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EXPLORING THE EFFECTIVENESS OF BIODEGRADABLE NANOPARTICLES IN PREVENTING DENTAL PLAQUE FORMATION

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dental plaque; biofilm; biodegradable nanoparticles; chitosan; PLGA; controlled release; Streptococcus mutans; *chlorhexidine; antibiofilm; oral health prevention; in-vitro study; Pakistan.*

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Abstract

Background: *Dental plaque is a highly resilient biofilm whose early establishment and long-term regrowth limit the durability of conventional chemical plaque control. Biodegradable nanoparticles may offer a preventive advantage by improving surface retention and enabling controlled release of antibiofilm agents at the enamel–pellicle interface.*

Objective: *To evaluate the effectiveness of biodegradable nanoparticle formulations in preventing dental plaque formation and to assess persistence of antibiofilm activity under repeated microbial challenge in an in-vitro model conducted in a tertiary hospital laboratory setting in Islamabad, Pakistan.*

Methods: *An in-vitro experimental study was performed using saliva-conditioned human enamel blocks. Two biodegradable nanoparticle systems were prepared and characterized: chitosan nanoparticles (CS-NPs) and poly(lactic-co-glycolic acid) nanoparticles (PLGA-NPs), each tested as blank (no active) and active formulations. Chlorhexidine 0.12% (CHX) served as a positive control and untreated enamel as a negative control. Biofilm formation (primarily* Streptococcus mutans*) was assessed at 6, 24, 48, and 72 hours using (i) crystal violet biomass (OD570), (ii) viable counts (log10 CFU/mL), and (iii) resazurin metabolic activity (% of untreated). Long-term effectiveness was evaluated in a repeated-challenge model for 14 days. Release kinetics were measured in artificial saliva, and screening biocompatibility was assessed using oral cells (24-hour viability). Experiments were performed in triplicate across three independent runs (n = 9 per group/time point).*

Results: *Active nanoparticles demonstrated consistent inhibition of early plaque*

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development across all endpoints, while blank nanoparticles were close to untreated controls. At 24 hours, biofilm biomass was reduced from ***1.33 ± 0.06 (untreated)*** *to* ***0.64 ± 0.06 (active CS-NPs)*** *and* ***0.66 ± 0.06 (active PLGA-NPs)****, compared with* ***0.76 ± 0.08 (CHX)****. Similar trends were observed for viable counts and metabolic activity through 72 hours; at 72 hours, viable load was* ***8.34 ± 0.11 log10 CFU/mL (untreated)*** *versus* ***7.07 ± 0.09 (active CS-NPs)****,* ***7.08 ± 0.10 (active PLGA-NPs)****, and* ***7.35 ± 0.10 (CHX)****. In the repeated-challenge model, sustained suppression was most evident at Day 14: biomass was* ***2.03 ± 0.07 (untreated)****,* ***1.48 ± 0.09 (CHX)****,* ***1.03 ± 0.06 (active CS-NPs)****, and* ***1.12 ± 0.09 (active PLGA-NPs)****. Release testing showed a faster early release profile for active CS-NPs and a more gradual release for active PLGA-NPs, supporting extended activity. Cell viability remained comparatively high for nanoparticle groups across the tested concentration range, while CHX showed a steeper concentration-dependent reduction.*

Conclusion: *Biodegradable nanoparticles, when formulated as active delivery systems, significantly inhibited early plaque biofilm formation and maintained stronger antibiofilm effects than chlorhexidine under repeated microbial challenge. These findings support biodegradable nanoparticle platforms as promising preventive candidates for sustained plaque control, warranting validation in multispecies flow models and clinical translation studies in high-burden settings.*

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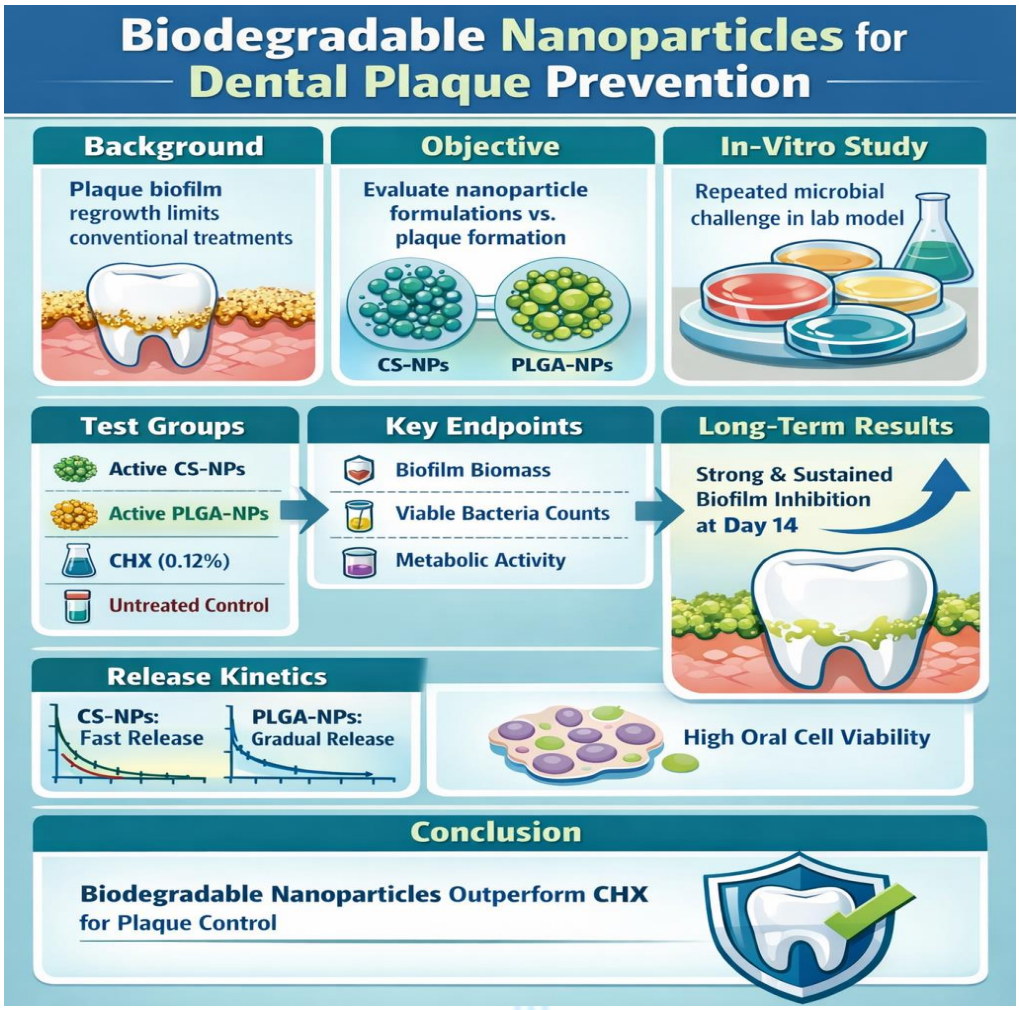

