Pulsed Laser Ablation Synthesis of TiO_2 Nanoparticles and their Enhanced Antimicrobial Activity under Femtosecond Laser Irradiation

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Received: 26 September 2025 | Revised: 3 November 2025 and 21 November 2025 | Accepted: 24 November 2025

 DOI: https://doi.org/10.48084/etasr.15159

ABSTRACT

This study analyzes the synthesis of titanium dioxide TiO_2-Nanoparticles (NPs), through Pulsed Laser Ablation in Liquid (PLAL). The combination was performed by irradiation of a titanium target at its second harmonic (532 nm, 10 ns pulse duration, and 10 Hz repetition rate), bv a Nd:YAG laser. The results of the analysis verified the antibacterial efficacy of the synthesized TiO_2-NPs against Methicillin-Resistant Staphylococcus Aureus (MRSA) and Pseudomonas aeruginosa, by reducing the bacterial growth, proving an effective strategy against ani-biotic resistant pathogens.

Keywords-femtosecond laser-based treatment; titanium dioxide nanoparticles (TiO_2-NPs); laser ablation synthesis technique

I. INTRODUCTION

The field of nanotechnology plays a significant role in medicine, biotechnology, food science, and material engineering [1-7]. The tip of this technological transformation are NPs, whose distinctive size- and shape-dependent properties provide them with unique physical, chemical, and optical characteristics, in contrast with other methods [8-10]. The large surface area-to-volume ratio of NPs enhances their reactivity and biocompatibility, enabling applications in drug delivery, biosensing, antibacterial therapies, diagnostic imaging, and tissue engineering [11-22]. Regarding infectious diseases, particularly those driven by antibiotic-resistant pathogens, the demand for new antimicrobial strategies has increased significantly [23]. Among the most critical clinical challenges are ocular infections, which can result in severe and often irreversible damage [24-27]. Metal oxide-based materials, such as TiO2, have antimicrobial properties [28-30], provide chemical stability, non-toxicity, and sustained antimicrobial efficacy [31-34], making them suitable for wound treatment, drug delivery, medical devices, and antimicrobial coatings [35, 36]. Authors in [31, 32] compared TiO_2 with other metal oxides, confirming low toxicity to living tissues, and broad-spectrum antimicrobial activity. These characteristics make the obtained crystalline TiO_2 phases the most suitable candidates for biomedical applications. The NP synthesis method is essential in determining their structural, optical, and biological performance. Although chemical and biological methods are well-established, PLAL has emerged as a clean, efficient, and versatile approach for generating NPs [37-40]. PLAL enables the synthesis of high-purity TiO_2-NPs without surfactants or hazardous chemicals. It also permits fine-tuning of particle size, morphology, and concentration by adjusting laser parameters [41-44]. The resulting colloidal dispersions are particularly well-suited for biomedical applications where purity and reproducibility are essential [45]. Although PLAL is widely used for NP fabrication, this study distinguishes itself by using the second harmonic (532 nm) of an Nd:YAG laser. This enhances ablation efficiency and allows for precise control over TiO_2 NP size and uniformity. This wavelength enables higher photon absorption by titanium than the fundamental 1064 nm wavelength does, yielding colloids of superior purity that are ideally suited for biomedical applications [44]. In addition, laser-based antimicrobial Photodynamic Therapy (aPDT) has proven efficient in eliminating pathogens [45, 46]. Authors in [47, 48] combining femtosecond laser irradiation with PLAL-generated NPs reported promising antibacterial results. This study examines the synthesis of high-purity TiO_2-NPs using PLAL at the second harmonic wavelength of 532 nm, with Transmission Electron Microscopy (TEM), Energy-Dispersive X-ray (EDX) spectroscopy, Ultraviolet–Visible (UV–Vis) spectroscopy and Inductively Coupled Plasma (ICP) methods. This study aims to evaluate whether TiO_2-NPs enhance the antibacterial efficacy against MRSA and Pseudomonas aeruginosa, considering that femtosecond laser excitation of PLAL-derived TiO_2-NPs will generate a higher yield of Reactive Oxygen Species (ROS) and cause greater damage to bacterial membranes than either treatment alone.

