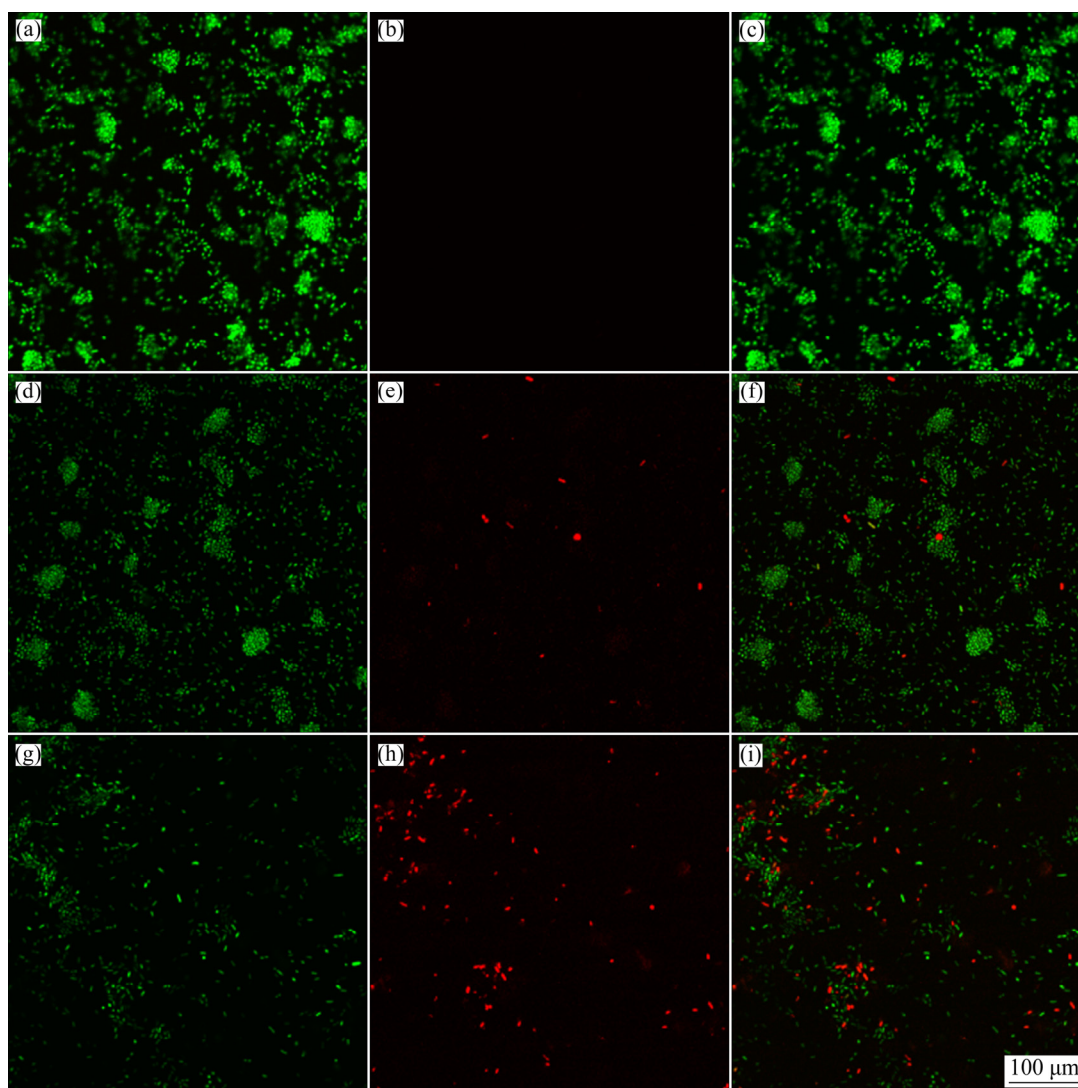


Fig. 6 Live/dead staining of *E. coli* after being cultured for 8 h on CP-Ti (a–c), AD30 (d–f) and AD50 (g–i)

red fluorescence indicates dead bacteria. After being cultured for 8 h, *E. coli* grown on CP-Ti shows green and clustered fluorescence, while almost no red fluorescence can be observed (see Figs. 6(a, b, c)). A significant decrease in live bacteria and an increase in dead bacteria are shown on the surface of the anodized samples, and the trend of bacterial inhibition is more obvious for AD50 (see Figs. 6(d, e, g, 6)). Besides, most dead bacteria are found around the bacteria cell clusters (see Figs. 6(f, i)).

