

## Article

# Micro-Pillar Arrays Coated with TiO<sub>2</sub> Nanotubes on Titanium Surface for Antibacterial Activity and Enhanced Bioactivity

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## Abstract

Titanium and its alloys are widely used for orthopedic and dental implants because of their favorable bone-matching elastic modulus, corrosion resistance, and biocompatibility. However, implant-associated infection and insufficient early osseointegration continue to compromise their long-term clinical performance. Here, inspired by natural micro- and nano-topography such as cicada wings and lotus leaves, we fabricated hierarchical surfaces comprising titanium micro-pillar arrays coated with TiO<sub>2</sub> nanotubes. Micro-pillar arrays with scanning pitches of 40, 60, and 80  $\mu\text{m}$  were fabricated via femtosecond laser ablation, followed by in situ anodization to grow TiO<sub>2</sub> nanotubes approximately 100 nm in diameter. The hierarchical architecture integrates micro-pillars and TiO<sub>2</sub> nanotubes to enhance antibacterial performance and bioactivity: the micro-pillar arrays coated with nanotubes provide a three-dimensional microenvironment for cell adhesion, and the TiO<sub>2</sub> nanotubes show contact-killing antibacterial function. The 40-MNTs sample (40  $\mu\text{m}$  micro-pillars coated with TiO<sub>2</sub> nanotubes) achieved the highest antibacterial efficiency while effectively supporting mesenchymal stem cell adhesion and proliferation. These findings show that combining micro-scale geometry with nano-topographical cues can improve antibacterial performance while maintaining cytocompatibility, providing a drug-free surface engineering strategy for titanium implants.

**Keywords:** micro-pillar arrays; femtosecond laser ablation; TiO<sub>2</sub> nanotubes; antibacteria; bioactivity

## 1. Introduction

Titanium and its alloys are the gold standard for orthopedic and dental implants because their relatively low elastic modulus reduces stress shielding [1,2]. In physiological environments, their surface spontaneously forms a stable and passive oxide layer, conferring exceptional corrosion resistance and biocompatibility [3,4]. These intrinsic characteristics enable their extensive clinical applications ranging from joint replacements and spinal instrumentation to bone-tissue engineering scaffolds [5,6]. Nevertheless, two persistent challenges limit implant performance. First, the relative bioinertness of untreated titanium often restricts osteogenic potential, leading to suboptimal early osseointegration in 5%–20% of cases and increasing the risk of mechanical loosening or long-term implant failure [7–10]. Secondly, bacterial adhesion and biofilm formation on the titanium surface can cause severe peri-implant infection [11,12]. Physical roughening and bioactive

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coatings have therefore been developed to improve osseointegration, whereas systemic or local antibiotics are commonly used to prevent or treat infection [13–16]. Systemic antibiotic delivery may not achieve effective concentrations in the poorly vascularized bone microenvironment, and conventional local delivery tends to cause burst release, cytotoxicity, and selective pressure for antimicrobial resistance [17–19]. Peri-implant bacterial colonization impairs osseointegration via sustained innate immune activation, osteoclastogenesis, and fibrous encapsulation, ultimately leading to implant failure. Therefore, developing implant surfaces that integrate potent antibacterial efficacy with robust osteogenic activity is crucial to address this pivotal challenge in orthopedic and dental practice [20–22].

Surface modification provides an effective route to confer antibacterial activity and enhance the bioactivity of titanium implants [23,24]. One common strategy involves functionalizing surfaces with metallic ions such as  $\text{Ag}^+$ ,  $\text{Cu}^{2+}$ , or metal oxides like  $\text{ZnO}$ , where sustained ion release induces bacterial death by disrupting cell membranes, denaturing proteins, and generating intracellular reactive oxygen species (ROS) [25–32]. For instance, Ma et al. demonstrated that surface pretreatments (sandblasting/acid etching) significantly enhance Ti roughness and wettability, thereby facilitating cell adhesion. Building on this, magnetron-sputtered Ag/Cu nanocomposite coatings leverage their high specific surface area to enable sustained ion release and ROS generation, optimizing the balance between antibacterial performance and cytocompatibility [33]. Similarly, Yadav et al. showed that functionally graded HA- $\text{Al}_2\text{O}_3$ - $\text{TiO}_2$  coatings fabricated via plasma spraying accelerate apatite precipitation by providing a bioactive calcium-phosphate-rich surface, thereby promoting MSC osteogenic differentiation [34], and Aksakal et al. reported that tailoring coating porosity via plasma spraying optimizes the surface topography, which promotes cell anchorage and nutrient transport and thereby creates a favorable microenvironment for osseointegration [35]. However, these coating-based strategies often suffer from poorly controlled release kinetics, which can cause localized cytotoxicity and compromise long-term osseointegration, in addition to processing complexity and stability concerns [36,37]. As an alternative, topographical modifications inspired by natural surfaces such as cicada wings and lotus leaves offer a complementary, drug-free approach that achieves physical contact-killing by compromising bacterial membrane integrity through specific nanostructures [38–41]. Among fabrication techniques, anodic oxidation has emerged as a versatile tool for generating  $\text{TiO}_2$  nanotube arrays, which increase surface area and wettability while exerting contact-dependent antibacterial effects. Guo et al. demonstrated that this technique can fabricate hierarchical micro-nano geometries that optimize peri-implant soft-tissue integration, while also leveraging  $\text{TiO}_2$  nanotube (TNT) arrays for structural contact-killing of bacteria [42]. Concurrently, Goli et al. successfully applied anodic oxidation to additively manufactured Ti-6Al-4V alloys; the resulting nanostructured oxide layers significantly enhanced bioactivity by increasing surface area and wettability, thereby fostering a conducive microenvironment for osteogenic activity [43]. Despite these advances, both coating-based and topography-based strategies face inherent limitations that prevent them from achieving both antibacterial activity and osteogenic bioactivity. Coatings suffer from poor release kinetics, which can cause cytotoxicity and compromise osseointegration [44,45]. Their long-term stability also remains a concern. Topography-based modifications avoid the issue of ion release but are mostly limited to single scales. Micro-scale structures have been shown to promote cell adhesion and tissue integration, but their relatively large interstitial spaces may facilitate bacterial colonization, while nanoscale features kill bacteria and may also constrain cell spreading and adhesion [46–48]. Thus, neither strategy in isolation provides a complete solution. Consequently, whether a hierarchically designed surface that integrates both micro- and nano-scale features can address this conflict by enhancing osseointegration while maintaining antibacterial performance remains to be elucidated.

This study combined femtosecond laser ablation and anodization to fabricate a biomimetic structure integrating micro-pillar arrays coated with TiO<sub>2</sub> nanotubes. Micro-pillar arrays were produced using scanning pitches of 40, 60, and 80 µm on titanium surfaces, and TiO<sub>2</sub> nanotubes approximately 100 nm in diameter were subsequently grown in situ, yielding a hierarchical micro-nano structure. We evaluated surface morphology, antibacterial activity against *Escherichia coli*, and the adhesion, viability, and proliferation of human bone marrow-derived mesenchymal stem cells. The results demonstrate that the hierarchical micro-pillar arrays with TiO<sub>2</sub> nanotubes achieve antibacterial performance and bioactivity, highlighting their potential as a drug-free strategy for enhancing the infection resistance and osseointegration of titanium implants.