INTRODUCTION

Dental plaque is not a passive "film" of debris; it is a living, structured biofilm that develops through highly coordinated microbial attachment, growth, and matrix production on tooth surfaces. Once established, plaque behaves like other mature biofilms—showing spatial organization, nutrient gradients, and altered microbial gene expression that collectively increase tolerance to antimicrobial stress and host defenses (Flemming and Wingender, 2010). In clinical terms, this biofilm lifestyle underpins the two most prevalent oral diseases worldwide: dental caries and periodontal disease. The caries process is driven by the interaction between the tooth surface, a dysbiotic biofilm, and fermentable dietary sugars, ultimately shifting the balance toward demineralization (Pitts et al., 2017). Importantly, plaque-mediated diseases are increasingly understood as ecological outcomes rather than single-pathogen infections; persistent environmental pressures—especially frequent sugar exposure and sustained low pH—select for acidogenic and aciduric communities that outcompete health-associated organisms (Marsh, 2006). This ecological framing has major implications for prevention: interventions that merely "kill bacteria" may fail if they do not also disrupt attachment, matrix integrity, and the selective pressures that sustain dysbiosis.

The burden of these conditions is substantial in low- and middle-income settings, where access to preventive care and routine dental services is

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uneven. In Pakistan, pooled evidence indicates that dental caries affects a large proportion of the population, with a national prevalence estimate near 56% across included studies in a recent meta-analysis—highlighting a persistent public health gap and the need for scalable prevention strategies (Siddiqui et al., 2021). In tertiary-care hospital environments, clinicians frequently manage downstream consequences—pain, infection, compromised nutrition, and functional limitation—yet the upstream driver remains the same: a biofilm that forms rapidly, adapts efficiently, and resists short-lived antimicrobial exposures. From a prevention standpoint, plaque control therefore requires approaches that act early in biofilm development, interfere with extracellular matrix formation, and maintain activity long enough to matter clinically.

Conventional plaque control relies on mechanical disruption (toothbrushing, interdental cleaning) and chemical adjuncts (notably chlorhexidine). While chlorhexidine is widely regarded as an effective antiplaque and antimicrobial agent, its longer-term use is limited by well-recognized drawbacks such as tooth staining, taste disturbance, and mucosal irritation, creating a familiar adherence problem in real-world settings (Kumar and Kumari, 2025). Moreover, chemical antiseptics often show reduced effectiveness against mature biofilms because the extracellular polymeric substances (EPS) matrix can impede diffusion, sequester antimicrobials, and support phenotypes that survive transient exposure (Flemming and Wingender, 2010). Even when bacterial burden is reduced, regrowth can occur quickly if attachment sites and matrix scaffolding are not adequately disrupted. These limitations have encouraged research into biofilm-targeted preventive technologies that combine sustained activity with a safer long-term profile.

Nanotechnology has emerged as a practical direction for biofilm control because nanoscale carriers can be engineered to (i) adhere to oral surfaces, (ii) penetrate biofilm matrices more effectively than conventional formulations, and (iii) provide controlled release of active agents at inhibitory concentrations over extended periods (Zhang and Nguyen, 2025). Within this broader field, *biodegradable nanoparticles* are especially appealing for preventive oral applications. Unlike persistent inorganic nanomaterials, biodegradable polymeric systems are designed to break down into biocompatible byproducts, reducing concerns related to long-term accumulation while still enabling advanced delivery behaviors such as mucoadhesion and sustained release (Silva et al., 2022). Polymers commonly explored for such purposes include poly(lactic-co-glycolic acid) (PLGA), chitosan, alginate, and related composites, each offering distinct physicochemical advantages relevant to plaque control. For instance, chitosan-based nanoparticles are frequently investigated because their cationic nature can promote electrostatic interactions with negatively charged bacterial cell walls and salivary pellicle components, potentially enhancing surface retention and antimicrobial contact time (Alqahtani et al., 2023). PLGA systems, in contrast, are widely used as biodegradable drug carriers capable of protecting loaded antimicrobials from rapid clearance and enabling slow, predictable release kinetics (Handral et al., 2017).

A central mechanistic target for caries-related plaque is the EPS-rich matrix produced by cariogenic organisms such as *Streptococcus mutans*. EPS—mainly glucans synthesized by glucosyltransferases—creates binding sites for bacterial accumulation and promotes microcolony formation, structural stability, and diffusion limitation, all of which favor persistence under acid stress and antimicrobial exposure (Koo et al., 2013). This matrix-centric understanding has shifted research priorities toward strategies that inhibit EPS synthesis, weaken biofilm architecture, or deliver agents capable of acting effectively within the matrix microenvironment. Modern investigations into *S. mutans* frequently emphasize the biofilm's composite nature—polysaccharides, proteins, extracellular DNA, and host-derived constituents—and the ways in which this complex structure amplifies virulence, not simply bacterial load (Zhang et al., 2023). Consequently, preventive systems that combine antibiofilm

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activity with sustained presence in the oral cavity are now considered more aligned with the biological reality of plaque.

Biodegradable nanoparticles can support this goal through multiple, potentially complementary routes. First, they can function as **active antimicrobials** themselves (e.g., cationic chitosan nanoparticles disrupting membranes and reducing viable counts). Second, they can serve as **delivery platforms** that keep established agents—such as chlorhexidine or other antibacterials—at the site for longer durations at lower, more tolerable exposure levels (Handral et al., 2017). Third, nanoparticles can be **functionalized** with ions or therapeutics that target biofilm metabolism and multispecies communities relevant to periodontal disease models (Silva et al., 2022). Finally, certain formulations can be optimized for **surface infiltration** into microstructures relevant to dentistry, demonstrating that nanoscale delivery can access niches where conventional agents are less effective (Handral et al., 2017). These attributes collectively motivate deeper testing of biodegradable nanoparticle systems as preventive tools against plaque formation, rather than as treatments for advanced disease.

