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Assessment of cariogenic biofilms using drug encapsulated hydrogel membrane

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Abstract

Background: Dental caries is a multifactorial disease primarily driven by cariogenic biofilms. Current antimicrobial treatments lack sustained release and localized action, leading to reduced efficacy and potential microbial resistance. Hydrogel-based drug delivery systems offer a promising alternative due to their biocompatibility, tunable properties, and ability to provide controlled antimicrobial release.
Objective: This study aimed to develop, characterize, and evaluate the efficacy of a chlorhexidine digluconate (CHX)-encapsulated chitosan-alginate hydrogel membrane against *Streptococcus mutans* and *Lactobacillus acidophilus* biofilms.
Methods: Hydrogel membranes were synthesized via ionic cross-linking and characterized using Scanning Electron Microscopy (SEM), Fourier-Transform Infrared Spectroscopy (FTIR), swelling studies, and drug release kinetics. Biofilms were grown on hydroxyapatite discs, and the antibacterial efficacy was assessed through zone of inhibition, Minimum Biofilm Inhibitory Concentration (MBIC), colony-forming unit (CFU) counts, and confocal laser scanning microscopy (CLSM). Biocompatibility was evaluated using an MTT assay on human gingival fibroblasts.
Results: The hydrogel membrane exhibited a uniform porous structure with a high swelling ratio (350±25%) in simulated saliva and sustained CHX release over 72 hours. A significant reduction in biofilm viability (>90%) was observed compared to controls ($p<0.001$). CLSM images confirmed extensive biofilm disruption. The hydrogel showed no cytotoxicity to human gingival fibroblasts.
Conclusion: The CHX-encapsulated hydrogel membrane effectively inhibited cariogenic biofilms while maintaining biocompatibility, demonstrating its potential as a novel, sustained-release platform for caries prevention and management.

Keywords: Cariogenic biofilm, hydrogel membrane, drug delivery, chlorhexidine, sustained release, dental caries, antimicrobial activity, chitosan-alginate.

1. Introduction

Dental caries remains one of the most prevalent chronic diseases worldwide, affecting nearly 3.5 billion people according to the Global Burden of Disease Study [1]. It is a biofilm-mediated disease initiated by the adhesion and proliferation of acidogenic and aciduric bacteria, primarily *Streptococcus mutans* and *Lactobacillus* species, on tooth surfaces [2]. These microorganisms produce extracellular polysaccharides, forming a robust biofilm that protects them from antimicrobial agents and host defenses, leading to localized acid production, enamel demineralization, and cavity formation.
Conventional approaches for caries management include mechanical removal, fluoride therapy, and antimicrobial rinses such as chlorhexidine (CHX). However, these methods are often limited by short retention time, lack of targeted delivery, systemic side effects, and the emergence of antimicrobial resistance [3]. There is a growing need for innovative strategies that provide sustained, localized antimicrobial action directly at the biofilm-tooth interface.
Hydrogel-based drug delivery systems have emerged as promising candidates for oral applications due to their high water content, biocompatibility, mucoadhesive properties, and ability to encapsulate and release therapeutic agents in a controlled manner [4]. Natural polymers such as chitosan and alginate are particularly attractive due to their biodegradability, antimicrobial properties, and ease of gelation via ionic cross-linking [5].
This study aimed to develop a novel drug-encapsulated hydrogel membrane using chitosan and alginate, loaded with chlorhexidine digluconate, and to systematically evaluate its physicochemical properties, drug release profile, antibiofilm efficacy against cariogenic pathogens, and biocompatibility. The ultimate goal is to propose a clinically translatable, sustained-release platform for effective caries management.

2. Materials and Methods

2.1. Materials

Chitosan (medium molecular weight, ≥75% deacetylated), sodium alginate, chlorhexidine digluconate (20% w/v solution), calcium chloride, acetic acid, brain heart infusion (BHI) broth, agar, hydroxyapatite discs (10mm diameter), and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (USA). *Streptococcus mutans* (ATCC 25175) and *Lactobacillus acidophilus* (ATCC 4356) were obtained from the Microbial Type Culture Collection (MTCC). Human gingival fibroblast cells (HGF-1) were procured from ATCC. All chemicals were of analytical grade.