## 2. Materials and Methods

### 2.1. Fabrication of Micro-Nano Structures

#### 2.1.1. Femtosecond Laser Ablation

Commercially pure titanium discs (purity > 99.7%; diameter, 14.5 mm; thickness, 2 mm, Jinzhou Fushibo New Material Research Co. Ltd., Jinzhou, China) were used as substrates. The discs were sequentially polished with 600-, 800-, and 2000-grit silicon carbide paper, then cleaned in acetone, ethanol, and deionized water before laser processing. Micropatterns were fabricated using a femtosecond laser (TruMicro 5000, TRUMPF, Schramberg, Germany) operating at a repetition rate of 200 kHz and a laser power of 6 W. These settings produced 30 µJ pulses and a single-pulse laser fluence of 2.1 J/cm<sup>2</sup>. The laser beam was focused to a 40 µm spot and directed using a SCANLAB galvanometer scanner. Samples were processed at a scanning speed of 400 mm/s, with line spacings of 40 µm, 60 µm, and 80 µm. Consequently, three distinct surface structures were fabricated and designated as 40M, 60M, and 80M, respectively.

#### 2.1.2. Anodization

Before anodization, samples were etched for 3 s in an HF:HNO<sub>3</sub>:H<sub>2</sub>O solution (1:4:2 by volume), rinsed with deionized water, and air-dried. The titanium sample and a platinum foil served as the anode and cathode, respectively. The electrolyte comprised ethylene glycol (EG) containing 2 vol% H<sub>2</sub>O and 2.5 g/L NH<sub>4</sub>F, and anodization was conducted at 40 V to fabricate titanium dioxide nanotubes on the surface of the samples. A two-step procedure was used to control nanotube diameter. Samples were first anodized at 40 V for 2 h and then treated under a reverse voltage of 4 V in 0.5 mol/L H<sub>2</sub>SO<sub>4</sub> solution. The initial oxide layer was removed by sonication in distilled water. For the second step, the samples were anodized under the same conditions as the first step for 1 h. Before bacterial and cell experiments, all anodized samples were annealed in air at 450 °C for 1 h, with a heating rate of 5 °C/min.

### 2.2. Characterization of Titanium Surface Topography

Titanium surface morphology was characterized using a field-emission Scanning Electron Microscope (FE-SEM; Nova 450, FEI, Hillsboro, OR, USA) at an accelerating voltage of 15 kV. Samples were sputter-coated with a thin nanoscale gold layer before imaging to improve conductivity. The three-dimensional geometry of the laser-patterned surfaces was characterized using a laser-scanning microscope (OLS4100, Olympus Corporation, Tokyo, Japan).

### 2.3. Bacterial Detection

#### 2.3.1. Antimicrobial Activity Assay

*Escherichia coli* (*E. coli*; ATCC 25923, Tianenze Biotechnology Co., Ltd., Beijing, China) was used to assess antibacterial activity, with polished titanium serving as the control. Bacteria were cultured in lysogeny broth (LB) containing tryptone (Aoboxing Biotechnology Co., Ltd., Beijing, China), yeast extract (Oxoid Ltd., Basingstoke, UK), and sodium chloride (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), and 1 mL *E. coli* with a concentration of  $10^4$  CFU/mL was added to each sample for incubation. After 24 h of culture, samples were washed three times with phosphate-buffered saline (PBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) to remove non-adherent bacteria. Then, the samples were subjected to ultrasonic vibration for 5 min to detach the adhered bacteria into 5 mL PBS. After a 1000-fold dilution with PBS, 100  $\mu$ L of bacterial suspension per sample was plated on tryptic soy agar (TSA; Oxoid Ltd., Basingstoke, UK) and incubated at 37 °C for 12 h before colonies were counted. Antibacterial effectiveness of samples was calculated as described previously. At the same time, the suspension amount of *E. coli* was measured by ultraviolet spectrophotometer at a wavelength of 300 nm after dilution in 10-fold steps with LB medium [49,50].

#### 2.3.2. Live/Dead Bacterial Detection

Samples were inoculated with *E. coli* at  $10^4$  CFU/mL. After 24 hours of incubation, the medium was removed, and bacterial viability was assessed using a live/dead staining kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. In this assay, viable bacteria were labeled with SYTO 9 (Green, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), whereas membrane-compromised bacteria were labeled with propidium iodide (Red, PI; Dojindo Laboratories, Kumamoto, Japan).

#### 2.3.3. Bacterial Morphology

Bacterial morphology was observed by FE-SEM (Nova 450, FEI) after 24 h of incubation. To maintain the morphology, all the samples were fixed in 2.5% glutaraldehyde for 2 h and then dehydrated with graded concentrations of ethanol (30%, 50%, 60%, 70%, 80%, 90%, 95%, and 100%). Before the observation, the samples were dried using a CO<sub>2</sub> critical point dryer and then sputter-coated with a nanoscale gold layer to improve the conductivity.

FE-SEM imaging was performed at a low accelerating voltage using a secondary electron detector. The gold coating thickness was optimized to prevent charging while preserving fine morphological details; no coating-induced artifacts were observed under the imaging conditions used.

### 2.4. MSC Culture

#### 2.4.1. Morphology of MSCs

Human mesenchymal stem cells (MSCs, Cyagen Biosciences, Ltd., Guangzhou, China) and culture medium containing 10% fetal bovine serum and 1% penicillin/streptomycin were obtained from Cyagen Bioscience Inc (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The stem cells were thawed and expanded to passage 6 before seeding on the samples. Similar to the observation of bacterial morphology, the samples with MSCs cultured on the surface for 2 d were fixed with 2.5% glutaraldehyde for 2 h and then dehydrated with graded concentrations of ethanol (30%, 50%, 60%, 70%, 80%, 90%, 95% and 100%). Finally, the samples were dried with a CO<sub>2</sub> critical point dryer. The samples were imaged with a field-emission SEM (MIRA3 LMH, TESCAN, Brno, Czech Republic) after being sputter-coated with a layer of Au.