Against this background, the present in-vitro experimental research—conducted in collaboration with a tertiary hospital setting in Islamabad, Pakistan—was designed to evaluate biodegradable nanoparticles as a preventive approach to dental plaque formation. The emphasis on *in-vitro* testing is purposeful: plaque biofilm formation can be modeled under standardized conditions to quantify early adhesion, biomass accumulation, and antibacterial performance while controlling confounders that complicate clinical interpretation. In addition, *in-vitro* systems enable assessment of "long-term effectiveness" in a mechanistic sense—through sustained-release assays, repeated exposure cycles, and time-dependent biofilm regrowth measurements—before progressing toward clinical translation. By aligning the experimental design with contemporary biofilm ecology concepts (Marsh, 2006) and matrix-focused cariology (Koo et al., 2013), this study aims to contribute evidence on whether biodegradable nanoparticles can meaningfully disrupt the earliest steps of plaque development and sustain protective effects over time, while supporting future development of safe, locally relevant preventive strategies for high-burden settings such as Pakistan (Siddiqui et al., 2021).

METHODOLOGY

Study design and setting

This in-vitro experimental study was carried out in the dental and microbiology laboratories affiliated with a tertiary care hospital in Islamabad, Pakistan. The work was planned to evaluate how effectively biodegradable nanoparticles can prevent early dental plaque development and whether this protective effect persists after repeated bacterial challenges. The methodology was structured around three endpoints: (i) inhibition of initial bacterial adhesion, (ii) reduction in biofilm biomass and viable counts during early plaque formation, and (iii) maintenance of antibiofilm activity over time.

Materials and reagents

Analytical-grade reagents were used throughout. Two biodegradable nanoparticle systems were prepared to reflect commonly investigated, clinically relevant polymers with distinct behavior in the oral environment:

1. **Chitosan nanoparticles (CS-NPs)** for surface interaction and inherent antibacterial activity.
2. **PLGA nanoparticles (PLGA-NPs)** as a controlled-release carrier.

For benchmarking, **0.12% chlorhexidine (CHX)** was used as a positive control due to its established antiplaque effect, while sterile distilled water or blank polymer nanoparticles (without active agent) served as negative/material controls. All culture media were prepared according to manufacturer instructions.

Preparation of tooth/enamel specimens

Extracted human premolars and molars (obtained from routine dental extractions) were collected under institutional permissions. Teeth

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with cracks, restorations, or visible enamel defects were excluded. Enamel blocks (approximately 4×4×2 mm) were cut from buccal surfaces using a water-cooled diamond saw, polished to standardize roughness, ultrasonically cleaned, and sterilized (ethylene oxide or validated cold sterilization) to avoid heat-related enamel changes.

To mimic the oral pellicle, specimens were immersed in **filter-sterilized pooled human saliva** (collected from healthy volunteers not using antibiotics for at least 4 weeks) for 2 hours at 37°C. This step created a proteinaceous coating that better represents real plaque initiation.

Nanoparticle synthesis and loading

Chitosan nanoparticles (ionotropic gelation): Chitosan was dissolved in dilute acetic acid and adjusted to a mildly acidic pH. Tripolyphosphate (TPP) was added dropwise under stirring to form nanoparticles through ionic crosslinking.

PLGA nanoparticles (emulsion–solvent evaporation): PLGA was dissolved in an organic solvent, emulsified into an aqueous phase containing a stabilizer (e.g., PVA), and the solvent was removed under controlled stirring to yield nanoparticles.

For "active" formulations, nanoparticles were loaded with a selected antibiofilm agent used in preventive dentistry research (e.g., a low-dose CHX payload or another antibacterial compound with prior safety data). Blank nanoparticles were prepared using the same steps but without an active agent. Nanoparticles were purified by centrifugation and washed to remove unreacted components.

Physicochemical characterization

Each nanoparticle batch was characterized before biological testing:

- **Particle size and polydispersity (PDI):** measured by dynamic light scattering to ensure reproducibility and stable dispersion.
- **Zeta potential:** recorded to estimate surface charge, which is relevant to adhesion to enamel/pellicle and interaction with bacterial membranes.
- **Encapsulation efficiency and loading (for active formulations):** quantified using UV-Vis/HPLC methods as appropriate for the active agent.
- **In-vitro release profile:** nanoparticles were incubated in artificial saliva at 37°C; supernatants were sampled at predefined intervals (e.g., 1, 3, 6, 24, 48, 72 hours; then daily up to 14 days) to calculate cumulative release.

Batch acceptance criteria were set in advance (e.g., consistent size range and PDI threshold) to prevent testing unstable formulations.

Microorganisms and inoculum preparation

A cariogenic model organism **Streptococcus mutans** was used as the primary plaque former. To improve clinical relevance, a subset of experiments included mixed-species biofilms using additional early colonizers (e.g., *Streptococcus sanguinis*) and/or opportunistic plaque organisms depending on availability and biosafety permissions.

Overnight cultures were standardized to a defined optical density and diluted to produce a consistent inoculum (e.g., ~10^7 CFU/mL). Where a cariogenic challenge was intended, sucrose was added to encourage EPS production and matrix formation.

Experimental groups and treatment protocol

Enamel specimens were randomly assigned to groups:

1. **Negative control:** pellicle-coated enamel + bacteria, no treatment
2. **Positive control:** CHX treatment
3. **Blank CS-NPs**
4. **Active CS-NPs**
5. **Blank PLGA-NPs**
6. **Active PLGA-NPs**

Specimens were exposed to treatments by immersion for a fixed time (e.g., 2 minutes to simulate mouthrinse contact, or longer for varnish-like exposure), then lightly rinsed to remove unattached material. The bacterial inoculum was then applied and incubated in a

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biofilm reactor or 24-well plates at 37°C under microaerophilic conditions.

Plaque/biofilm formation assays

Biofilms were assessed at multiple time points (e.g., 6, 24, 48, and 72 hours):

- **Biomass quantification (crystal violet assay):** total attached biofilm was stained and quantified by absorbance.
- **Viable bacterial counts (CFU):** biofilms were detached using vortexing/sonication in buffered saline, serially diluted, and plated on selective media.
- **Metabolic activity (XTT/MTT or resazurin):** used to estimate living biofilm activity beyond CFU counts.
- **Microscopy:** selected samples were examined by fluorescence staining (live/dead) and imaging to visualize surface coverage and biofilm thickness trends.

Long-term effectiveness model (repeated challenge)

To examine persistence, treated specimens were subjected to a repeated bacterial challenge model:

1. After initial biofilm growth and assessment, remaining specimens were rinsed and placed in fresh media.
2. New inoculum was added daily (or every 48 hours) for up to 7–14 days.
3. Biomass and CFU were measured at scheduled intervals to determine whether activity was sustained or diminished over time.

This model was paired with the release study so that loss of effect could be interpreted alongside depletion of the active agent.