II. MATERIALS AND METHODS

A. *Preparation of TiO2-NPs Using Pulsed Laser Ablation*

Figure 1 shows the synthesis of TiO_2-NPs through PLA of a titanium target immersed in distilled water. To prepare the NPs, a titanium plate (99.99% pure) with rectangular dimensions of 20 mm × 40 mm × 2 mm was used. The titanium surface was polished to eliminate the oxide layer formed during exposure to air, ensuring a clean, smooth surface. Then, the plate was cleaned with ethanol and deionized water to remove any organic residues or NPs, using the ultrasonic method and then immersed in a glass vessel containing 10 mL of distilled water. A second-harmonic Nd:YAG laser system (Quanta-Ray PRO 350) from Spectra-Physics with a wavelength of 532 nm, pulse duration of 10 ns, and repetition rate of 10 Hz was used for ablation. The laser beam was directed onto the titanium surface using highly reflective mirrors and an L-convex lens with a focal length of 10.5 cm to focus the beam. To prevent NPs from obstructing the laser beam path and absorbing incident energy, the beaker containing the sample was placed on a speed-controlled stirrer to ensure solution homogeneity. A motorized rotating spinner rotated the beaker containing the sample at 177 RPM to homogenize the colloids and prevent deterioration during ablation. The effects of ablation times ranging from 5 min to 20 min on the synthesized NPs were examined at an average laser power of 150 mW. The synthesized TiO_2-NPs underwent various characterization analyses, such as TEM, UV-Vis absorption spectroscopy, EDX, and ICP, to understand their structural, optical, and physical properties, elemental composition, and NP concentrations.

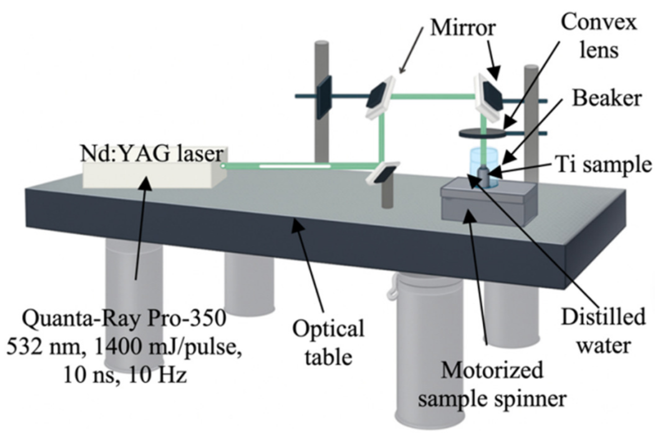


Fig. 1. Schematic diagram of the laser ablation in distilled water for the synthesis of TiO2-NPs using the 2nd harmonic of the Nd: YAG at 532 nm pulsed laser.

B. *Microorganism and Culture Conditions*

The bacterial pathogens selected for this study were MRSA (ATCC 43300) and Pseudomonas aeruginosa (ATCC 90257). The strains were grown in Brain Heart Infusion (BHI) broth and stored at 37°C. Before treatment, the turbidity of the microbial suspension was adjusted to the 0.5 McFarland standard (approximately 1.5×10^8 Colony-Forming Units (CFUs)/mL). Then, 100 µL aliquots from these standardized suspensions were transferred into assigned wells of a 96-well microtiter plate.

C. Femtosecond Laser Treatment System Preparation

Femtosecond laser irradiation was performed using an INSPIRE HF100 laser system (Spectra-Physics), which was powered by a mode-locked femtosecond Ti:sapphire laser (Mai Tai HP, Spectra-Physics). The system delivers pulses at a wavelength of 400 nm with an average power of 1.5–2.9 W and a repetition rate of 80 MHz. It covers a wavelength range from 690 nm to 1040 nm. The laser output power was monitored using a Newport 843R power meter. As depicted in Figure 2, the laser beam was positioned approximately 10 cm above the microorganisms grown overnight in the 96-well microtiter plate to ensure homogeneous irradiation. The initial 2-mm laser beam diameter was expanded to 10 mm using a beam expander consisting of two converging lenses. A laser attenuator (A) controlled the power of the laser reaching the samples (set at 50 mW), and an adjustable iris (I) modified the diameter of the laser beam to 6 mm. Mirrors (M_1 and M_2) directed the laser beam toward the samples.

