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Antibiofilm efficacy of clindamycin-loaded gold nanoparticles coated guttapercha evaluated by crystal violet biomass assay and surface roughness analysis by atomic force microscopy

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Abstract:
Aims: This study explores the development and evaluation of gutta-percha (GP) cones coated with clindamycin-loaded gold nanoparticles (Clindamycin-AuNPs) to enhance antibiofilm efficacy against Enterococcus faecalis and to characterize surface modifications.
Methods and Material: Gold nanoparticles were prepared by reducing $HAuCl_4$ with ascorbic acid under alkaline conditions, then loaded with clindamycin to form brick-red colloids (λ = 250–260 nm). Ten ISO 70 gutta-percha (GP)cones were soaked in this Clindamycin-AuNP suspension for 24 h (n = 10), with ten uncoated cones as controls. To test antibiofilm activity, 48 h E. faecalis *biofilms (ATCC 51299) were grown in 96-well plates, exposed to each cone, stained with crystal violet, and quantified by OD readings to calculate viability. Surface roughness (Ra) of coated versus uncoated GP was measured by atomic force microscopy*
Statistical analysis used: Data were analyzed in SPSS 26.0. Normality of all variables was confirmed by Kolmogorov–Smirnov and Shapiro–Wilk tests ($p > 0.05$). Surface roughness and bacterial viability between two groups were compared using independent samples t-tests. Biofilm biomass (OD readings) across coated GP, uncoated GP, and culture-only controls was assessed by one-way ANOVA with Bonferroni post hoc comparisons. Statistical significance was set at $\alpha = 0.05$.
Results: These findings demonstrate that Clindamycin-AuNP functionalization of GP cones substantially inhibits E. faecalis biofilm formation and alters surface morphology to potentially improve sealer adhesion.
Conclusions: Clindamycin-AuNP functionalization of GP cones significantly impedes E. faecalis *biofilms and alters surface topography, offering a promising strategy to improve root-canal sealing and antimicrobial performance.*
Key-words: *AuNP- Gold Nanoparticle, AFM- Atomic force Microscopy, E Faecalis*

INTRODUCTION

Endodontic therapy aims to eliminate microorganisms from the root canal system and achieve a hermetic seal to prevent reinfection, which remains critical for long-term treatment success [1]. Despite advances in rotary instrumentation and irrigant activation techniques, persistent infections continue to challenge clinicians, often due to the complex anatomy of root canals and the resilience of endodontic pathogens [2]

Enterococcus faecalis form dense biofilms within dentinal tubules [3]. Biofilm communities confer enhanced resistance to antimicrobial agents and mechanical removal, making *E. faecalis* a gold-standard model for evaluating novel disinfection strategies [4]

GuttaPercha is inherently inert and lacks antimicrobial properties; its smooth surface may limit sealer adhesion, potentially leading to microgaps at the GP–sealer interface and subsequent reinfection [5]. Surface roughness has been shown to play a critical role in improving sealer bond strength, as increased nanoscale texturing enhances mechanical interlocking and wettability at the GP–sealer interface.

Gold nanoparticles (AuNPs) are particularly attractive due to their biocompatibility, tunable surface chemistry, and unique optical and plasmonic properties [6]. By adjusting particle size, shape, and surface ligands, AuNPs can be engineered for controlled therapeutic agent release and enhanced interaction with microbial biofilms [7].

Clindamycin- loaded AuNPs enable targeted high local antibiotic concentrations, overcoming biofilm barriers and reducing systemic exposure.

METHOD

Synthesis Of Clindamysin Loaded Gold Nanoparticles

A 0.5 mM solution of auric chloride tetrahydrate was prepared by dissolving it in 50 mL of deionized water. The pH of the solution was adjusted to above 8 using 1 N NaOH. Ascorbic acid (0.1 M) was added as a reducing agent. Subsequently, 1 mL of clindamycin was added dropwise. Upon the formation of clindamycin-incorporated gold nanoparticles, the solution changed color to brick red. The particles were centrifuged and dried for further procedures.