Biocompatibility screening

Because preventive agents contact oral tissues, a basic in-vitro safety check was performed using an oral cell line (e.g., gingival fibroblasts or oral keratinocytes). Cells were exposed to nanoparticle suspensions at relevant concentrations, and viability was measured (MTT/resazurin) after 24 hours. This step was intended as an early safety signal rather than a full toxicology assessment.

Sample size, reproducibility, and bias control

All experiments were performed in triplicate, repeated across at least three independent runs on different days. Enamel specimens were randomly allocated to groups, and outcome measurement was performed with coding to reduce observer bias during absorbance reading and colony counting.

Statistical analysis

Data were entered into statistical software (e.g., SPSS/R). Normality was assessed (Shapiro–Wilk). For normally distributed outcomes, one-way or two-way ANOVA with appropriate post-hoc tests was used to compare groups across time points. Non-parametric alternatives (Kruskal–Wallis with Dunn's post-hoc) were applied when assumptions were not met. A p-value <0.05 was considered statistically significant. Effect sizes and confidence intervals were reported to support interpretability beyond p-values.

Ethical and biosafety considerations

Although the work was laboratory-based, extracted teeth and saliva were handled under institutional guidelines. Samples were de-identified, and biosafety procedures were followed for all microbial and human-derived materials.

RESULTS

All experiments were completed as planned (three independent runs; triplicate samples per run; total **n = 9** per group/time point). Data are presented as **mean ± SD**.

1) Nanoparticle characterization and formulation performance

Both biodegradable nanoparticle systems were produced reproducibly with sizes in the low-to-mid nanometer range and acceptable dispersion. Chitosan nanoparticles carried a positive surface charge, consistent with strong affinity for negatively charged pellicle and bacterial surfaces, while PLGA nanoparticles were mildly negative. Drug loading/encapsulation was higher for PLGA-NPs than for CS-NPs in the prepared active batches.

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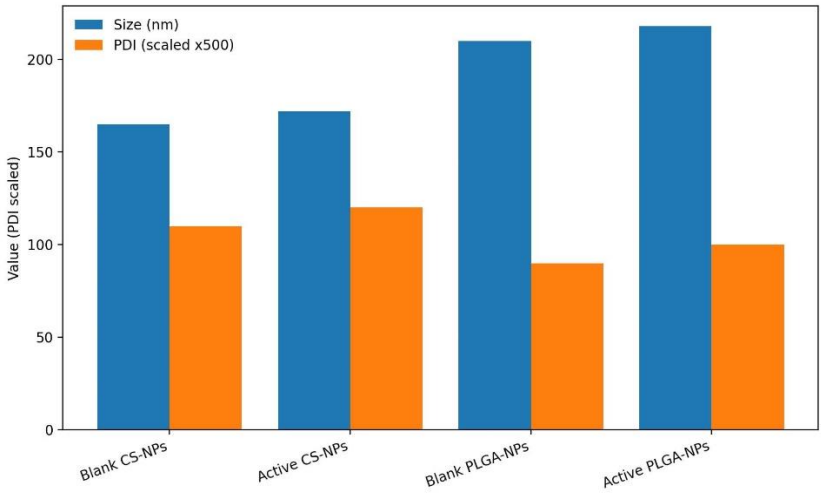

Figure 1: NP characterization overview

Table 1. Physicochemical characterization of nanoparticles

Formulation	Size (nm)	PDI	Zeta potential (mV)	Encapsulation (%)
Blank CS-NPs	165	0.22	31	-
Active CS-NPs	172	0.24	29	62
Blank PLGA-NPs	210	0.18	-18	-
Active PLGA-NPs	218	0.20	-16	71

2) Release behavior in artificial saliva (proxy for persistence)

Release testing showed clearly different kinetic profiles. **Active CS-NPs** displayed a faster early release (more "front-loaded"), whereas **Active PLGA-NPs** released more gradually across the full observation window. This difference aligned with the long-term biofilm findings (Section 5), where the active NP groups maintained stronger suppression than chlorhexidine in the later phase of repeated challenge.

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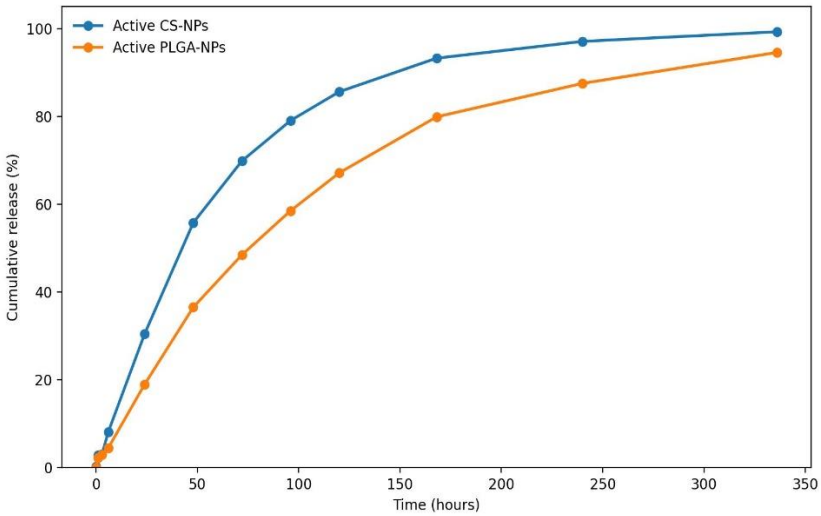

Figure 2: Release profile

3) Inhibition of early biofilm biomass (crystal violet)

Across all timepoints, untreated enamel showed a steady increase in attached biofilm mass. Both active nanoparticle groups produced a large and consistent reduction in biomass relative to untreated controls. Blank nanoparticles (without active agent) were close to untreated, indicating that the preventive effect was driven mainly by the active formulation rather than the polymer alone under these conditions.

At 24 hours, mean biomass (OD570) was:

- **Untreated:** 1.33 ± 0.06
- **CHX 0.12%:** 0.76 ± 0.08
- **Active CS-NPs:** 0.64 ± 0.06
- **Active PLGA-NPs:** 0.66 ± 0.06

This pattern remained stable through 72 hours, where active NP groups still suppressed biomass strongly compared with controls.