#### 2.4.2. MSC Cytoskeleton

F-actin of the stem cells was also stained to observe the morphology after 2 d of culture. Generally, 4% formaldehyde was used to fix the samples at 4 °C for 2 h, and then 0.1% Triton-X-100 was used to permeabilize for 5 min after washing with PBS three times. After three further PBS washes, samples were blocked with 1% bovine serum albumin for 1 h at room temperature. After the removal of bovine serum albumin (BSA; Thermo Fisher Scientific, Waltham, MA, USA) solution, F-actin was stained with rhodamine-phalloidin (Dojindo Laboratories, Kumamoto, Japan) for 40 min at room temperature, and nuclei were labelled with 4', 6-diamidino-2-phenylindole (DAPI, Dojindo Laboratories, Kumamoto, Japan) after three PBS washes. Images were acquired using a confocal laser-scanning microscope (LSM 710, Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

#### 2.4.3. MSC Viability Assay

Cell viability was assessed using a live/dead assay according to the manufacturer's instructions. After 2 d of culture, samples were stained with a calcein acetoxymethyl ester (calcein AM; Dojindo Laboratories, Kumamoto, Japan): propidium iodide (PI) solution (2 M: 10 µM, Dojindo Laboratories, Kumamoto, Japan) for 10 min at room temperature. Then, the samples were washed three times with PBS, and the cells were observed with an inverted fluorescence microscope (Axio Vert. A1, Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Representative images were captured for further analysis.

#### 2.4.4. Proliferation Experiment

Cell proliferation was quantified using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). MSCs were seeded on all samples at an initial density of  $2 \times 10^4$  cells/mL, which was confirmed by cell counting prior to seeding. After cells were cultured on the samples for 1, 3, and 5 days, CCK-8 working solution (10% CCK-8 solution in cell culture medium) was added for 1 h of culture at 37 °C. Absorbance at 450 nm was then measured using a multimode plate reader (EnSpire, PerkinElmer Inc, Waltham, MA, USA).

#### 2.5. Statistical Analysis

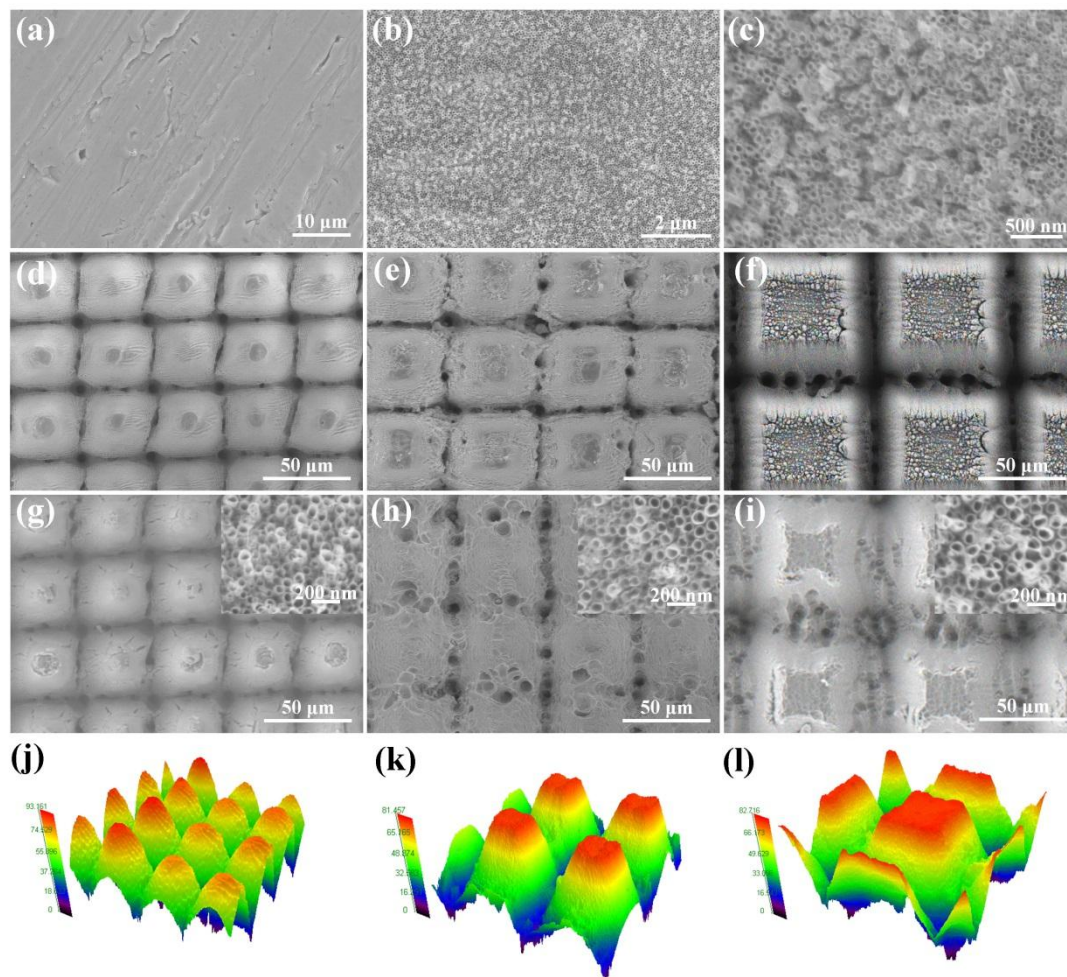
Data are presented as the mean  $\pm$  standard deviation (SD), with four samples per group. Two-group comparisons were performed using Student's *t*-test.  $p < 0.05$  and  $p < 0.01$  were considered statistically significant and are denoted by \* and \*\*, respectively.

### 3. Results and Discussion

#### 3.1. Characterization of Micro/Nano Structures on the Ti Surface

FE-SEM and laser-scanning microscopy were used to characterize the fabricated surface morphology of the micro-pillar arrays coated with TiO<sub>2</sub> nanotubes (approx. 100 nm in diameter), as shown in Figure 1b,c. Relative to the flat titanium sheet (Figure 1a), anodized samples displayed vertically aligned TiO<sub>2</sub> nanotubes that were uniformly distributed across the surface (Figure 1b,c). In addition to anodic oxidation, a femtosecond laser was used to fabricate micro-scale structures. Different sizes of micro-cube arrays (40 µm: Figure 1d; 60 µm: Figure 1e; and 80 µm: Figure 1f) were formed on the titanium surface by controlling the femtosecond laser scanning distance. As the scanning distance decreased from 80 µm to 40 µm, the top surface area of each cube decreased and the unit morphology transitioned from micro-cube to micro-pillar. Laser-scanning microscopy indicated a structure depth of approximately 80 µm for all three arrays (Figure 1j–l). Based on the periodic microarrays, anodization was performed on the surface to form the TiO<sub>2</sub> nanotubes. As shown in Figure 1g–i and the high-magnification insets, the nanotube