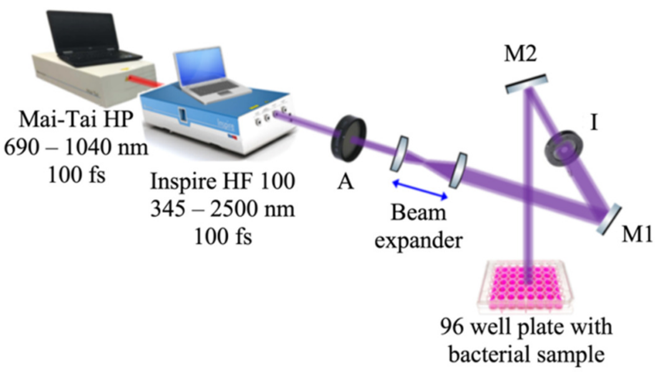


Fig. 2. Schematic diagram of the experimental setup for the antimicrobial femtosecond laser treatment. A: attenuator, M_1, and M_2: highly reflective mirrors, I: iris.

D. Evaluation of Bacterial Growth Kinetics after Treatment

After treatment, the bacterial samples were kept in an incubator at 37°C for 16 h. Optical Density (OD) was measured every 30 min using a microplate reader at a wavelength of 620 nm. Growth curves and maximum growth kinetics (μ_{max}) were determined to evaluate the antibacterial effects of TiO_2-NPs and/or femtosecond laser treatment. The positive control wells contained microorganisms that were not treated, and the negative control wells contained only BHI broth.

E. Data Analysis

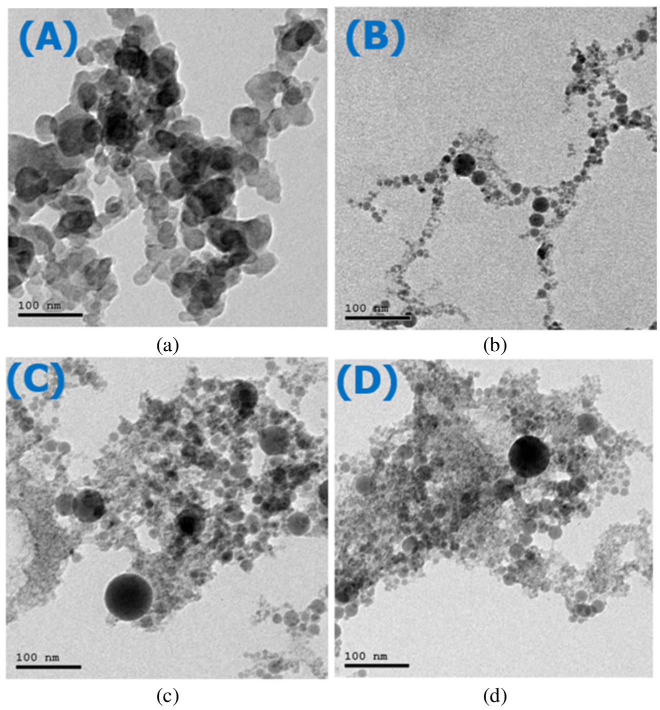
Growth curves were generated to track microbial multiplication over time. These curves were then subjected to detailed growth kinetics analyses. The data were expressed as the mean ± standard error. One-way Analysis of Variance (ANOVA), followed by Tukey's post hoc test, was used to evaluate statistical significance for multi-group comparisons, and GraphPad Prism 7 software was utilized for the analysis. A p-value of less than 0.05 was considered statistically significant. All experiments were conducted in triplicate within a Class II biological safety cabinet (MSC-Advantage™).

III. RESULTS AND DISCUSSION

A. Characterization of TiO_2-NP Colloids

1) TiO_2-NP Shape and Size Distribution Analysis by TEM

Figure 3 illustrates TEM images of TiO_2-NPs produced by laser ablation to determine their size distribution and morphology. A high-resolution TEM image shows spherical TiO_2-NPs at various ablation times and an ablation laser fluence of 1.24 J/cm². NP density increases with greater ablation time and more NPs are formed, as displayed in Table I. Figure 4 presents the size distribution histogram and the average sizes of the TiO_2 NP colloids: 19.11 nm, 11.96 nm, 8.33 nm, and 6.4 nm for laser ablation times of 5 min, 10 min, 15 min, and 20 min, respectively. The size of the spherical NPs decreases as the laser ablation time increases. Since laser ablation generates NPs that are dispersed in the solution. Most of them are irradiated, resulting in size reduction due to photofragmentation. The concentration of TiO_2-NP colloids, prepared at various laser ablation times, was determined using an ICP (Agilent 5100 Synchronous Vertical Dual View (SVDV) ICP-OES with an Agilent Vapor Generation Accessory (VGA 77)). Increasing the ablation time from 5 min to 20 min increased the concentration of TiO_2 NP colloids from 1.9 mg/L to 6 mg/L, showing that as ablation time increases, so does ablation efficiency.