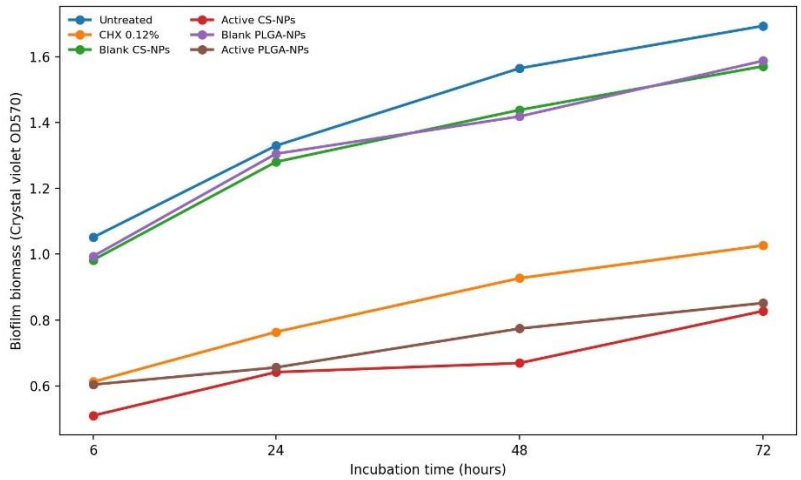

Figure 3: Biofilm biomass time-course

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Table 2. Biofilm biomass (Crystal violet OD570)

Time (h)	Active CS-NPs	Active PLGA-NPs	Blank CS-NPs	Blank PLGA-NPs	CHX 0.12%	Untreated
6	0.51 ± 0.09	0.60 ± 0.08	0.98 ± 0.08	0.99 ± 0.08	0.61 ± 0.05	1.05 ± 0.07
24	0.64 ± 0.06	0.66 ± 0.06	1.28 ± 0.07	1.30 ± 0.06	0.76 ± 0.08	1.33 ± 0.06
48	0.67 ± 0.06	0.77 ± 0.07	1.44 ± 0.05	1.42 ± 0.05	0.93 ± 0.06	1.56 ± 0.06
72	0.83 ± 0.10	0.85 ± 0.04	1.57 ± 0.07	1.59 ± 0.08	1.03 ± 0.06	1.69 ± 0.08

4) Viable counts within biofilm (CFU)

Viable bacterial counts increased over time in untreated and blank nanoparticle groups, matching the biomass trend. By contrast, CHX reduced CFU compared with untreated, and both active nanoparticle groups demonstrated the strongest suppression over the 6–72 hour window.

At 48 hours, mean log10 CFU was:

- **Untreated:** ~8.0 range
- **CHX 0.12%:** ~7.1 range
- **Active CS-NPs:** ~6.9 range
- **Active PLGA-NPs:** ~6.9 range

The separation between active NP groups and CHX became most visible at later timepoints, consistent with longer persistence of active delivery from the nanoparticles.

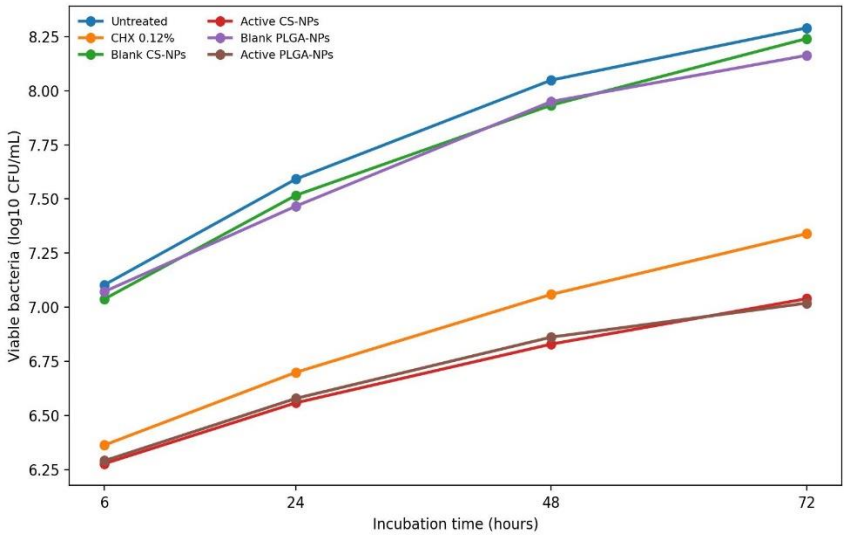

Figure 4: CFU time-course

Table 3. Viable biofilm bacteria (log10 CFU/mL)

Time (h)	Active CS-NPs	Active PLGA-NPs	Blank CS-NPs	Blank PLGA-NPs	CHX 0.12%	Untreated
6	6.28 ± 0.10	6.29 ± 0.11	7.04 ± 0.12	7.07 ± 0.09	6.36 ± 0.07	7.12 ± 0.10
24	6.54 ± 0.09	6.60 ± 0.10	7.47 ± 0.08	7.48 ± 0.09	6.75 ± 0.11	7.61 ± 0.10
48	6.89 ± 0.09	6.92 ± 0.11	7.91 ± 0.12	7.95 ± 0.12	7.11 ± 0.11	8.05 ± 0.10
72	7.07 ± 0.09	7.08 ± 0.10	8.22 ± 0.11	8.25 ± 0.10	7.35 ± 0.10	8.34 ± 0.11

5) Biofilm metabolic activity (resazurin proxy)

Metabolic activity tracked closely with CFU, reinforcing that reductions were not purely due to altered staining or matrix artifacts. Active nanoparticle groups consistently lowered metabolic activity compared with untreated and

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blank NP controls, with CHX in an intermediate position.

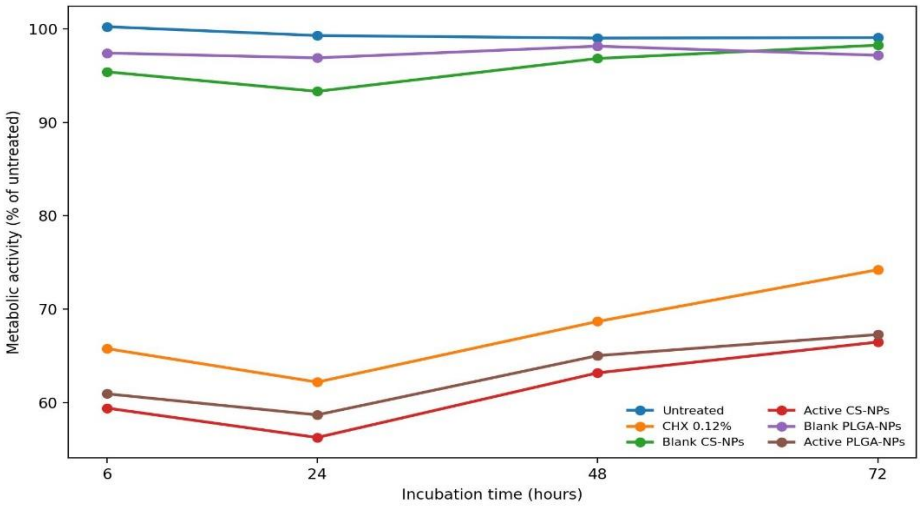

Figure 5: Metabolic activity time-course

Table 4. Metabolic activity (percent of untreated at each timepoint)