Characterization of clindamycin AuNP was done using UV Visible spectrophotometer. UV-Visible (UV-Vis) spectrophotometry is one of the primary techniques used to confirm the synthesis, stability, and drug loading of gold nanoparticles (AuNPs), including clindamycin-loaded AuNPs. Gold nanoparticles exhibit a strong surface plasmon resonance (SPR) — a collective oscillation of conduction electrons at specific wavelengths when excited by light. SPR peak typically appears around 520–550 nm depending on particle size, shape, and the surrounding medium. Upon drug loading, there may be a shift in the SPR peak (either redshift or blueshift), and/or changes in absorbance intensity or peak broadness. The spectrum shows a sharp, intense peak near 250–260 nm, with a maximum absorbance above 0.8 A. The spectrum aligns with successful clindamycin incorporation onto AuNPs.

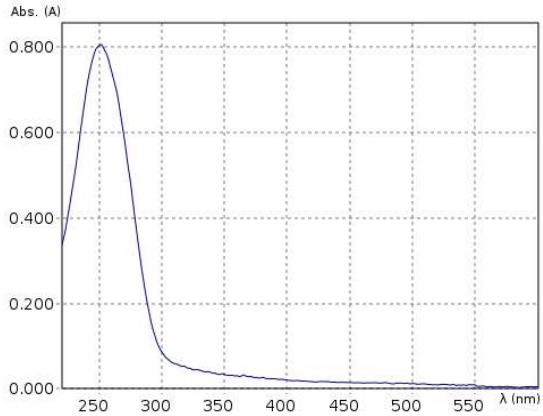

Figure 1 UV visible spectroscopy absorbance of Clindamycin coated gold nanoparticles

Coating Guttapercha With Clindamycin Loaded Gold Nanoparticles

Pure Clindamycin -loaded Au nanoparticles were incorporated in PVA-polyvinyl acetate polymer which acted as a medium to coat guttapercha surface. GP cones (Size 70, Diadent) from freshly opened boxes were organized into two groups-(A-Uncoated GP, B-Coated GP). Guttapercha cones are coated with Clindamycin loaded Au nanoparticles by dipping in PVA polymer which is incorporated with nanoparticles. Dip coating helps to get 3-dimensional coating of Guttapercha cones with nanoparticles.

Gp cones (ISO 70) DiaDent from freshly opened boxed were organized into 2 groups. Group 1 –functionalized gp , group 2 – Uncoated gp . Group 1 gpcones were immersed in the clindamycin-AuNps containing beaker for 24 hours

Preparation of inoculum and contamination of specimens

On confirmation of sterility by incubating in 5 mL sterile Mueller Hinton broth at 37°C for 24h the samples were transferred to fresh sterile Mueller Hinton broth 5 mL to be inoculated with 200 µL of an Enterococcus faecalis (ATCC 51299) suspension containing 108 cells mL^{-1}. The specimens were incubated at 37 °C and the nutritional broth changed every second day for a period of 8 weeks. The purity of the infection was checked by culturing and gram staining at days 28 and 56.

Crystal Violet biomass assay

The crystal violet assay is a widely used method for quantifying biofilm formation or adherent cell biomass in a 96-well microtiter plate. To begin the procedure, each well is inoculated with 100–200 µL of a bacterial suspension (typically diluted to an optical density of 0.05–0.1 at 600 nm) and incubated under suitable conditions, such as 24–48 hours at 37 °C, to allow for biofilm development. After incubation, the planktonic (non-adherent) cells are gently removed by aspirating the culture medium, and the wells are washed two to three times with phosphate-buffered saline (PBS) to eliminate residual free-floating cells. Optionally, the adherent biofilms or cells can be fixed by adding 100 µL of methanol or ethanol to each well and incubating for 15–20 minutes, followed by air drying. Subsequently, 100 µL of 0.1% (w/v) crystal violet solution is added to each well and incubated for 15–20 minutes at room temperature to stain the biofilms. The excess stain is then discarded, and the wells are washed three to four times with distilled water until no free dye remains, after which the plate is left to dry completely. To quantify the biomass, the bound dye is solubilized using 100–200 µL of 95% ethanol or 30% acetic acid per well, and the absorbance is measured at 570 nm or 590 nm using a microplate reader. The absorbance directly correlates with the amount of adherent biomass present in each well. Proper controls (such as blank wells with media only) and technical replicates are essential for accurate interpretation of results.