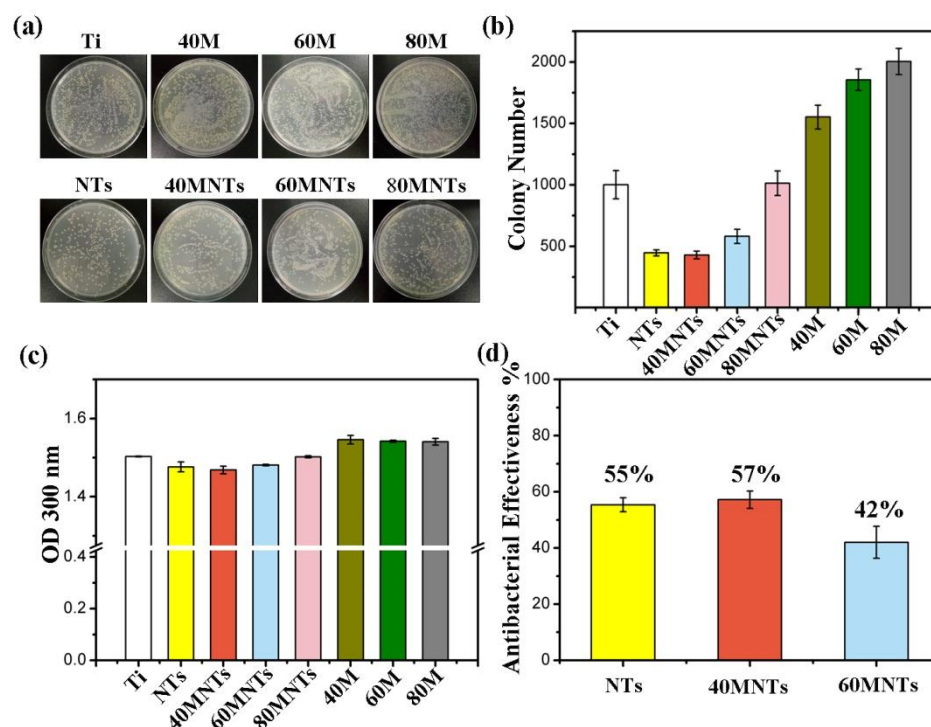
arrays were uniformly distributed across the microstructure surface, and the anodization process sharpened the micro units. The average depth of the microarrays with nanotubes on the surface remained approximately 80  $\mu\text{m}$  because the anodization process formed only a thin nanotube layer on the surface [51,52].



**Figure 1.** The topography and typical 3D view of the samples: (a) polished Ti plate; (b) and (c) TNTs; (d–f) micro pillars fabricated with different line spacing 40M (d), 60M (e) and 80M (f); (g–i) micro/nano structure formed via nanotube on the surface of micro pillars 40MNTs (g), 60MNTs (h), and 80MNTs (i), and the insets image show the nanotube arrays on the micro pillar surface; (j–l) laser microscopy images of the micro pillars fabricated with different line spacing 40M (j), 60M (k), and 80M (l).

### 3.2. Antimicrobial Performance

Plate counting was used to quantify adherent *E. coli* after 24 h (Figure 2a). The smooth titanium surface exhibited a uniform distribution with a mean colony count of  $1002 \pm 115$ , confirming extensive bacterial adhesion on the pure surface. In contrast, the microstructure surfaces supported higher colony densities, and bacterial colony numbers increased as the pitch widened from 40  $\mu\text{m}$  to 80  $\mu\text{m}$ . These results indicate that the laser-patterned microstructure arrays alone did not inhibit bacterial adhesion and proliferation and instead provided additional area and interstitial space for colonization. Additionally, anti-adhesion performance decreased progressively with increasing spacing of 40  $\mu\text{m}$ , 60  $\mu\text{m}$ , and 80  $\mu\text{m}$ .



**Figure 2.** Antibacterial activity of the micro/nano structure. (a) Typical image of *E. coli* colonies from the sample surface after 1 day of culture; (b) the statistical results of *E. coli* colony number for figure (a); (c) the bacterial density in the supernatant after 1 d incubation; (d) the antibacterial effectiveness of the micro/nano structure.

Following modification with nanotubular structures, the nanotube array surfaces (NTs) and most micro-pillar arrays with TiO<sub>2</sub> nanotube groups (40MNT and 60MNT, except for the 80 µm spacing group) showed lower colony densities than the smooth titanium surface. Quantitative analysis of bacterial colony counts (Figure 2b) and the calculation of antibacterial efficacy relative to the smooth titanium control (Figure 2d) confirmed the antibacterial performance of the NTs, 40MNTs, and 60MNTs groups. Statistical analysis revealed a mean colony count of  $447 \pm 25$  on the NTs. For the 40MNTs and 60MNTs arrays, the colony counts were  $420 \pm 31$  and  $591 \pm 57$ , respectively. By contrast, the 80MNT surface produced  $1,014 \pm 100$  colonies, similar to the polished control ( $1002 \pm 115$ ). These results demonstrate that larger pillar spacings attenuate the antibacterial efficacy of the nanotube layers. The 40MNT surface produced the largest reduction in adherent bacteria, corresponding to an antibacterial effectiveness of 57%. Furthermore, the optical density (OD) of *E. coli* in the supernatant was measured at 300 nm using a UV-Vis spectrophotometer (Figure 2c). These results indicated comparable bacterial densities across all groups, with no observable antibacterial activity against planktonic bacteria.

The OD values reflect the density of planktonic bacteria suspended in the supernatant, which do not contact the sample surface. The TiO<sub>2</sub> nanotube arrays exert their antibacterial effect primarily through a contact-killing mechanism that requires direct physical interaction between bacterial cells and the nanotopographies to disrupt membrane integrity. Consequently, planktonic bacteria in the supernatant that do not contact the surface remain unaffected, resulting in comparable OD values across all groups. In contrast, the colony-counting method specifically quantifies viable bacteria that were adhered to and subsequently detached from the sample surfaces, thereby directly reflecting the surface-contact antibacterial efficacy of the nanotube arrays. The OD results indicate that no soluble antibacterial agents were released into the supernatant, confirming that the antibacterial effect is confined to the material surface.