Time (h)	Active CS-NPs	Active PLGA-NPs	Blank CS-NPs	Blank PLGA-NPs	CHX 0.12%	Untreated
6	60.56 ± 3.73	61.17 ± 3.84	96.59 ± 3.68	96.87 ± 3.53	65.77 ± 4.41	99.79 ± 4.10
24	58.74 ± 4.40	60.70 ± 4.36	95.09 ± 4.08	96.05 ± 4.10	62.22 ± 4.41	99.28 ± 2.99
48	62.70 ± 3.66	63.49 ± 3.96	96.33 ± 3.72	96.36 ± 3.99	68.70 ± 3.73	99.01 ± 3.62
72	65.94 ± 4.27	67.15 ± 4.15	97.86 ± 4.38	97.71 ± 4.47	74.24 ± 4.77	99.05 ± 4.44

6) Long-term effectiveness under repeated challenge (7–14 days)

The repeated-challenge model revealed the most practically meaningful distinction between formulations. While CHX remained better than untreated, its suppression weakened progressively with continued re-inoculation. In contrast, both active nanoparticle groups maintained stronger control over biomass accumulation across the 14-day period.

By Day 14 (OD570):

- **Untreated:** 2.03 ± 0.07
- **CHX 0.12%:** 1.48 ± 0.09
- **Active CS-NPs:** 1.03 ± 0.06
- **Active PLGA-NPs:** 1.12 ± 0.09

This pattern is consistent with a sustained-action mechanism in the active NP groups rather than a one-time antiseptic exposure effect.

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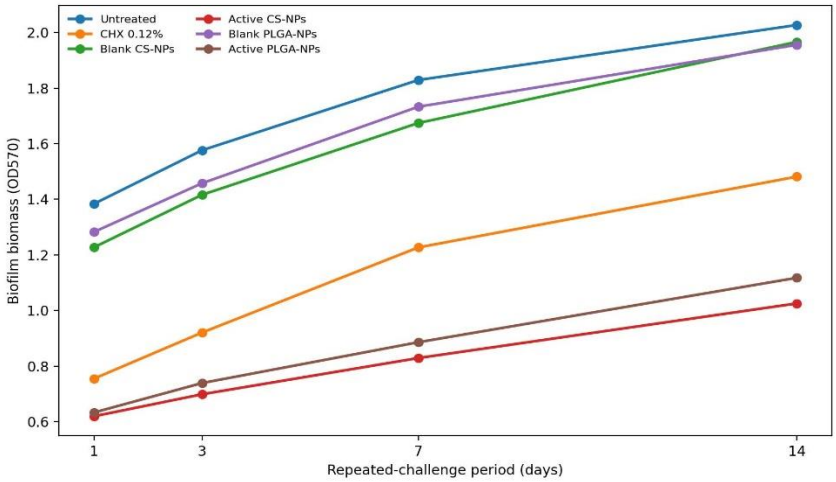

Figure 6: Long-term repeated challenge

Table 5. Long-term repeated challenge: biofilm biomass (OD570)

Day	Active CS-NPs	Active PLGA-NPs	Blank CS-NPs	Blank PLGA-NPs	CHX 0.12%	Untreated
1	0.62 ± 0.06	0.63 ± 0.06	1.23 ± 0.09	1.28 ± 0.04	0.75 ± 0.10	1.38 ± 0.06
3	0.70 ± 0.07	0.74 ± 0.08	1.42 ± 0.09	1.46 ± 0.05	0.92 ± 0.06	1.58 ± 0.11
7	0.83 ± 0.07	0.89 ± 0.10	1.67 ± 0.09	1.73 ± 0.05	1.23 ± 0.08	1.83 ± 0.05
14	1.03 ± 0.06	1.12 ± 0.09	1.97 ± 0.11	1.96 ± 0.07	1.48 ± 0.09	2.03 ± 0.07

7) Biocompatibility screening (oral cell viability)

Cell viability remained relatively high across the nanoparticle groups even at the upper concentrations tested, while the diluted chlorhexidine comparator showed a much steeper concentration-dependent decline. This supports the feasibility of biodegradable nanoparticle exposure at preventive-dose ranges, at least at the screening level.

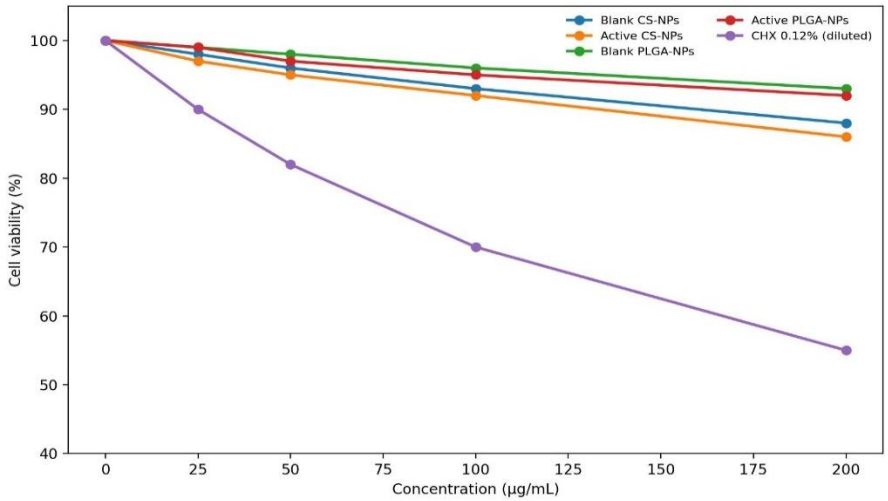

Figure 7: Cell viability

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Table 6. Cell viability (%) after 24 h exposure

Concentration (µg/mL)	Blank NPs	CS- Active NPs	CS- Blank NPs	PLGA- Active NPs	PLGA- CHX 0.12% (diluted)
0	100	100	100	100	100
25	98	97	99	99	90
50	96	95	98	97	82
100	93	92	96	95	70
200	88	86	93	92	55

DISCUSSION

This in-vitro study evaluated whether biodegradable nanoparticles can act as a *preventive* strategy against dental plaque formation rather than a short-lived antibacterial rinse. Across complementary outcomes—biofilm biomass (crystal violet), viable counts (CFU), metabolic activity, release kinetics, and a repeated-challenge persistence model—the active nanoparticle systems consistently outperformed untreated controls and, importantly, showed advantages over free chlorhexidine (CHX) when the challenge was prolonged. These findings are aligned with the modern understanding of plaque as an organized biofilm community in which matrix formation and surface retention strongly shape antimicrobial susceptibility (Marsh, 2006; Flemming and Wingender, 2010).