Surface roughness analysis by AFM

To determine the surface roughness of gutta-percha using atomic force microscopy (AFM), a systematic and precise approach is required. First, gutta-percha samples are cut into small, flat sections and mounted securely on a specimen holder, often using a double-sided adhesive or epoxy to ensure stability during scanning. The surface should be clean and free from debris or contaminants that could interfere with imaging. AFM is typically operated in tapping mode or non-contact mode to prevent damage to the soft gutta-percha surface while accurately capturing its topography. During scanning, a sharp probe at the end of a cantilever interacts with the surface at nanometer resolution, and deflections are measured by a laser reflected off the cantilever into a photodetector. The resulting topographical data is rendered into a 2D or 3D image, from which quantitative surface roughness parameters can be extracted using AFM software. The most commonly reported roughness values include Ra (arithmetical mean roughness) and Rq (root mean square roughness), which provide insight into the average height deviations and overall surface texture.In this study Bruker Dimension Edge Scanning Probe Microscope was used to obtain the Ra values. Multiple scans from different areas of the sample are typically analyzed to ensure reproducibility and statistical reliability. These roughness measurements are crucial for evaluating how surface characteristics of gutta-percha may influence properties such as adhesion, wettability, and interaction with sealing materials in endodontic applications.

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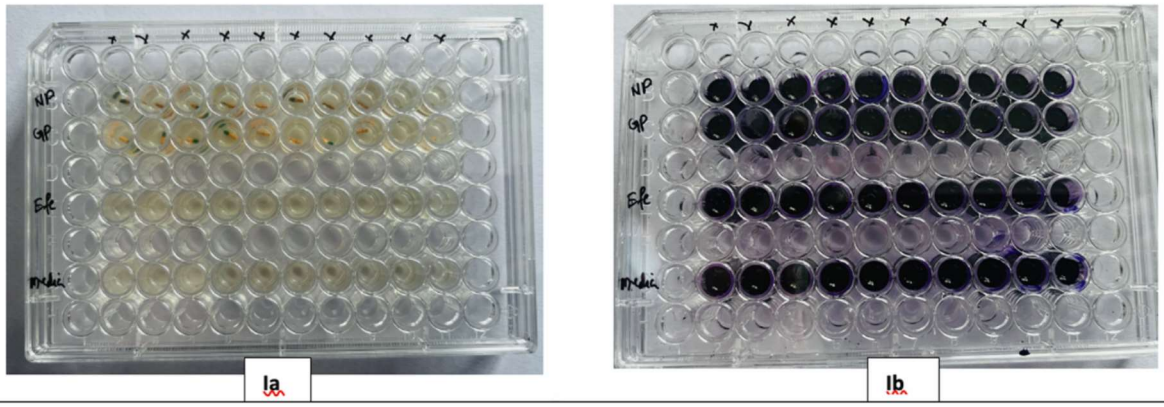


Figure 2- Crystal-violet biomass assay of *E. faecalis* biofilms after exposure to gutta-percha cones.
(a) 96-well plate immediately after removal of GP cones (Group 1 = uncoated, Group 2 = Clindamycin–AuNP-coated; NP = no-GP control; M = medium-only control).
(b) The same plate after staining with 0.1% crystal violet and gentle washing: wells with more biofilm biomass retain deeper purple color.