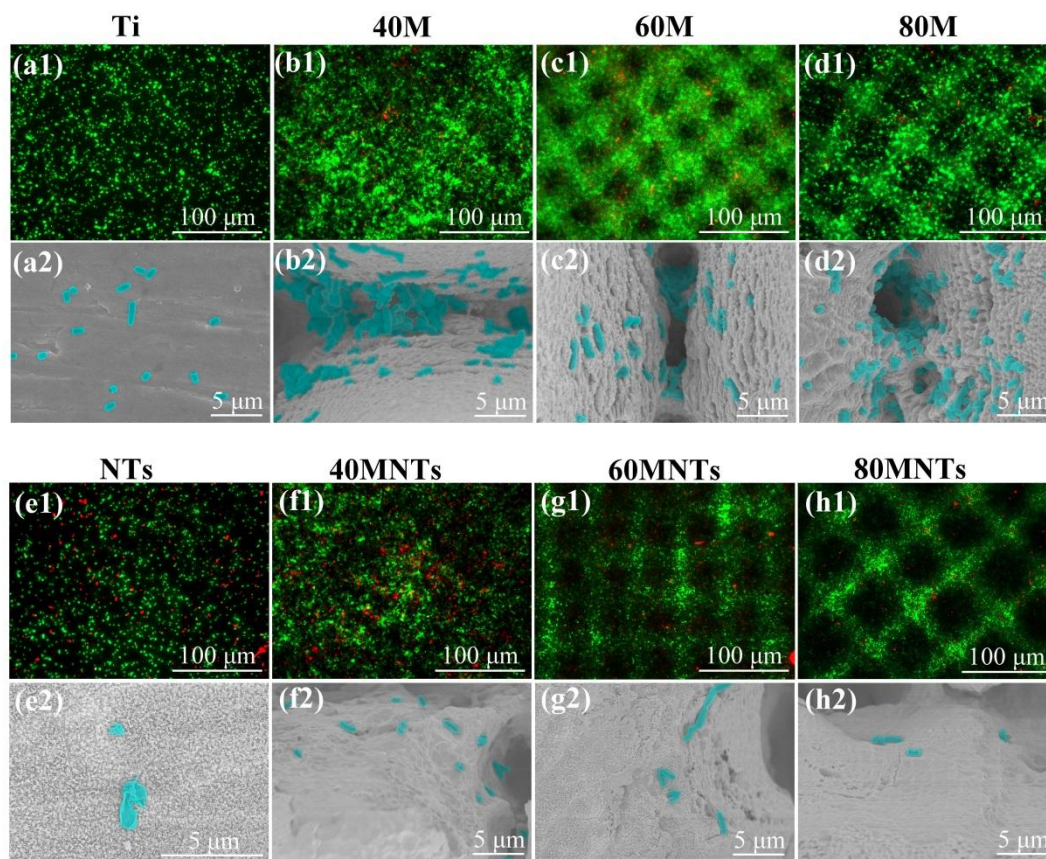
Quantitative antibacterial assays showed that the cubic microstructures failed to eliminate *E. coli*; instead, they actively promoted bacterial adhesion and proliferation. The grooves and interstitial spaces of these microstructures provide a three-dimensional space that increases the number of stable attachment sites and aggregation zones for bacteria. This topographical configuration enhanced inter-bacterial connectivity, thereby facilitating biofilm formation and maturation [53,54]. Nanostructures exhibited significant antibacterial efficacy primarily through contact-mediated killing mechanisms. The high aspect ratio and sharp morphology of TiO<sub>2</sub> nanotube arrays facilitate the physical perforation of bacterial membranes; this mechanical stress compromises membrane integrity, triggering the leakage of intracellular contents and subsequent bacterial inactivation [55–58]. At a pitch of 40 µm, the hierarchical architecture maximized the specific surface area and the density of TiO<sub>2</sub> nanotubes, which synergized with the spatial confinement to exert optimal antibacterial response [59]. Conversely, increasing the pitch to 80 µm significantly reduced the effective density of the nanotube-covered surface area and created larger regions in which bacteria could attach and form biofilms, attenuating antibacterial performance [60,61].

TiO<sub>2</sub> nanotube arrays can kill bacteria through a contact-killing mechanism, but do not affect the viability of mesenchymal stem cells (MSCs). This phenomenon arises from size-dependent biophysical interactions between the nanostructures and the two distinct cell types. For bacteria (typically 1–2 µm in diameter), the nanotube diameter (~100 nm) and tip curvature are geometrically comparable to the bacterial size, enabling these sharp nanotopographies to penetrate the rigid peptidoglycan cell wall, leading to membrane rupture and cytoplasmic leakage, thereby killing the bacteria. In contrast, compared to bacteria, MSCs (20–30 µm in diameter) are an order of magnitude larger. Their flexible plasma membrane, coupled with a dynamic cortical actin cytoskeleton, allows the cells to envelop the nanotube protrusions rather than being pierced by nanotubes. The nanotubes do not cause mechanical damage to cells; instead, they serve as abundant nanoscale adhesion sites that promote cell adhesion [62–65].

### 3.3. Activity and Morphology of the *E. coli*

Live/dead staining and FE-SEM were used to examine bacterial viability and morphology on the different surfaces (Figure 3). For the live/dead stain, two agents, SYTO-9 and PI, were used to stain the bacteria on different surfaces. The SYTO-9 stain is able to penetrate the cell walls of living bacteria, while the PI stain is not, meaning that living bacteria will be green under a fluorescence microscope. The PI stain is only able to attach to dead bacteria and is commonly used to identify dead cells, which will be red under a fluorescence microscope.

Polished smooth titanium and the uncoated micro-pillar surfaces showed abundant green fluorescence and little red fluorescence, indicating high bacterial viability and insufficient antibacterial activity for these structures alone (Figure 3(a1–d1)). The number of dead bacteria decreased as the micro-pillar pitch increased from 40 µm to 80 µm. In contrast, the TiO<sub>2</sub> nanotube arrays (NTs) showed substantially more red fluorescence (Figure 3(e1)), consistent with nanotube-associated bactericidal activity, confirming their potent bactericidal effect. For the hierarchical micro-nano structures, red signal density was notably higher than that on the micro-pillar surfaces, and the bactericidal intensity decreased as pillar spacing increased (40MNTs > 60MNTs > 80MNTs), consistent with the colony-counting results. The 40MNTs displayed the most effective antibacterial performance, followed by the 60MNTs group. The 80MNTs showed negligible antibacterial activity. These observations confirm that while the nanotube modification provides effective antibacterial defense, the bactericidal efficacy is progressively attenuated as the micro-pillar spacing widens, which simultaneously promotes bacterial colonization.



**Figure 3.** Activity and morphology of the *E. coli* cultured on different samples. Fluorescence images show the live/dead assays: (a1) the polished Ti plate; (b1–d1) the micro pillars 40M, 60M, and 80M, respectively; (e1) NTs surface; (f1–h1) the micro/nano structure 40MNTs, 60MNTs, and 80MNTs. The SEM images of *E. coli*: (a2) the polished Ti plate; (b2–d2) the micro pillars 40M, 60M, and 80M, respectively; (e2) NTs surface; (f2–h2) the micro/nano structure 40MNTs, 60MNTs, and 80MNTs. In the SEM images (a2–h2), the blue color represents the adhered bacterial cells, applied as pseudo-coloring to enhance contrast for visualizing bacterial distribution and adhesion morphology on different surfaces; no chemical staining was involved.

FE-SEM micrographs (Figure 3(a2–d2)) provided further detail on bacterial adhesion morphology. The SEM images (Figure 3(b2–d2)) were taken at the sidewalls and inter-pillar grooves of the micro-pillar arrays, where bacteria preferentially colonized. The SEM images (Figure 3(f2–h2)) were taken on the nanotube-coated micro-pillar surfaces, showing the distribution and morphological alterations of bacterial cells. Bacteria on smooth Ti and micro-pillar surfaces retained an intact morphology. On the micro-pillar surfaces, bacteria preferentially colonized the sidewalls and inter-pillar grooves, forming dense clusters (Figure 3(b2–d2)). In contrast, the micro-nano composite surfaces significantly inhibited colonization. Bacteria on the nanotube-coated surfaces were more sparsely distributed and frequently appeared shriveled, deformed, or ruptured, as highlighted in the magnified views of Figure 3(e2–h2), failing to form the dense aggregates observed on the micro-pillar arrays. Specifically, representative deformed and ruptured bacterial cells are visible in the micro-nano composite groups (Figure 3(f2–h2)), where the loss of intact rod-shaped morphology and membrane disruption are evident. These alterations indicate that the nanotube structures effectively disrupted bacterial cell membranes via a contact-killing mechanism, resulting in a pronounced antibacterial effect.