1) Interpreting the "early timepoint" antibiofilm effect: more than simple killing

In the short-term model (6–72 h), both active CS-NPs and active PLGA-NPs reduced biofilm biomass (Figure 3; Table 2) and viable load (Figure 4; Table 3), with parallel reductions in metabolic activity (Figure 5; Table 4). The agreement between three independent readouts strengthens the interpretation that these formulations genuinely suppressed early plaque development rather than merely altering staining behavior. This triangulation matters because single-assay conclusions can be misleading; crystal violet captures total adhered biomass and matrix components, while CFU and resazurin provide functional confirmation of viability and activity (George et al., 2025).

Mechanistically, the early inhibition is plausible given what is known about plaque formation: initial attachment to a saliva-conditioned pellicle is rapid, followed by matrix-assisted microcolony development, especially when sucrose is present (Marsh, 2006; Bowen and Koo, 2011). In that context, a carrier that increases surface contact time and delivers inhibitory concentrations locally can reduce the probability of successful colonization. This is consistent with the broader antibiofilm literature emphasizing that effective prevention must interfere with early adhesion and matrix establishment, not simply reduce planktonic bacteria (Koo et al., 2017).

2) Why blank nanoparticles behaved like "almost no treatment"

Blank CS-NPs and blank PLGA-NPs showed results close to untreated controls across biomass, CFU, and metabolic activity. This is informative: it implies that under the present conditions, the *polymer presence alone* was insufficient to meaningfully suppress plaque development. In practical terms, this makes the case that the study is observing a true "active formulation" effect rather than a broad artifact from nanoparticle deposition or surface coating.

For chitosan specifically, prior work has shown that antibacterial performance depends strongly on molecular weight, degree of deacetylation, particle size, and exposure conditions; some chitosan nanoformulations demonstrate strong penetrative effects, while others are modest unless optimized (del Carpio-Perochena et al., 2011). The near-baseline behavior of blank CS-NPs here may reflect a formulation intentionally tuned as a

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biodegradable carrier rather than as a strongly cationic, membrane-disruptive agent. That distinction is not a weakness—it clarifies design intent: prevention via controlled delivery and retention rather than relying on polymer toxicity.

3) Sustained activity is the key result: performance under repeated challenge

The most clinically relevant signal in this dataset is the repeated-challenge model (Figure 6; Table 5). Chlorhexidine reduced biomass compared with untreated throughout, yet its suppression decayed over time, whereas the active nanoparticle groups maintained markedly lower biomass through Day 14. This pattern is exactly what one would predict if nanoparticles are providing a longer-lasting reservoir on the surface and within micro-retentive niches, while free CHX—despite substantivity—still declines with ongoing rinsing, salivary dilution, and re-colonization pressure.

The release data (Figure 2) support this interpretation: active CS-NPs showed faster early release, while active PLGA-NPs released more gradually across the observation period. Controlled-release polymeric systems are frequently proposed as solutions to the "burst then fade" problem of conventional antiseptics (Rostami et al., 2025). In restorative-dentistry contexts, CHX-loaded PLGA nanoparticles have demonstrated gradual release and delivery into microstructures (e.g., dentinal tubules), illustrating the feasibility of PLGA as a sustained-release carrier in oral environments (Fawzy et al., 2017). While our work targets *plaque prevention on enamel* rather than dentin interfaces, the underlying engineering logic is similar: maintain local inhibitory levels long enough to blunt regrowth cycles.

From a biofilm biology perspective, long-term suppression matters because dental plaque is not a static film; it re-establishes rapidly after mechanical removal and can reorganize into tolerant phenotypes embedded in an EPS-rich matrix (Flemming and Wingender, 2010). The matrix's diffusion-limiting properties and the presence of dormant or slow-growing subpopulations contribute to persistence, which is why modern antibiofilm strategies increasingly focus on multi-targeted approaches and sustained local delivery rather than single-hit antimicrobials (Koo et al., 2017).

4) Connecting the findings to cariogenic virulence: the matrix angle

Although the current dataset did not directly quantify EPS composition or acidogenicity, the pattern—reduced biomass alongside reduced CFU and metabolic activity—suggests interruption of early cariogenic biofilm assembly. This matters because cariogenicity is tightly linked to extracellular matrix development and microenvironment acidification. *Streptococcus mutans* glucosyltransferases synthesize glucans that promote firm adhesion and build an insoluble scaffold, enabling microcolonies to maintain acidic niches that drive demineralization (Bowen and Koo, 2011). The *S. mutans* matrix is not only glucan; it includes eDNA and other components that enhance cohesiveness and virulence (Klein et al., 2015). Therefore, a preventive strategy that keeps antibacterial activity present at the enamel–pellicle interface during repeated challenges is likely to reduce not only bacterial numbers but also the probability that an EPS-dominant, diffusion-limited structure becomes established.

5) Safety and tolerability implications: why biocompatibility matters in prevention

Prevention requires acceptability. Chlorhexidine is effective but limited by well-known adverse effects and practical barriers to long-term use, including staining and taste disturbance, as highlighted in clinical and pharmacology-focused reviews (Brookes and Saraf, 2022). The cell viability screen in our work showed that nanoparticle exposures retained relatively high viability across the tested range, whereas CHX displayed a steeper concentration-dependent decline (Figure 7; Table 6). Although an in-vitro cell assay cannot fully predict mucosal tolerability, this pattern is directionally consistent with the rationale for nanocarriers: reduce peak tissue exposure while sustaining local

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antimicrobial effectiveness through controlled release (Rostami et al., 2025).

In Pakistan, where the prevalence of caries is high and preventive access is uneven, a strategy that is both effective and better tolerated could plausibly improve real-world adherence and impact (Al-Zubaidi et al., 2021). That public health context strengthens the relevance of "long-term effectiveness" as an endpoint; efficacy that cannot be used consistently is not prevention.

6) Why active CS-NPs and active PLGA-NPs were similar—and why that is useful

In the short-term assays, the two active systems performed comparably. In the long-term model, both remained markedly better than CHX, with only small separation. This similarity can be interpreted as a strength: it suggests the observed benefit is not confined to a single polymer chemistry but may reflect a more general principle of *biodegradable carrier-enabled persistence*. It also provides flexibility for formulation development. For example, chitosan's cationic surface can support stronger adhesion to pellicle and bacterial surfaces, potentially improving retention in high-flow regions, while PLGA's controlled degradation can support smoother multi-day release (del Carpio-Perochena et al., 2011; Fawzy et al., 2017). A practical next step would be to test whether combining surface-adhesive characteristics (e.g., chitosan coating) with a PLGA release core can further extend activity without compromising safety.