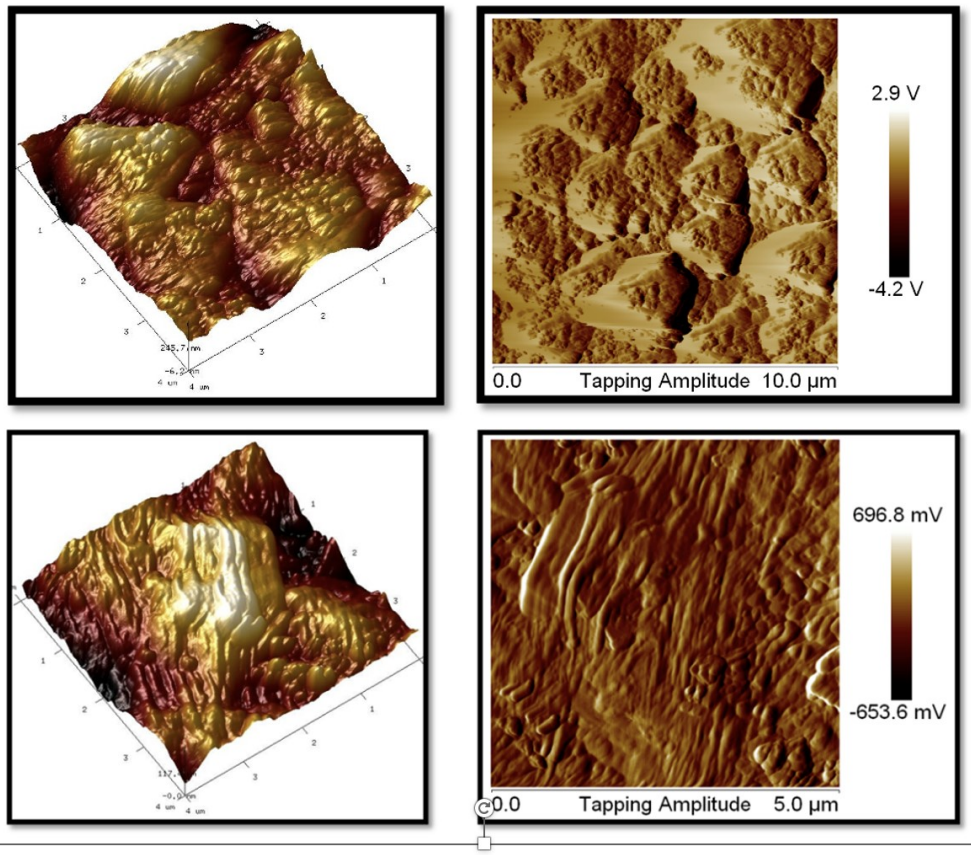


Figure 3 -AFM analysis of gutta-percha (GP) cone surfaces.
Top panels (Group 1): Uncoated GP; **Bottom panels (Group 2):** Clindamycin–AuNP–coated GP.

RESULTS

Assessment of antibacterial efficacy by crystal violet assay The microtiter plate assay using crystal violet done on 1-day-old biofilm of E. faecalis strains ATCC 51299 provides information on the effects of the nanoparticles on the biofilm biomass. The OD obtained for the test materials against two E. faecalis strains was demonstrated in table 1. Crystal violet assay against E. faecalis strain ATCC 51299 showed that Group 1 showed statistically significant ($P < 0.05$) reduction in OD (0.4817) than Group 2 (0.6198). Crystal violet assay against E. faecalis showed that Group 1 showed the highest reduction in OD (0.6252
Data was analyzed using the statistical package SPSS 26.0 (SPSS Inc., Chicago, IL). Descriptive statistics was performed to assess the mean standard deviation, Median and Proportion of the respective groups. Independent T test /one way Anova test followed by Bonferroniposthoc test used for comparison of variables between the groups