These observations are consistent with Figure 1. The substantial inter-pillar spaces in the micro-pillar topographies promoted *E. coli* adhesion and proliferation. Bacteria tend

to colonize the sidewalls and the base of these grooves, where they proliferate and form interconnected networks between adjacent pillars, promoting colony maturation. As the pillar spacing increases from 40  $\mu\text{m}$  to 80  $\mu\text{m}$ , the available volume for colonization expands. Thus, the micro-interstitial architecture enhanced bacterial colonization by increasing the potential for inter-colony connectivity. The  $\text{TiO}_2$  nanotubes synergistically enhance antibacterial efficacy through two mechanisms: first, the dense, high-aspect-ratio nanotube arrays reduce the effective surface contact area available for stable bacterial attachment, thereby preventing the initial adhesion required for colony maturation. Second, the sharp edges of the nanotubes exert mechanical stress on bacterial cell membranes upon contact, causing membrane deformation, rupture, and subsequent cytoplasmic leakage [66].

The antimicrobial efficacy of the micro-nano composite depends on the balance between micro-scale geometric confinement and nano-scale bactericidal action. In the 40MNTs, the narrow pillar spacing optimizes nanotube density relative to the available colonization area, achieving superior antimicrobial activity while maintaining a favorable surface for osseointegration. Conversely, increasing the spacing to 80  $\mu\text{m}$  reduces the local density of nanotubes and enlarges the interstitial volume, providing bacteria with space to evade contact-killing and aggregate within the gaps [67,68]. The micro-nano composite morphology achieves superior antibacterial performance compared to single-scale micro-structures by integrating spatial confinement with nanoscale contact-mediated bactericidal action.

### 3.4. Morphology and Cytoskeleton of Mesenchymal Stem Cells

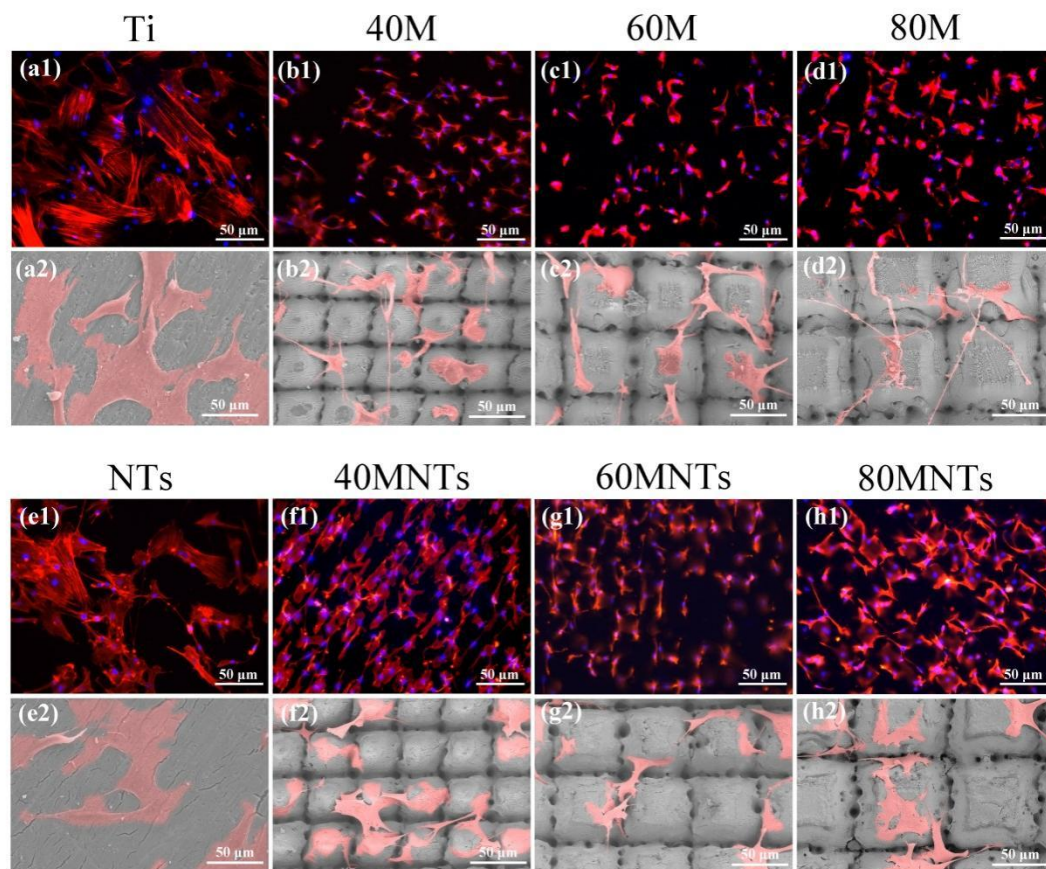
To investigate how different surface topographies influence MSC behavior, MSC morphology and cytoskeletal organization were characterized after two days of culture on various substrates via fluorescent staining and FE-SEM (Figure 4). Fluorescence microscopy showed red F-actin and blue cell nuclei, clearly illustrating cell spreading and cytoskeletal architecture.

The FE-SEM and fluorescent staining results revealed that surface morphology regulates MSC adhesion and growth. On the smooth titanium surface (Figure 4(a1,a2)), MSCs appeared spindle-shaped and spread two-dimensionally. This flat surface lacked three-dimensional topological anchor points, resulting in limited cell attachment sites and a restricted cell-surface contact area.

FE-SEM observations revealed that cells on the micro-pillar arrays and hierarchical micro-nano structures exhibited a clear three-dimensional interweaving morphology. Cell filopodia extended and wrapped tightly around the micro-pillars, forming a robust 3D mechanical anchorage. These findings suggest active proliferation and differentiation potential. Fluorescence staining confirmed these observations, showing increased cell density on all micro-pillar spacings (40–80  $\mu\text{m}$ ). Notably, compared to the single micro-pillar structures, the micro-nano structure surfaces promoted wider cell filopodia spreading and higher nuclear density. These findings indicate that the hierarchical architecture, with its combination of 3D micro-space and nanoscale adhesion sites, significantly enhances cell adhesion and provides an optimal biophysical environment that supports proliferation and osteogenic differentiation.

The FE-SEM and fluorescence staining results agreed well. The micro-pillar arrays provide a 3D physical space that improves cell adhesion compared to traditional 2D flat surfaces. As reported by Nguyen et al. [69], these micro-scale topographies significantly increase the specific surface area for cell spreading. This 3D microenvironment facilitates intracellular force transmission, enhances cell adhesion area and cytoskeletal tension, providing a favorable basis for osteogenic differentiation. The  $\text{TiO}_2$  nanotube arrays further optimize the cell-material interface. Huang et al. [70] suggested that nanostructures

provide abundant nanoscale adhesion sites. This high-density distribution improves cell adhesion and synergistically enhances cell activity and proliferation by promoting integrin clustering.