(a) (b) (c) (d)

Fig. 3. TEM images of TiO2 NPs synthesized via laser ablation with an average laser power of 150 mW, using different ablation times: (a) 5 min, (b) 10 min, (c) 15 min, and (d) 20 min.

TABLE I. PHYSICOCHEMICAL CHARACTERISTICS OF TiO_2-NPS SYNTHESIZED AT DIFFERENT ABLATION TIMES

Ablation time (min)	Average particle size (nm)	Concentration (mg L⁻¹, ICP)	Observed morphology (TEM)
5 min	19.11 ± 0.8	1.9 ± 0.1	Spherical, slightly polydisperse
10 min	11.96 ± 0.6	3.2 ± 0.2	Uniform spherical particles
15 min	8.33 ± 0.5	4.8 ± 0.3	Highly uniform, dense distribution
20 min	6.40 ± 0.4	6.0 ± 0.2	Smallest, well-dispersed NPs

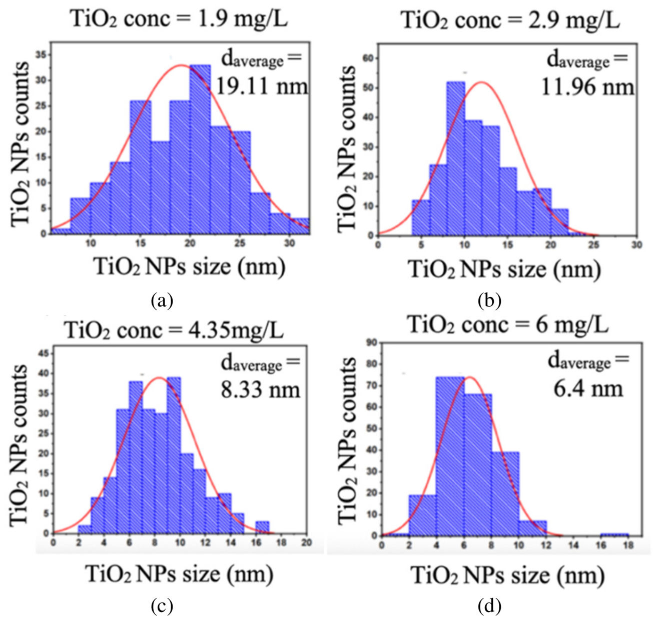


Fig. 4. Size distribution of TiO_2 NPs synthesized via laser ablation with an average laser power of 150 mW, using different ablation times: (a) 5 min, (b) 10 min, (c) 15 min, and (d) 20 min.

2) *Optical Absorption Characteristics of TiO2-NPs Using UV-Vis Spectroscopy*

Figure 5 shows the UV-visible absorption spectra of TiO_2-NPs measured using a UV-visible spectrophotometer (model C-7200) with a wavelength range of 200 nm–1,100 nm. As portrayed in Table II, the TiO_2 colloidal solutions absorb light strongly in the UV region. Absorbance intensity indicates the concentration of NPs in the solution. According to the Beer–Lambert law, the amount of light absorbed is proportional to the substance's concentration. As ablation time increases, TiO_2 NP absorbance increases, indicating that more NPs are present in the solution after 20 min of ablation. Colloidal TiO_2-NPs exhibit a Surface Plasmon Resonance (SPR) peak around 227 nm. The size, shape, and concentration of the NPs can affect the position of the SPR peak. As laser ablation time increases, the peak position shifts toward shorter wavelengths due to the synthesis of smaller particle sizes. The SPR peaks also become sharper with increasing ablation time, as seen with a 20-min ablation time.

3) *Elemental Analysis of TiO_2-NPs Using EDX*

To confirm the production of TiO_2-NPs, elemental analysis using an EDX detector (JSM6510LA, Oxford) was performed. The EDX results show the presence of titanium (Ti) and oxygen (O), and their respective atomic and weight fractions are depicted in the EDX spectrum in Figure 6. The ZAF (Atomic Number, Absorption, Fluorescence) approach was used to measure the atomic composition of TiO_2-NP colloids. The inset table summarizes the results, demonstrating the atomic and weight ratios of Ti and O in the solution, and confirming the high purity of the synthesized TiO_2-NPs.