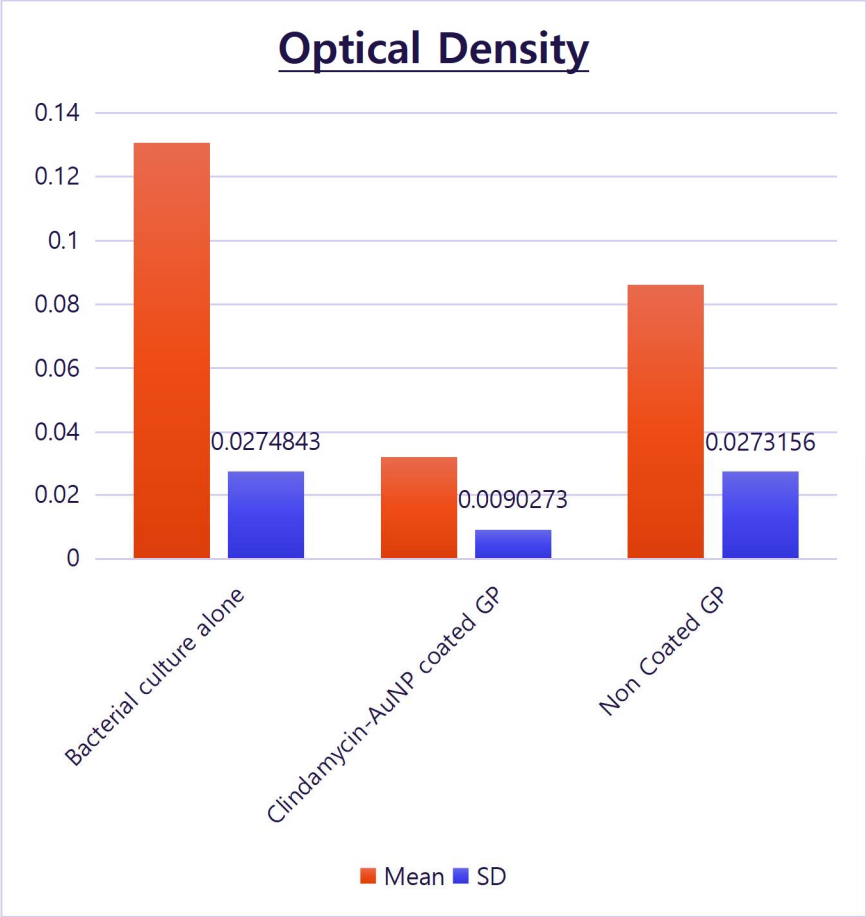
The mean OD was significantly lowest in the Clindamycin-AuNP coated gutta-percha (GP) group (0.0322 ± 0.0090), followed by the non-coated GP group (0.0862 ± 0.0273), and highest in the bacterial culture alone (0.1308 ± 0.0275). The ANOVA F value of 46.19 with a P value of 0.0001 indicates a highly significant difference among the groups. This demonstrates that the Clindamycin-AuNP coating substantially reduced bacterial growth

	N	Mean	SD	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
Bacterial culture alone	**10**	**.13075**	**.0274843**	**.00869**	**.111089**	**.150411**	**.0894**	**.1637**
Clindamycin-AuNP coated GP	**10**	**.03219**	**.0090273**	**.00285**	**.025732**	**.038648**	**.0207**	**.0472**
Non Coated GP	**10**	**.08622**	**.0273156**	**.00863**	**.066680**	**.105760**	**.0559**	**.1550**
Anova (F Value)				**46.19**				
P Value				**0.0001***				

The ANOVA F value of 46.19 with a P value of 0.0001 indicates a highly significant difference among the groups. This demonstrates that the Clindamycin-AuNP coating substantially reduced bacterial growth

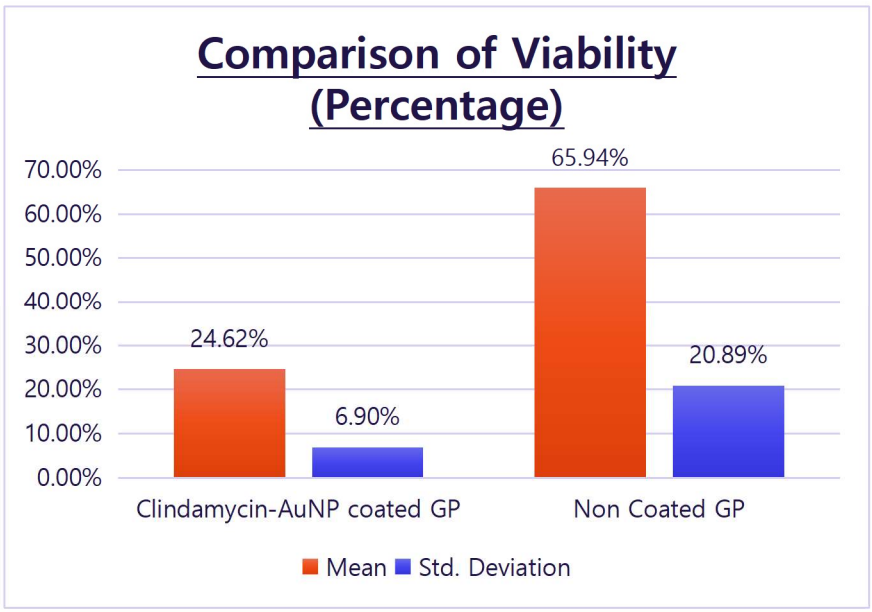
Groups	N	Mean	Std. Deviation	Std error	P Value
Clindamycin-AuNP coated GP	10	.057190	.0061055	.0019307	0.0001*
Non Coated GP	10	.030120	.0049177	.0015551	