**Figure 4.** The fluorescence micrographs and SEM images of MSCs cultured on the samples after 2 d of culture. (a1,a2) Polished Ti plate, the micro surface: (b1,b2) 40M, (c1,c2) 60M, and (d1,d2) 80M; (e1,e2) NTs, the micro/nano surface: (f1,f2) 40MNTs, (g1,g2) 60MNTs, and (h1,h2) 80MNTs.

The micro-nano structure enhances early-stage cellular responses through synergy between micro-scale 3D adhesion spaces and nano-scale adhesion sites. Importantly, the nanotube arrays do not cause physical damage to MSCs. Instead, the high-density nanotopographies offer numerous adhesive sites that facilitate cellular responses, as evidenced by the well-spread cytoskeleton and filopodia extending around the micro pillars (Figure 4). Maher et al. similarly confirmed this dual-scale modification strategy, showing that micro-scale structures act as scaffolds for cell colonization, while nanostructures enhance bioactivity by providing additional 3D adhesion sites [66]. In our study, the optimal bioactivity of the 40MNTs confirms that the micro-pillar arrays provide sufficient 3D adhesion space, while the nanoscale sites enhance the osteogenic response for efficient bone formation.

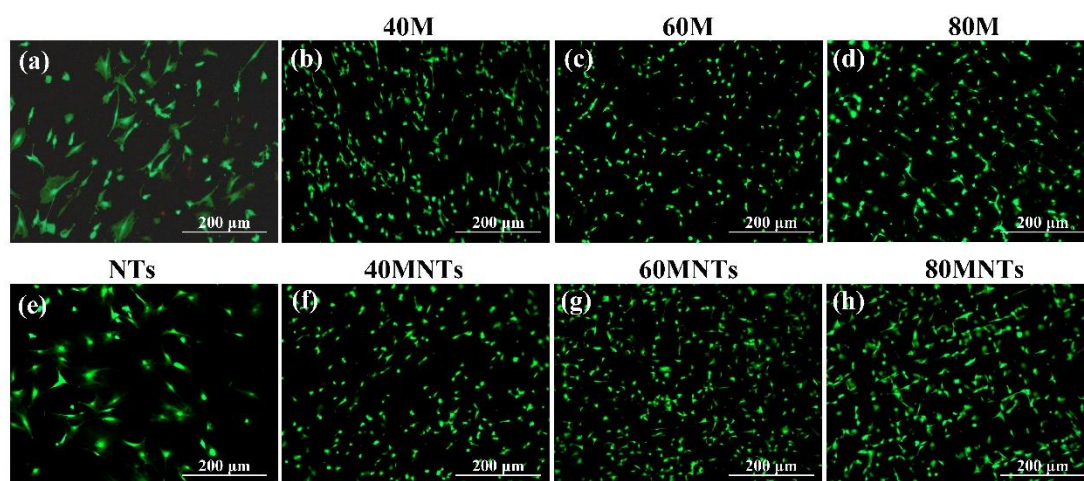
Notably, while the fluorescence images (Figure 4(a1–h1)) revealed a clear trend of higher cell density on the hierarchical micro-nano surfaces, the corresponding SEM images (Figure 4(a2–h2)) showed a less pronounced difference. This discrepancy primarily arises from the distinct sample preparation procedures of the two techniques. Fluorescence staining is performed with minimal manipulation, preserving overall cell distribution with high fidelity, whereas SEM preparation involves multiple steps, including fixation, dehydration, and drying, which can cause detachment of less firmly adhered cells.

Consequently, the SEM images reflect cell adhesion strength and morphological features rather than serving as a direct quantitative measure of cell density. Furthermore, the fluorescence images (low magnification, large field of view) reflect the overall cell population, while the SEM images (high magnification, localized regions) focus on detailed morphological features of individual cells. These two datasets are complementary: the fluorescence images provide an overview of cell distribution and density, and the SEM images offer detailed insight into cell morphology and spreading. The enhanced filopodia extension and robust three-dimensional wrapping observed in the SEM images (Figure 4(b2–h2)) indicate that surviving cells exhibit stronger adhesion and more extensive spreading on the hierarchical surfaces, consistent with the overall trend shown by the fluorescence images.

### 3.5. Mesenchymal Stem Cell Viability and Proliferation

To investigate the influence of surface topography on mesenchymal stem cell (MSC) viability and proliferation, we evaluated the cellular behavior via live/dead fluorescence staining and CCK-8 assays.

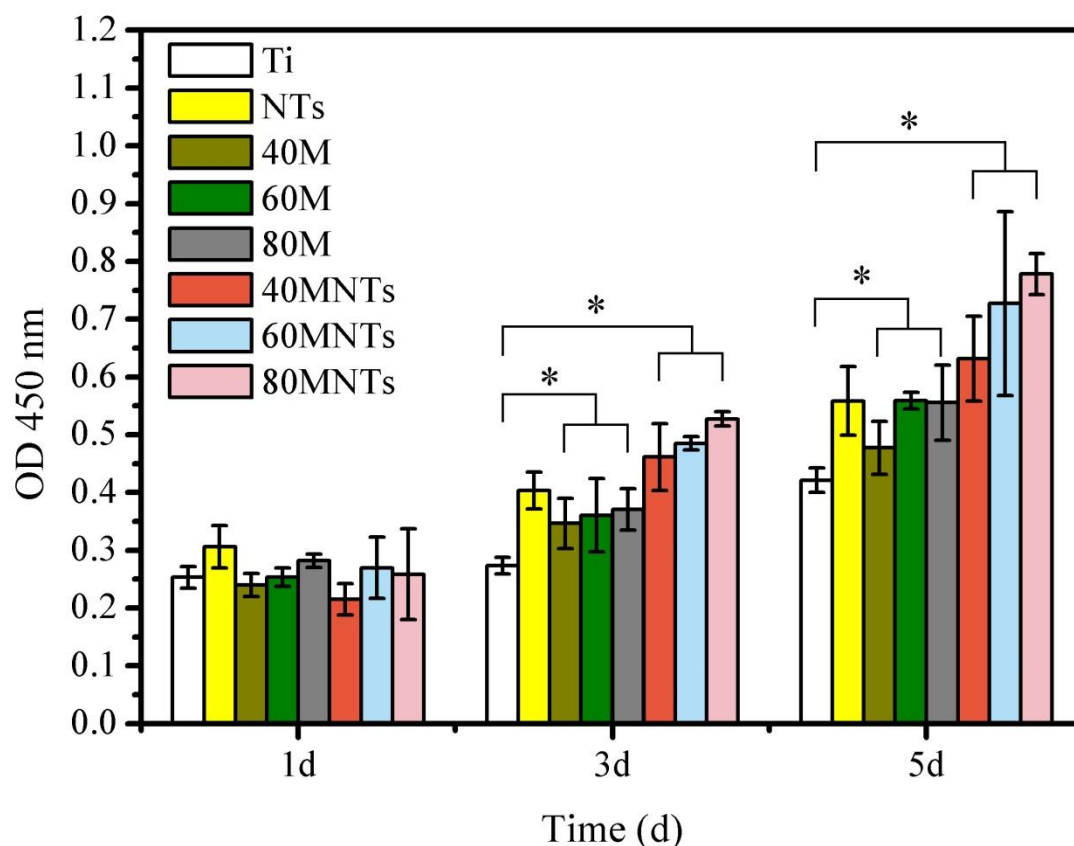
Live/dead staining (Figure 5) revealed predominantly green fluorescence and negligible red fluorescence across all samples, indicating good biocompatibility and high cell viability on all surfaces tested. Both the micro-pillar arrays (Figure 5b–d) and hierarchical micro-nano structures (Figure 5f–h) exhibited higher cell proliferation density than the smooth Ti control (Figure 5a). The micro-pillar architecture provides a three-dimensional space that increases the effective surface area and offers suitable geometric cues for cell anchoring. Compared with flat substrates, this 3D microenvironment effectively promotes cell adhesion, spreading, and proliferation. The nano-scale structures provide additional adhesion sites that also facilitate cell adhesion. Thus, the hierarchical micro-nano structure effectively supports cell growth while maintaining good biocompatibility.