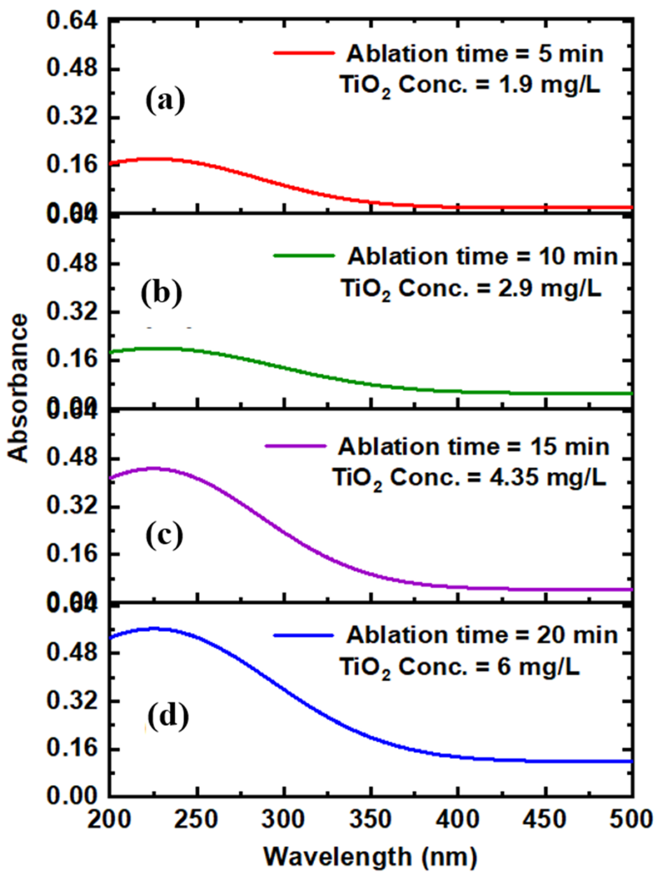


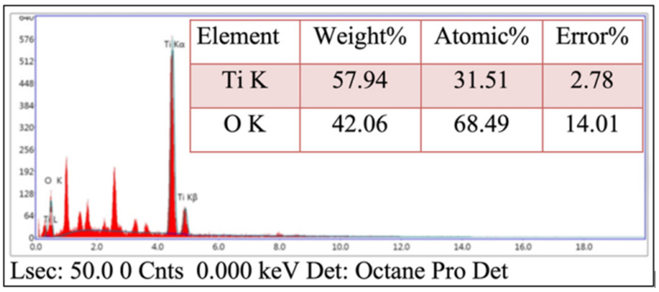
Fig. 5. The optical absorption spectrum of TiO2-NP colloidal solutions at different ablation times and a constant average power of 150 mW: (a) 5 min, (b) 10 min, (c) 15 min, and (d) 20 min.

TABLE II. UV–VIS ABSORPTION CHARACTERISTICS OF TiO_2-NPS AT DIFFERENT ABLATION TIMES

Ablation time (min)	λ_{max} (nm)	Spectral shift (nm)	Observation
5	233	Reference	Broader SPR peak
10	230	-3	Slight blue shift
15	228	-5	Sharper peak
20	227	-6	Strongest blue shift; smallest particles

B. *Growth Kinetics of S. aureus and P. aeruginosa after TiO_2-NPs and/or Femtosecond Laser Treatment*

The antibacterial efficacy of TiO_2-NPs, both alone and in combination with femtosecond laser irradiation, was evaluated by monitoring the growth kinetics of MRSA and Pseudomonas aeruginosa. As presented in Figure 7, the experimental data reveal the impact of increasing TiO2-NP concentration on the growth rate (μ_{Max}) of these bacterial strains.



Element	Weight%	Atomic%	Error%
Ti K	57.94	31.51	2.78
O K	42.06	68.49	14.01

Fig. 6. EDX analysis of the elemental composition of TiO2-NP colloid. Inset table exhibiting ZAF method standardless quantitative analysis of TiO2-NP colloids.