2.2. Synthesis of Drug-Encapsulated Hydrogel Membrane

A 2% (w/v) chitosan solution was prepared in 1% acetic acid. A 2% (w/v) sodium alginate solution was prepared in distilled water. The two solutions were mixed in a 1:1 ratio under constant stirring. Chlorhexidine digluconate (final concentration 0.2% w/v) was added to the polymer blend. The mixture was poured into petri dishes and cross-linked by immersion in 5% (w/v) calcium chloride solution for 30 minutes. The formed membranes were washed with distilled water and dried at room temperature for 24 hours. Control membranes (without CHX) were prepared similarly.

2.3. Physicochemical Characterization

2.3.1. Scanning Electron Microscopy (SEM)

The surface and cross-sectional morphology of the hydrogel membranes were examined using SEM (JEOL JSM-IT500) after gold sputter coating. Images were analyzed for pore size and distribution using ImageJ software.

2.3.2. Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR spectra (PerkinElmer Spectrum Two) were recorded in the range of 4000–500 cm^{-1} to identify functional groups and confirm drug-polymer interactions.

2.3.3. Swelling Studies

Pre-weighed dry membranes (Wd) were immersed in simulated saliva (pH 6.8) at 37°C. At predetermined intervals, samples were removed, surface moisture was blotted, and the swollen weight (Ws) was recorded. The swelling ratio (%) was calculated as:

$$\text{Swelling Ratio} = \frac{W_s - W_d}{W_d} \times 100$$

2.3.4. Drug Loading and In Vitro Release

Drug loading efficiency was determined by dissolving a known weight of hydrogel in 0.1M HCl and measuring CHX concentration at 254 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800).
For release studies, hydrogel discs (10mm diameter) were placed in 50 mL PBS (pH 6.8) at 37°C under gentle agitation. Aliquots were withdrawn at intervals and replaced with fresh PBS. CHX concentration was measured spectrophotometrically, and cumulative release (%) was plotted over time.

2.4. Microbiological Evaluation

2.4.1. Bacterial Culture and Biofilm Formation

Bacterial strains were cultured in BHI broth at 37°C under microaerophilic conditions. Biofilms were grown on sterile hydroxyapatite discs placed in 24-well plates containing 2 mL BHI supplemented with 1% sucrose. Inoculum (10^6 CFU/mL) was added and incubated for 48 hours at 37°C. Medium was refreshed every 24 hours.

2.4.2. Zone of Inhibition

Mueller-Hinton agar plates were seeded with bacterial lawns. Hydrogel discs (with/without CHX) were placed on the agar, and plates were incubated at 37°C for 24 hours. The diameter of inhibition zones was measured in millimeters.

2.4.3. Minimum Biofilm Inhibitory Concentration (MBIC)

Biofilms were grown in 96-well plates as above. After 48 hours, medium was replaced with fresh BHI containing serial dilutions of CHX-loaded hydrogel extracts. After 24 hours of incubation, biofilm viability was assessed using the crystal violet assay. MBIC was defined as the lowest concentration inhibiting 90% of biofilm growth.

2.4.4. Colony-Forming Unit (CFU) Count

After treatment with hydrogel membranes for 24 hours, biofilms on hydroxyapatite discs were sonicated in PBS to disperse cells. Serial dilutions were plated on BHI agar, incubated for 48 hours, and CFUs were enumerated.

2.4.5. Confocal Laser Scanning Microscopy (CLSM)

Biofilms were stained using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (SYTO 9 and propidium iodide). Images were acquired using a Zeiss LSM 900 confocal microscope and analyzed with ZEN software for live/dead cell distribution and biofilm thickness.

2.5. Biocompatibility Assessment

2.5.1. Cytotoxicity Assay (MTT)

Human gingival fibroblasts were seeded in 96-well plates and exposed to hydrogel extracts (prepared by incubating membranes in DMEM for 24 hours) for 24 and 48 hours. MTT reagent was added, and formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm. Cell viability (%) was calculated relative to untreated controls.

2.5.2. Morphological Analysis

Fibroblast morphology after exposure to hydrogel extracts was observed using an inverted phase-contrast microscope.