The mean surface roughness was significantly higher in the coated group (0.05719 ± 0.0061) compared to the non-coated group (0.03012 ± 0.0049), with a P value of 0.0001, indicating a statistically significant difference.



The Clindamycin-AuNP coated GP group had a significantly lower OD than both the bacterial culture alone (mean difference = 0.0986, p = 0.0001) and the non-coated GP group (mean difference = 0.0540, p = 0.0001). Additionally, the non-coated GP group showed significantly lower OD than the bacterial culture alone (mean difference = 0.0445, p = 0.001). These results confirm that Clindamycin-AuNP coating significantly inhibits bacterial growth compared to the other groups.

The mean cell viability was significantly lower in the coated group (24.62% ± 6.90) compared to the non-coated group (65.94% ± 20.89), with a P value of 0.0001, indicating a highly significant reduction in viability due to the Clindamycin-AuNP coating.



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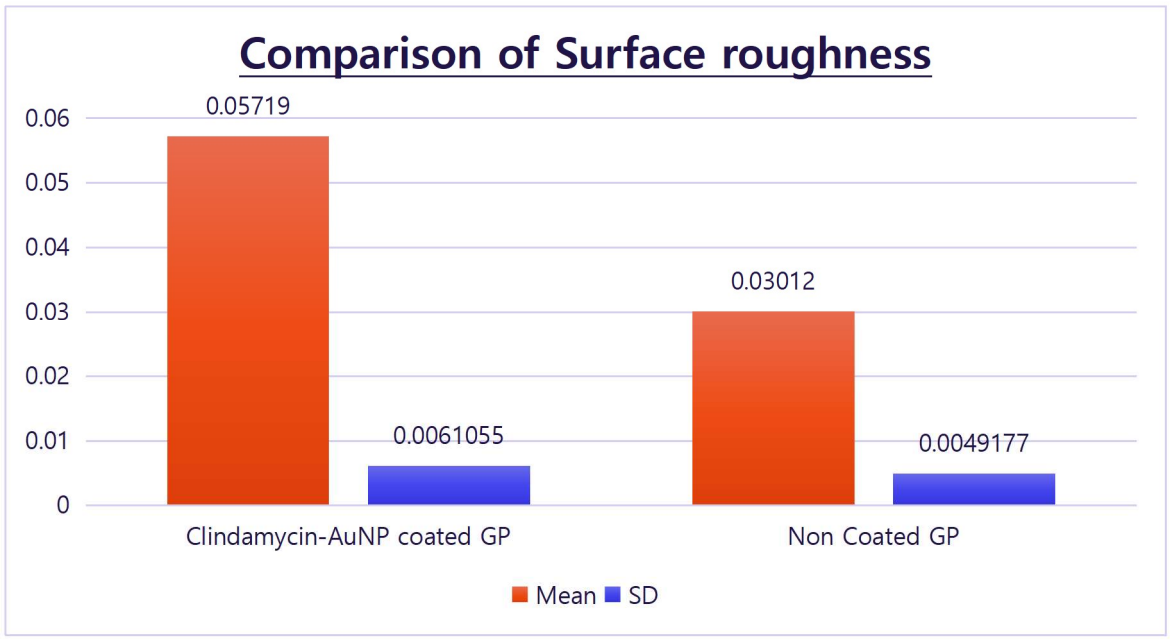

DISCUSSION

Persistent endodontic infections remain a formidable challenge, largely due to the persistence of *Enterococcus faecalis*, a Gram- positive, facultative anaerobe commonly isolated from failed root canal treatments because of its multifactorial virulence and antibiotic- resistance profiles[16]. Its pathogenic success is multifactorial: it readily adheres to dentin and produces extracellular polymeric substances that form dense biofilms resistant to conventional irrigants and medicaments [4]; it survives nutrient starvation and high-pH environments—such as those created by calcium hydroxide dressings—by entering a quiescent state[8]; and it can penetrate dentinal tubules up to 300–400 µm deep, evading mechanical removal during canal preparation[6][19][18]. These characteristics make *E. faecalis* an exacting test organism for evaluating novel antimicrobial strategies.