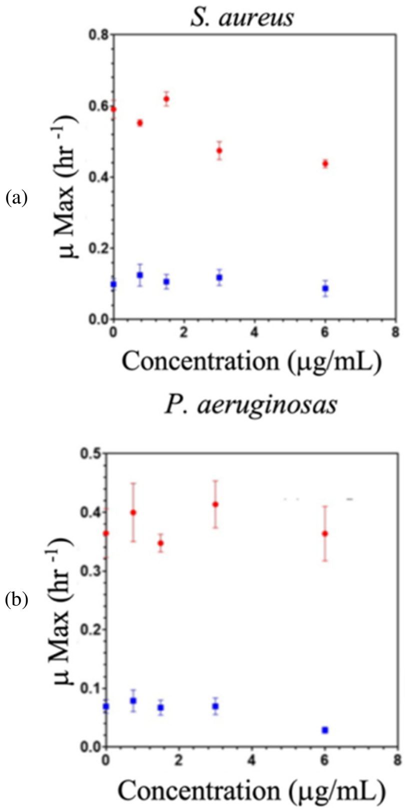


Fig. 7. Growth kinetics analysis of methicillin-resistant *S. aureus* and *P. aeruginosa* after femtosecond Laser and/or TiO2-NP treatment: (a) methicillin-resistant *S. aureus* and (b) *P. aeruginosa*.

Figure 7 (a) shows that the S. aureus control group treated with only TiO_2-NPs (red circles) exhibited a concentration-dependent reduction in growth rate. There was an initial decrease, followed by a slight increase at higher concentrations. This suggests that TiO_2-NPs alone possess some antibacterial activity, but their effectiveness may be influenced by concentration-dependent factors or aggregation at higher doses. In contrast, the group treated with both a femtosecond laser and TiO_2-NPs (blue squares) consistently exhibited a significantly lower growth rate across all tested concentrations. This indicates a potent synergistic or additive antibacterial effect. The μMax values for the laser-treated groups remained consistently low, demonstrating strong inhibition of bacterial growth. Figure 7 (b) illustrates the growth kinetics for P. aeruginosa, exhibiting analogous behavior to that of S. aureus. The control group treated with only TiO_2-NPs (red circles) exhibited a fluctuating growth rate, suggesting an inconsistent antibacterial effect when TiO_2-NPs are used alone. However, the combination of a femtosecond laser and TiO_2-NPs resulted in suppressed growth rates and maintained low μ_(Max) values across the concentration range. These results further support the enhanced antibacterial activity achieved through combined treatment. The primary antibacterial mechanism of TiO_2 involves direct physical contact between TiO_2-NPs and the bacterial cell surface. This contact causes mechanical damage.

Due to their nanoscale size and high surface area-to-volume ratio, TiO_2-NPs can adhere to the bacterial cell wall. This adhesion can cause significant physical or mechanical stress and can disrupt the integrity of the cell membrane, leading to increased permeability. This allows for the leakage of essential intracellular components, such as ions, metabolites, proteins, and RNA. This leakage compromises cellular function and ultimately leads to cell death. Secondly it can lead to electrostatic attraction: The surface of bacteria is typically negatively charged. TiO_2-NPs can possess a localized positive charge, leading to strong electrostatic attraction. This binding brings NPs closer to the cell membrane, intensifying the mechanical stress and facilitating other damaging interactions [23, 49-51]. TiO_2-NPs that penetrate or damage the cell membrane can interfere with the bacterial respiratory chain. This disruption can lead to incomplete oxygen reduction, generating endogenous superoxide radicals ($O_2^{\bullet -}$) and other ROS inside the bacterium. The resulting internal oxidative stress can overwhelm the bacteria's natural antioxidant defenses, causing damage to DNA, proteins, and lipids. Furthermore, once the cell membrane is compromised, NPs can enter the cytoplasm and interfere with essential cellular functions. One such function is the inhibition of enzyme activity. NPs can bind to critical enzymes, altering their conformation and inhibiting their function. This is particularly true for enzymes containing thiol groups (-SH), which are vital for many metabolic processes. Disruption of nutrient transport is another consequence. By damaging the cell membrane and binding to transport proteins, TiO_2-NPs can disrupt the uptake of essential nutrients, effectively starve the bacterium and inhibit its growth and replication [52-54]. The multiple antimicrobial mechanisms of NPs would require multiple gene mutations in the same bacterial cell for the cell to develop resistance. This makes it difficult for bacterial cells to become resistant to NPs. The antimicrobial efficacy of laser irradiation primarily stems from the generation of ROS, the fundamental mechanism of light-mediated bacterial inactivation. Under normal physiological conditions, ROS perform beneficial cellular functions. However, external stimuli can dramatically increase ROS levels, which can have detrimental effects on cellular differentiation, signaling pathways, and viability [54, 55]. ROS production occurs through two concurrent photochemical pathways: electron transfer reactions (Type I), which generate oxygen, peroxide, or hydroxide radicals; and energy transfer processes (Type II), which produce singlet oxygen ($^1O_2$) [56, 57]. These mechanisms depend on the photochemical interaction between laser radiation and cellular chromophores, which facilitates the conversion of light energy into chemical energy [58, 59]. The efficacy of laser-induced ROS generation depends on multiple irradiation parameters, such as power, intensity, fluence, exposure duration, operational mode, and critically, wavelength [60, 61].