Figure 7 shows the gene expressions of *ompA*, *ompF*, *csgA*, and *csgG* of *E. coli* cultured for 8 h on the CP-Ti, AD30, and AD50 samples. The qRT-PCR results indicate that compared to the CP-Ti group, the expressions of *csgA* and *csgG* are significantly down-regulated on the AD30 and

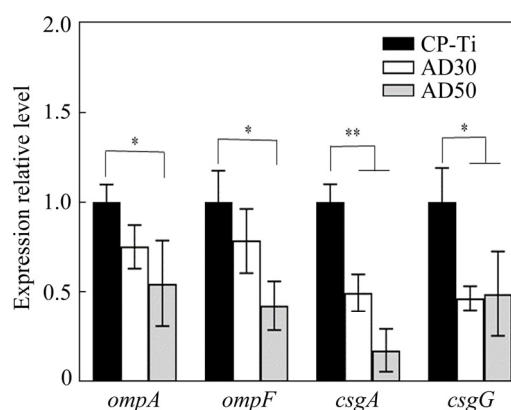


Fig. 7 *E. coli* gene expression after being cultured for 8 h on CP-Ti, AD30, and AD50 (* $p < 0.05$, ** $p < 0.01$)

AD50 samples, while the expression of *ompA* and *ompF* is only significantly down-regulated on AD50.

4 Discussion

Implants are most susceptible to bacteria at the early adhesion stage, and thus one of the most efficient ways to cope with BRI should be inhibiting the early adhesion of bacteria [21,22]. However, few studies aim at the anti-adhesion behavior of the TNT arrays on Ti-based implants and some of them show inconsistent conclusions, which may lead to the confusion in the future development of implants [23,24]. Consequently, the present work is focused on the interaction mechanisms of *E. coli* and the TNT arrays on Ti substrates until the incubation of 12 h, when the logarithmic phase ends and the stationary phase starts, as presented in Fig. 3.

According to Fig. 4, generally speaking, the adhesion of *E. coli* is significantly inhibited on the bare TNT arrays with different average diameters compared with that on CP-Ti and blank control, and the samples with larger TNT diameter exhibit better antibacterial behavior, which is consistent with previous studies [25,26]. However, the antimicrobial mechanism of TNT has not been studied according to the growth phases of bacteria. Therefore, the interaction mechanisms between bacteria and TNT in the three early growth phases will be discussed.

As shown in Fig. 3, there are three phases within the incubation of 24 h of *E. coli*, i.e., 0–4 h for the lag phase, 4–12 h for the logarithmic phase, and >12 h for the stationary phase. Hence, the results after culturing *E. coli* for 4, 8, and 12 h indicate the bacteria–material interactions at the end of the lag phase, at the middle of the logarithmic phase, and at the beginning of the stationary phase.

Bacteria in the lag phase are primarily adapted to their new environment and prepared for proliferation [27], and thus the density and viability of bacteria are generally determined by the initial attachment on the material surface. As shown in Fig. 4, the presence of significantly different OD values between the CP-Ti group and the anodized groups instead of between the AD30 and AD50 groups after incubation for 4 h suggests that the initial bacterial adhesion on the anodized substrates is strongly inhibited due to the presence of TNT arrays, while the nanotube geometry does not play a

key role in determining the anti-adhesion behavior at the lag phase. The interactions between biomaterials and bacteria (or cells) always start from the displacement of water from the interface [10], so the wettability of material surfaces is critical to the initial adhesion. It is well accepted that the increase of surface hydrophilicity usually leads to the inhibition of bacterial initial adhesion [28]. Therefore, it is reasonable that the hydrophilic surface of TNT significantly inhibits the initial adhesion of *E. coli* (see Figs. 5(a–c)). In fact, the wettability of a material surface is determined by the surface free energy [29]. Generally, it is believed that a higher surface free energy results in better adhesion [30]. However, ZHANG et al [31] revealed that the bacterial initial adhesion not only is influenced by the material surface free energy, but also highly depends on the surface free energy of the bacteria themselves, i.e., with decreasing surface free energy difference between biomaterials and bacteria, a better bacterial adhesion is usually observed. In this study, by considering the low surface free energy of *E. coli* (~35 mN/m) [32], the lower surface free energy difference (~10 mN/m) between the polished samples and *E. coli*, compared with that (~35 mN/m) between the anodized ones and *E. coli*, should be responsible for the different bacterial adhesion behavior on the polished and anodized samples, as shown in Figs. 4 and 5. Besides the influence of initial attachment, TNT arrays can also affect bacterial activities. The aggregation and the regular rod morphology of *E. coli* as shown in Fig. 5(a) indicate the good bacterial activity on CP-Ti, which is in accordance with previous studies [33,34]. It is important to note that the corrugating surfaces of bacteria on the anodized samples suggest that *E. coli* starts to be damaged (see the red arrows in Figs. 5(b, c)).