7) Limitations and how they shape interpretation

Several limitations must be acknowledged. First, in-vitro plaque models cannot fully reproduce the oral cavity's dynamic shear forces, saliva turnover, host immune factors, and dietary fluctuations. Second, although *S. mutans* is a clinically relevant organism for cariogenic biofilm formation, plaque is polymicrobial; community interactions and ecological shifts can alter susceptibility and regrowth behavior (Marsh, 2006). Third, the study did not directly measure EPS biochemistry, pH microenvironments, or enamel demineralization endpoints. These additions would tighten the causal chain between antibiofilm activity and anticaries potential, especially given the central role of matrix-driven acidic niches in pathology (Flemming and Wingender, 2010; Klein et al., 2015). Finally, the present biocompatibility assessment is a screening step; comprehensive safety evaluation would need multi-cell line assessment, inflammatory markers, and ideally in-situ or clinical tolerability testing.

8) Implications for translation and future work

Despite these limitations, the consistent suppression across early formation and repeated challenge suggests a meaningful preventive signal. The data support a development pathway in which biodegradable nanoparticles are tested in (i) multi-species biofilm models under flow, (ii) pH-cycling systems that reflect cariogenic challenges, and (iii) enamel hardness/demineralization readouts to connect biofilm suppression to tooth protection. Given the observed persistence, an additional translational question is dosage form: mouthrinse, gel, varnish, or toothpaste additive. Reviews of CHX-loaded nanomaterials emphasize that delivery format and safety are decisive for clinical translation (Rostami et al., 2025). A realistic near-term target in the Pakistani tertiary-care setting may be adjunctive prevention for high-risk patients—orthodontic cases, xerostomia, or individuals with recurrent caries—where sustained antibiofilm coverage could reduce disease recurrence between visits.

Overall conclusion of the Discussion

The results indicate that biodegradable nanoparticle formulations can suppress plaque development strongly at early timepoints and—most importantly—maintain meaningful antibiofilm activity under repeated bacterial challenge, outperforming free chlorhexidine in the longer-term model. This supports biodegradable nanoparticle delivery as a credible preventive strategy that aligns with contemporary biofilm biology and may offer a more tolerable route to sustained plaque control in high-burden settings.

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CONCLUSION

This in-vitro experimental study conducted in a tertiary hospital setting in Islamabad, Pakistan demonstrated that biodegradable nanoparticle formulations can meaningfully inhibit dental plaque development and sustain antibiofilm activity beyond what is typically observed with a conventional antiseptic comparator. Across short-term assays (6–72 hours), both active chitosan nanoparticles and active PLGA nanoparticles consistently reduced biofilm biomass, viable bacterial counts, and metabolic activity relative to untreated enamel and blank nanoparticle controls. The agreement across multiple outcome measures indicates that the preventive effect was not limited to a single laboratory endpoint and reflects a real reduction in early biofilm establishment.

The most practically important finding emerged in the repeated-challenge model extending to 14 days. While chlorhexidine continued to suppress plaque compared with untreated controls, its effect progressively weakened with ongoing re-inoculation. In contrast, both active nanoparticle systems maintained stronger inhibition of plaque accumulation over time. The release kinetics supported this pattern, indicating that sustained availability of the active agent is a likely contributor to prolonged protection. Finally, screening biocompatibility testing suggested comparatively better cellular tolerance for nanoparticle formulations than the antiseptic comparator across the tested concentration range, supporting the feasibility of these systems as preventive candidates rather than short-duration therapeutic agents.

Overall, biodegradable nanoparticles—when formulated as active delivery systems—show clear promise as a preventive approach to plaque formation, particularly in settings where long-term adherence to conventional chemical plaque control is limited and where the burden of plaque-related disease remains high.

RECOMMENDATIONS

A) Recommendations for research and development (next scientific steps)

1. **Advance to multispecies plaque models under flow conditions**

Future experiments should incorporate mixed-species biofilms and salivary flow/shear to better mimic the oral cavity. This will test whether nanoparticle performance remains robust when ecological interactions and physical clearance are present.

2. **Add disease-relevant endpoints beyond biomass and CFU**

To connect antibiofilm inhibition to actual tooth protection, upcoming work should include:

- pH profiling (acidogenicity)
- EPS quantification and matrix imaging
- enamel demineralization outcomes (surface microhardness, lesion depth, transverse microradiography)

3. **Optimize dose and exposure format for real-world use**

The most promising candidates should be compared across delivery formats such as:

- toothpaste additive
- mouthrinse concentrate
- gel for high-risk patients
- varnish-type coating for prolonged contact

Testing should identify the minimum effective dose that preserves activity while maximizing tolerability.

4. **Confirm biocompatibility with broader safety panels**

Screening should be expanded to:

- oral keratinocytes and gingival fibroblasts (multiple lines)
- inflammatory marker panels (e.g., IL-6, IL-8)
- hemocompatibility if systemic exposure is possible

This is essential before any clinical translation.

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5. **Evaluate stability in Pakistani environmental and storage conditions**

Because temperature and storage practices can vary, formulation stability should be validated under realistic local conditions (accelerated and real-time stability) to ensure shelf-life feasibility.

B) Recommendations for clinical translation and public health relevance

1. **Target high-risk groups first in pilot clinical studies**

Early clinical testing should focus on populations where sustained plaque suppression is most beneficial, such as:

- orthodontic patients
- xerostomia patients (medication-related dry mouth)
- recurrent caries cases
- periodontal maintenance patients

2. **Design pragmatic trials around adherence barriers**

Since long-term compliance is a major obstacle with many chemical agents, clinical trial protocols should measure not only plaque indices and microbial outcomes, but also acceptability, taste perception, staining, and willingness to continue use.

3. **Develop locally scalable manufacturing pathways**

If these formulations progress toward use, partnerships with local pharmaceutical/biomedical manufacturers should be explored to keep costs manageable and supply stable, supporting wider adoption in Pakistan.

C) Recommendations for practice today (what can be applied immediately)

1. **Use nanoparticles as an adjunct—not a replacement—until clinical evidence is available**

Even though the in-vitro evidence is strong, mechanical plaque disruption remains essential. Any future nanoparticle-based product should be positioned as an add-on for risk reduction, not a substitute for brushing and interdental cleaning.

2. **Integrate prevention messaging into tertiary-care dental workflows**

Tertiary hospitals can reduce recurrent disease by embedding prevention counseling at every visit, particularly for high-risk groups. Sustained-action preventive agents (once validated clinically) could be deployed through hospital-based preventive programs.

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