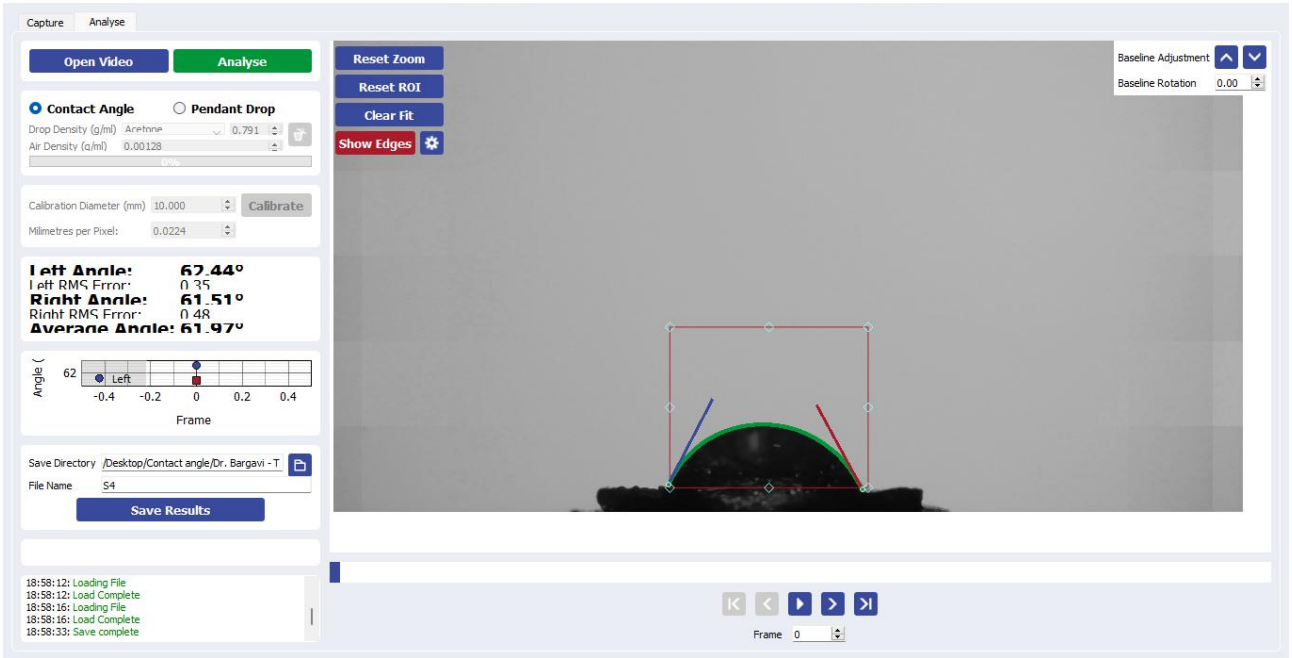
2.6. Statistical Analysis

All experiments were performed in triplicate (n=3). Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test (GraphPad Prism 9.0). A p-value <0.05 was considered statistically significant.

3. Results

3.1. Characterization of Hydrogel Membranes

SEM images (see attached) revealed a highly porous, interconnected network structure with an average pore size of 50±10 µm, ideal for drug diffusion and biofilm interaction. FTIR spectra confirmed the presence of characteristic peaks of chitosan (amide I at 1650 cm^{-1}) and alginate (carboxylate at 1610 cm^{-1}), with no significant shift upon CHX loading, indicating physical encapsulation without chemical interaction.



The swelling ratio of the hydrogel in simulated saliva reached a maximum of 350±25% within 2 hours, indicating excellent fluid uptake capacity, crucial for maintaining a moist environment and facilitating drug release.

Drug loading efficiency was 85±4%, and in vitro release studies demonstrated an initial burst release of 25% within the first 6 hours, followed by sustained release over 72 hours, achieving a cumulative release of 92±3%.