The logarithmic phase is usually considered as the most important stage for the bactericidal effect because the exponential growth leads to significantly higher sensitivity of bacteria to the environmental factors [35]. In this phase, the dependence of the activity of *E. coli* on the implant surfaces (see Fig. 4) should include two different mechanisms: the influence on bacterial proliferation and the bactericidal effect. The increasing number of bacteria as shown in Fig. 5(d) compared with that in Fig. 5(a) suggests the strong proliferation of

E. coli on CP-Ti. However, with increasing the anodization voltage, much fewer bacteria clusters are observed (see Figs. 5(e, f)), indicating the inhibition effect of bacterial proliferation by the TNT arrays. In order to further investigate this effect, qRT-PCR was performed as presented in Fig. 7. *csgA* and *csgG* are non-structural proteins related to the biogenesis of curli. Curli is a class of thin and coiled amyloid fibers expressed on the surface of *E. coli*, and is associated with adhesion and proliferation on abiotic surfaces and biofilm formation [36,37]. The down-regulation of *csgA* and *csgG* on the anodized groups demonstrates that the TNT arrays can significantly influence the proliferation of *E. coli*. The inhibition of bacterial proliferation is probably related to the nano-roughness of the TNT arrays (see Figs. 2(b, c)), because nano-roughness surfaces can significantly inhibit the proliferation of bacteria compared to flat surfaces [38]. The bactericidal effect of the anodized samples is also apparent, as presented in Figs. 6(e, h). The nanotube geometry can alter the osmotic pressure between the inner and outer subshells of bacteria by a simple biomechanical process, i.e., stretching the cell membrane, which ultimately leads to bacterial death [39]. Therefore, the influence of the TNT arrays on the bacterial membrane has been paid much attention to in this work. The two genes, i.e., *ompA* and *ompF*, play key roles in maintaining outer membrane stability and determining the transmembrane transports of hydrophilic solutes in *E. coli*, respectively [40–42]. So, the down-regulation of *ompA* in the anodized groups (see Fig. 7) implies the less stable bacterial membrane on TNT arrays, and the down-regulation of *ompF* in the anodized groups suggests the damage permeation of substrate molecules across the porin. In a word, the lower expression of these two genes on the anodized substrates indicates that the rupture and death of *E. coli* should be attributed to the destructive influence of the nanotube geometry on the bacteria membrane.

The stationary phase starts when the further growth of bacteria ceases due to the limited nutrients or some other conditions [43]. The comparable bacterial activity of CP-Ti and AD30 after culturing for 12 h (see Fig. 4) indicates that *E. coli* on the two groups meets a similar proliferation–death balance, which is probably due to the overspreading bacteria on the sample surface.

However, the lower OD values of AD50 after incubation for 12 h imply a longer-term antibacterial potential for larger nanotubular diameter.

5 Conclusions

(1) After electrochemical anodization, the sample surfaces show titania nanotubular structures, and the anodized samples present better hydrophilicity, higher surface free energy, and rougher surface compared with the polished CP-Ti samples.

(2) The TNT arrays exhibit significant early antibacterial behavior, and a stronger antibacterial effect is observed on the substrates with higher anodization voltage.

(3) The underlying mechanisms of the interactions between bacteria and anodized samples are different at various growth phases of *E. coli*. At the lag phase, the anti-initial-attachment effect is dominant, which may be related to the fact that the surface free energy difference between *E. coli* and anodized samples is higher than that between *E. coli* and the polished CP-Ti. At the logarithmic phase, both anti-proliferation and physical bactericidal effects contribute to the antibacterial behavior, which should be mainly attributed to the nano-topography of TNT arrays. At the stationary phase, the overgrowth of *E. coli* on TNT arrays may significantly undermine the antibacterial properties, especially for the samples with lower nanotubular diameter.

(4) This work provides a new vision upon the early interactions between bacteria and TNT arrays, which can probably be used to improve the antibacterial assessment of biomaterials in the future.

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二氧化钛纳米管阵列在大肠杆菌不同生长阶段的抗菌机理

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摘要: 为了明确二氧化钛纳米管(TNT)阵列在早期粘附阶段的抗菌行为, 在抛光的商业纯钛(Ti)表面制备两种 TNT 阵列, 并探究 TNT 阵列和模式菌(大肠杆菌)之间的相互作用机制。结果显示, TNT 阵列具有明显的早期抗菌性能, 且这种抗菌性能与 TNT 阵列的表面自由能以及纳米拓扑结构密切相关。TNT 阵列的抗菌机理包括: (1)在迟缓期(0~2 h)抑制细菌初始粘附效应; (2)在对数期(4~12 h)抑制增殖和物理杀菌作用; (3)在稳定期(>12 h)可能由于 TNT 阵列表面细菌的过度生长而导致抗菌性能降低。

关键词: 二氧化钛纳米管阵列; 大肠杆菌; 抗菌机制; 细菌粘附; 细菌增殖

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