**Figure 5.** Fluorescence images showing the live/dead assays of MSCs. (a) Polished Ti plate, the micro surface: (b) 40M, (c) 60M, and (d) 80M; (e) NTs, the micro/nano surface: (f) 40MNTs, (g) 60MNTs, and (h) 80MNTs.

The CCK-8 assay (Figure 6) revealed that MSC proliferation increased significantly over time on all surfaces from day 1 to day 5, confirming that all fabricated structures exhibited excellent cytocompatibility without cytotoxicity. Prior to seeding, cell counting confirmed an identical initial density of  $2 \times 10^4$  cells/mL across all groups, establishing a consistent day 0 baseline. The time-dependent increase in OD values from day 1 to day 5 reflects active cell proliferation, demonstrating that all fabricated surfaces support MSC survival and growth. These results agree with the live/dead staining observations.

Compared with smooth Ti surfaces, the micro-pillar arrays (40M, 60M, 80M) exhibited higher OD values. This suggests that the micro-pillar architecture provides a 3D space with a larger effective surface area that facilitates cell attachment and spreading, while the TiO<sub>2</sub> nanotube arrays (NTs) promote proliferation through nanoscale adhesion sites that enhance cell-substrate interactions. Notably, the hierarchical micro-nano structures (40MNTs, 60MNTs, 80MNTs) achieved the highest proliferation rates. Comparing the day 1 cell viability results (Figure 6) with the day 1 antibacterial performance (Figure 2) reveals a positive correlation: the 40MNTs group, which exhibited the highest antibacterial efficacy against *E. coli* among all tested surfaces, also showed a high level of MSC proliferation at day 1. This correlation suggests that the 40MNTs architecture achieves effective bacterial killing and provides a favorable microenvironment for MSC adhesion and proliferation.



**Figure 6.** MSC proliferation on various sample surfaces evaluated by the CCK-8 assay. All groups were seeded at an identical initial density of  $2 \times 10^4$  cells/mL. Error bars represent the standard deviation of three specimens for each data point. Statistical significance ( $p < 0.05$ ).

It is well documented that micro- and nano-scale topographies on titanium surfaces can regulate the osteogenic differentiation of mesenchymal stem cells through mechanotransduction pathways [71]. In particular, TiO<sub>2</sub> nanotubes with diameters of approximately 100 nm have been shown to enhance the osteogenic differentiation of BMSCs via the Kindlin-2/Integrin  $\beta 1$ /YAP pathway [64], and TiO<sub>2</sub> nanotube surfaces have been demonstrated to effectively modulate the osteo-adipogenic balance of MSCs [72]. Hierarchical structures combining micropits with nanotubes have been proven to simultaneously promote MSC proliferation and osteogenic differentiation in the absence of osteogenic supplements [73], and femtosecond laser-generated micron/submicron hybrid topographies have also been confirmed to enhance MSC adhesion as well as osteogenic and chondrogenic differentiation [74]. Furthermore, Li et al., reported that 3D-printed

hierarchical titanium implants significantly promote osseointegration in vivo, achieving a Bone Volume/Total Volume (BV/TV) ratio of 43.4% at 8 weeks, which was 1.5 times that of the untreated controls [75].

Although the present study did not directly evaluate osteogenic differentiation markers, such as ALP activity, osteogenic gene expression (e.g., Runx2, osteocalcin), or Alizarin Red S staining for matrix mineralization, our results demonstrate that micro-pillar arrays coated with TiO<sub>2</sub> nanotubes on titanium surfaces significantly enhance the early-stage cellular events that are essential for subsequent osteogenesis. These events include MSC adhesion, spreading, cytoskeletal organization, and proliferation. These cellular responses are well-recognized precursors to osteogenic differentiation in the literature. The micro-pillar arrays provide a three-dimensional framework for cell colonization, and the TiO<sub>2</sub> nanotubes offer additional nanoscale adhesion sites. This structural combination effectively optimizes the cell-material interface and creates a favorable biophysical microenvironment for bone-tissue integration. Therefore, although direct evidence of osteogenic activity requires future investigation, our findings strongly suggest that the 40MNTs surface creates a favorable biophysical microenvironment that is conducive to osteogenesis and, consequently, is an important prerequisite for successful osseointegration.

#### 4. Conclusions

In this study, we combined femtosecond laser ablation and in situ anodization to fabricate micro-pillar arrays coated with TiO<sub>2</sub> nanotubes on titanium surfaces, achieving both antibacterial and enhanced bioactivity. Surface topography is a key factor in modulating the biological microenvironment. Micro-pillar arrays provide three-dimensional space to enhance cell adhesion and bioactivity, but their grooves also facilitate bacterial colonization, which compromises antibacterial performance. Conversely, TiO<sub>2</sub> nanotubes provide nano-scale cell adhesion sites for cells and exert mechanical contact-killing effects on bacteria. By integrating these two features, the hierarchical structure resolves the trade-off between antimicrobial defense and bioactivity. Specifically, the 40-MNTs (40 µm pillar spacing coated with TiO<sub>2</sub> nanotubes) showed optimal performance, inhibiting *E. coli* by 57% and significantly promoting the adhesion, spreading, and proliferation of mesenchymal stem cells (MSCs). These findings provide a drug-free surface engineering strategy for medical-grade titanium implants. With efficient antibacterial defense and enhanced support for early-stage cellular events, this hierarchical micro-nano structure holds promise for future clinical applications in orthopedic and dental implants.

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