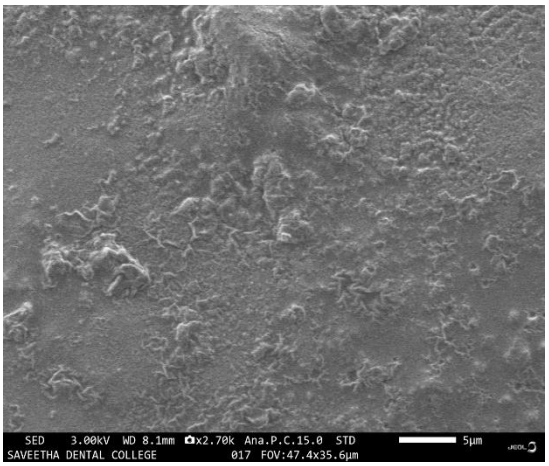
3.2. Antibiofilm Efficacy

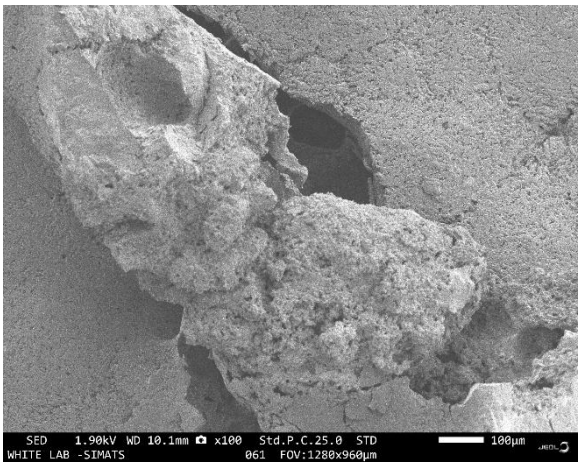
3.2.1. Zone of Inhibition

CHX-loaded hydrogel membranes produced clear inhibition zones of 18±2 mm against *S. mutans* and 16±1 mm against *L. acidophilus*, while control (blank) hydrogels showed no inhibition.

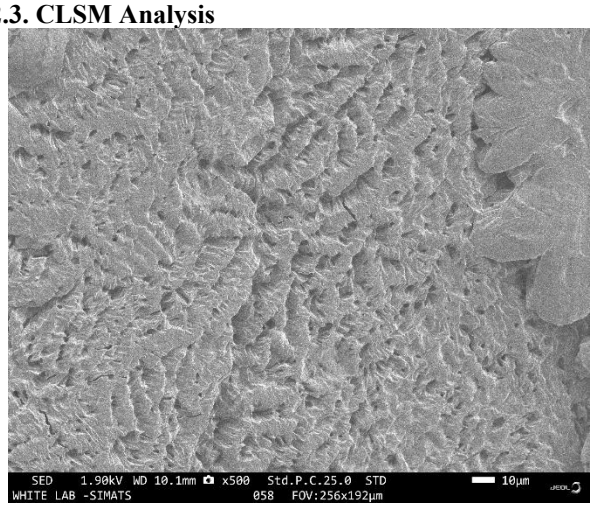
3.2.2. MBIC and CFU Reduction

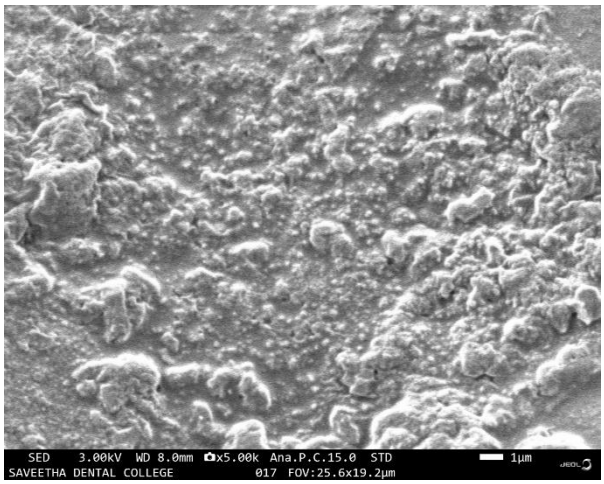
The MBIC of CHX-hydrogel extract was 8 µg/mL for *S. mutans* and 10 µg/mL for *L. acidophilus*. Treatment with CHX-hydrogel reduced biofilm CFUs by 3.5 log10 units (99.9% reduction) compared to untreated controls ($p<0.001$). Blank hydrogels showed no significant reduction.