Conventional GP points, while prized for their thermoplasticity, dimensional stability, and ease of handling, are fundamentally inert and lack several key properties needed for optimal obturation. First, they possess no intrinsic

antimicrobial activity, leaving any residual bacteria beneath the filling free to survive and potentially recolonize the canal space [15]. Second, their relatively smooth, hydrophobic surface results in low surface energy and poor wettability, which can hinder sealer penetration and adhesion, leading to microgaps at the GP–sealer interface and increased risk of microleakage[9]. Third, unmodified GP provides no bioactive cues to the surrounding dentin or periapical tissues, offering no support for tissue healing or regeneration [10]. Finally, conventional GP cannot be tailored for controlled drug release, limiting the ability to deliver sustained, localized antimicrobial or anti-inflammatory agents directly within the root canal system. Together, these deficiencies have driven the development of functionalized GP materials—such as those coated with antibiotic-loaded nanoparticles—to combine effective sealing with targeted antibacterial and bioactive properties. Such approaches build on earlier work with silver- and calcium- hydroxide nanoparticle medicament [17].

Gold nanoparticles (AuNPs) have emerged as versatile drug carriers in biomedical applications, offering several advantages for antimicrobial delivery. Their inherent biocompatibility ensures minimal cytotoxicity to mammalian cells, while surface functionalization via thiol or amine linkers allows stable conjugation of therapeutic agents and targeting ligands [7][11]. The high surface-to-volume ratio of AuNPs facilitates controlled, sustained release of loaded drugs, and their intrinsic antimicrobial effects—stemming from membrane disruption and localized oxidative stress—can further potentiate antibacterial activity . In this study, AuNPs with an average hydrodynamic diameter of ~20 nm and a zeta potential of –25 mV were synthesized by reducing $HAuCl_4$ with ascorbic acid in the presence of clindamycin, yielding a brick-red colloid characterized by a surface plasmon resonance peak at ~520 nm.

Clindamycin, a lincosamide antibiotic, inhibits bacterial protein synthesis by binding the 50S ribosomal subunit and is effective against Gram-positive cocci and anaerobes [12]. However, its penetration into established biofilms is limited, and systemic administration may not achieve sufficiently high local concentrations within the root canal environment. Conjugating clindamycin to gold nanoparticles creates a multifunctional platform in which the antibiotic is stably loaded onto the nanoparticle surface, ensuring high local concentrations and limiting premature systemic release. The nanoscale dimensions of the AuNPs promote sustained desorption of clindamycin over time, maintaining therapeutic levels directly at the gutta-percha–biofilm interface far longer than bulk antibiotic application. In addition, the AuNP core itself contributes to antibacterial activity by perturbing bacterial membranes and generating localized oxidative stress, which weakens the biofilm matrix and enhances antibiotic penetration. As the nanoparticle coating deposits onto the gutta-percha, it also roughens the surface at the nanoscale, improving wetting and mechanical interlocking of sealers. By marrying sustained, localized antibiotic delivery with enhanced surface topography, this approach offers a synergistic strategy to both eradicate persistent endodontic pathogens and reinforce the hermetic seal of root canal obturations.

To assess antibiofilm efficacy, we employed the crystal violet assay, which quantifies total biofilm biomass, including cells and extracellular matrix, via staining with 0.1% crystal violet and measurement of optical density at 595 nm. After incubating coated and uncoated gutta-percha cones with an *E. faecalis* suspension (10^8 CFU/mL) for 48 hours, we observed mean OD_{595} values of 0.0322 ± 0.0090 for coated cones, 0.0862 ± 0.0273 for uncoated controls, and 0.1308 ± 0.0275 for bacterial culture alone. One-way ANOVA ($F = 46.19$; $p < 0.0001$) with Bonferroni post hoc tests ($p \leq 0.001$) confirmed highly significant reductions in biofilm biomass for the coated group[13], indicating that clindamycin-AuNP functionalization drastically inhibits biofilm formation.