This study revealed that pathogens subjected to combined femtosecond laser irradiation and NP treatment exhibited enhanced antimicrobial activity. These results align with those in [47, 48, 62-64], which presented evidence of the antimicrobial potential of metal oxide NPs and laser irradiation. The observed reduction in bacterial growth can be attributed to several mechanisms. Due to its unique temporal characteristics, femtosecond laser technology offers distinct advantages in biological applications. These ultrashort pulses deposit energy into microscopic volumes within extremely brief timeframes without affecting the surrounding tissues. The combination of pulse width and repetition rate determines thermal accumulation in biological specimens. Short pulse durations enable rapid thermal diffusion, and adequate interpulse intervals allow for complete heat dissipation, which prevents thermal accumulation and minimizes collateral damage [65, 66]. This work's novelty is that it bridges material synthesis with functional biological applications. While TiO_2-NPs have been produced by PLAL in prior studies, coupling them with femtosecond laser excitation at 400 nm for antimicrobial purposes has not been previously explored. This integration enhances ROS generation and yields a pronounced, synergistic antibacterial response against clinically relevant ocular isolates. Although PLAL is a well-established method for generating NPs, using the second harmonic wavelength (532 nm) enhances ablation efficiency and enables more precise control of NP size than the more common 1064 nm wavelength. Using this wavelength also minimizes contamination, resulting in highly pure TiO_2 colloids, which are important for biomedical applications. Unlike most previous studies, which proposed using PLAL-synthesized TiO_2 for photocatalytic or environmental applications, this study addresses its antimicrobial potential against clinically relevant ocular pathogens for the first time. In addition, this research combines PLAL-synthesized TiO_2-NPs with femtosecond laser irradiation. While both femtosecond laser exposure and metal oxide NPs have antibacterial effects, their combined application results in a significant synergistic response that suppresses bacterial growth more effectively than either treatment alone. This is one of the first reports on the synergistic action of TiO_2-NPs synthesized at 532 nm and femtosecond laser irradiation against drug-resistant ocular bacteria. Further optimization of laser parameters and NP concentration is required to maximize the antibacterial effect. The significant and consistent reduction in bacterial proliferation by the combined treatment highlights the potential of this approach as a promising strategy against antibiotic-resistant infections.

IV. CONCLUSIONS

This work examines the synthesis of titanium dioxide (TiO_2)-Nanoparticles (NPs) using Pulsed Laser Ablation (PLA) at 532 nm. This method yields highly pure colloids with tunable size, enhancing their potential integration into biomedical applications. The antibacterial efficacy of the NPs against methicillin-resistant S. aureus and P. aeruginosa was evaluated. The findings show that TiO_2-NPs alone exhibit some antibacterial activity, but combining them with femtosecond laser irradiation leads to a significantly greater and more consistent reduction in bacterial growth. This synergistic effect highlights the potential of this combined approach as a powerful tool for addressing the growing challenge of antibiotic resistance. The consistent, potent antibacterial effects observed indicate that laser-ablated TiO_2-NPs, particularly when coupled with femtosecond laser treatment, offer a promising alternative for various antimicrobial applications. Future research will focus on optimizing TiO_2-NP synthesis parameters, elucidating the precise mechanisms of their combined action, and exploring their efficacy in more complex biological systems. This work contributes to the growing body of knowledge on nanotechnology-driven antimicrobial strategies and paves the way for novel therapeutic interventions.

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