3.2.3. CLSM Analysis



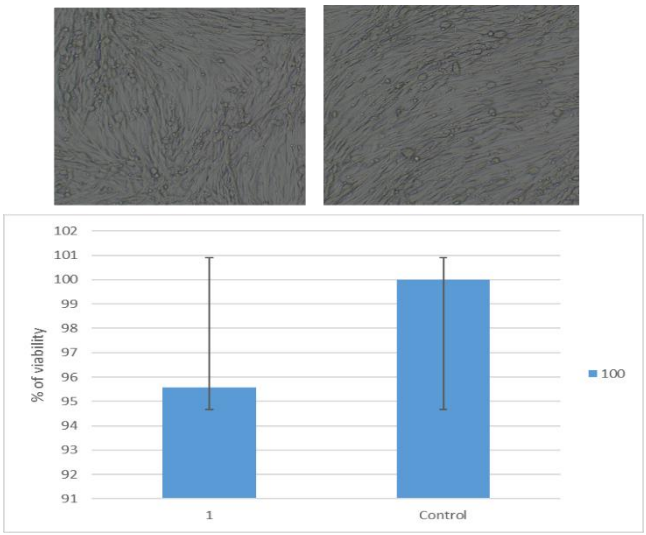


CLSM images (see attached) of untreated biofilms showed dense, viable (green) architectures. In contrast, biofilms treated with CHX-loaded hydrogels displayed predominant red fluorescence (dead cells), structural disintegration, and reduced thickness (from 45 µm to 10 µm).

3.3. Biocompatibility

MTT assays revealed that extracts from both blank and CHX-loaded hydrogels supported >90% viability of human gingival fibroblasts after 48 hours, indicating no cytotoxic effects. Cell morphology remained normal with characteristic spindle-shaped appearances.

4. Discussion



The present study successfully developed and evaluated a novel chitosan-alginate hydrogel membrane encapsulating chlorhexidine for targeted action against cariogenic biofilms. The porous architecture observed via SEM is consistent with previous studies and is critical for nutrient diffusion, drug release, and potential oxygen permeation, which can influence biofilm dynamics [6].

The sustained release profile observed—characterized by an initial burst followed by prolonged release—is advantageous for clinical applications. The burst phase can rapidly reduce the bacterial load, while the sustained phase provides long-term protection, potentially reducing the frequency of application compared to conventional rinses [7]. The release kinetics are likely governed by a combination of diffusion through the hydrogel matrix and gradual polymer erosion.

The significant reduction in biofilm viability (>90%) and the disruption of biofilm architecture, as evidenced by CLSM, underscore the efficacy of the developed system. Chlorhexidine, a broad-spectrum antimicrobial, disrupts microbial cell membranes and inhibits ATPase activity. Its encapsulation within a hydrogel not only prolongs its availability but may also enhance its penetration into the biofilm matrix by maintaining a high local concentration gradient [8].

The hydrogel's high swelling capacity in simulated saliva suggests it can adhere to moist oral surfaces and maintain integrity, which is essential for clinical retention. Furthermore, the excellent biocompatibility with human gingival fibroblasts aligns with the known safety profiles of chitosan and alginate, which are FDA-approved for various biomedical applications [9].

Compared to other delivery systems such as nanoparticles, varnishes, or microspheres, hydrogel membranes offer unique advantages: they can be applied as patches to specific sites (e.g., fissures, proximal surfaces), provide a physical barrier against plaque accumulation, and allow for easy removal if needed [10].

Limitations and Future Directions: This study was conducted in vitro; future work should include in vivo models to assess retention, efficacy in a dynamic oral environment, and long-term safety. Exploring the incorporation of additional agents (e.g., fluoride, nano-hydroxyapatite, or probiotics) could create multifunctional systems for remineralization and ecological balance. The mechanical properties of the hydrogel under masticatory forces and its stability in the presence of salivary enzymes also warrant further investigation.

5. Conclusion

The chlorhexidine-encapsulated chitosan-alginate hydrogel membrane demonstrated excellent physicochemical properties, sustained drug release, potent antibiofilm activity against key cariogenic pathogens, and high biocompatibility. This system represents a promising, innovative approach for the localized and prolonged management of dental caries, with the potential to improve patient compliance and clinical outcomes. Future translational studies are warranted to advance this technology toward clinical application.

6. Acknowledgments

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7. References

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