Surface roughness refers to the microscopic peaks and valleys on a material's surface, typically quantified by the average roughness parameter, Ra, which represents the arithmetic mean of absolute deviations from the mean surface plane. In endodontics, Ra is particularly important because a rougher gutta-percha (GP) surface increases its effective contact area with sealers, promoting both mechanical interlocking and improved wettability. Atomic force microscopy (AFM) in tapping mode is the gold-standard technique for measuring Ra on GP: a sharp cantilever tip raster-scans a defined region (commonly 10 μm × 10 μm), and high-resolution height data are recorded to compute Ra values. Studies have shown that increasing Ra from ~0.02 μm (typical of unmodified GP) to ~0.05–0.1 μm (after nanoparticle or acid-etch treatments) can double sealer bond strengths and significantly reduce microleakage under simulated pressure conditions. Using atomic force microscopy in tapping mode over 10 μm × 10 μm areas, we measured mean Ra values of 0.05719 ± 0.00611 μm for coated cones versus 0.03012 ± 0.00492 μm for uncoated cones—a nearly twofold increase ($p = 0.0001$) . This augmented roughness, resulting from AuNP deposition, is likely to translate into stronger sealer bonds in clinical applications. [14]

In summary, the functionalization of gutta-percha with clindamycin-loaded gold nanoparticles offers a dual benefit: potent inhibition of *E. faecalis* biofilm formation and significant enhancement of surface roughness conducive to improved sealer adhesion. The sustained, localized release of clindamycin from the AuNP matrix maintains inhibitory concentrations at the dentin–filling interface, while the nanoparticles themselves disrupt biofilm integrity and deposit a textured nanoscale layer that augments mechanical interlocking. Together, these effects promise to reduce residual bacterial contamination and microleakage—two principal causes of endodontic failure. Future work should focus on in situ release-kinetics under dynamic conditions, quantitative bond-strength testing with various sealers, and comprehensive biocompatibility and in vivo efficacy studies. If these investigations confirm safety and performance, Clindamycin–AuNP-coated gutta-percha could represent a significant advance toward more durable, infection-resistant root canal obturation.

Future research should first elucidate the in situ release dynamics of clindamycin from AuNP-coated gutta-percha under simulated intracanal flow and pressure, ensuring sustained antimicrobial delivery throughout treatment. Parallel studies must examine how various endodontic sealers interact with the nanoparticle-roughened surface—quantifying bond strength, microleakage, and long-term stability—to confirm compatibility and sealing performance. Expanding the antimicrobial repertoire by loading AuNPs with alternative or combination antibiotics (for example, metronidazole or doxycycline) and testing against multispecies biofilms will determine whether this approach can address the polymicrobial nature of persistent infections. Crucially, comprehensive biocompatibility and safety assays on periapical cell lines and organotypic tissue models are needed to rule out cytotoxic or inflammatory effects. Finally, in vivo validation in animal models of endodontic infection, coupled with optimization of nanoparticle synthesis and coating protocols for reproducibility, storage stability, and cost-effective scale-up, will be essential steps toward clinical translation.

CONCLUSION

Clindamycin–AuNP functionalization of gutta-percha achieves a synergistic enhancement of endodontic obturation by simultaneously delivering potent, localized antibacterial activity and engineering a nanoscale-textured surface that promotes superior sealer adhesion. The AuNP–clindamycin conjugate dramatically reduced *E. faecalis* biofilm biomass (65% decrease in OD_{595}) and biofilm viability (41% absolute reduction) compared with unmodified GP, confirming its antibiofilm efficacy. Concurrently, the nanoparticle coating increased surface roughness (Ra) nearly twofold, from 0.030 μm to 0.057 μm, a change known to improve mechanical interlocking and wettability of sealers. These dual benefits have the potential to minimize residual bacterial contamination and microleakage—key drivers of endodontic failure.

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