DEVELOPING AN ANTIBIOTIC-INFUSED PERIODONTAL FILM FOR EARLY INTERVENTION OF PERIODONTITIS

By

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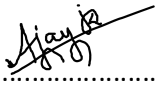
ABSTRACT

Globally about one billion people suffer from periodontitis. Early intervention prevents the progression of periodontitis. In this research, the antimicrobial activity of the metronidazole-loaded Poly (ε-caprolactone (PCL) thin films was aimed at the anaerobic bacteria (*P. gingivalis*) isolated from the close-flap periodontal pocket and vancomycin-loaded PCL thin film for aerobic bacteria (*S. aureus*) isolated from an open-flap periodontal pocket. Medium molecular weight (48 kDa) PCL, tween 20, and vancomycin or metronidazole were used for the fabrication of the films. The vancomycin films gave a good zone of inhibition (ZOI) with and without the addition of silk sericin. Similarly, a good zone of inhibition was observed with metronidazole films with and without silk sericin. The addition of silk sericin was to get a better slow release of antibiotics from the films. The aggregate release of vancomycin from film having silk sericin after 72 hours was 42.2% versus 77.7% for vancomycin without silk sericin. There was a 26.6 percent release of metronidazole without silk sericin as against 10.38 percent with silk sericin. In live/dead assay, cell viability was increased when comparing the silk sericin and control groups showing controlled antibiotic release, while more than 90% of bacteria were killed. Modified PCL films had reductions of 3-log CFU/mL compared to plain PCL films. Human gingival fibroblast viability for vancomycin and metronidazole films with silk sericin was at 104% and 101%, whereas that for plain PCL films was at 93.8%. At 72hr, mass loss was 10.71% for film vancomycin with silk sericin in lipase while in control, the mass loss was 1.52%. The lipase induced four and a half percent metronidazole thin film at 72 hours compared with the one percent in PBS alone. The water contact angle decreased from 85.4° for plain PCL films to 54.6° for vancomycin films and 52.7° for metronidazole films, reflecting increased hydrophilicity. To conclude PCL films loaded with antibiotics and silk sericin demonstrate periodontal disease intervention through controlled drug release and tissue compatibility.

DECLARATION

I confirm that this thesis:

- Contains no material from previous degree or diploma submissions from other universities unless the originals are referenced adequately.
- No part of this research will be presented for any subsequent degree or diploma at Flinders University unless it is approved first by Flinders University.
- As far as it is possible to understand, there are no previously published works of another author here apart from those cited in the text.



Signed..... Ajay Jose

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LIST OF ABBREVIATIONS

Abbreviation	Full name
CFU	Colony forming unit
EMP	Enamel matrix derivatives
HGF	Human gingival fibroblasts
LC	Loading capacity
MBC	Minimum bactericidal concentration
Met	Metronidazole
MIC	Minimum inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
PCL	Polycaprolactone
SRP	Scaling and root planing
SS	Silk sericin
Van	Vancomycin
ZOI	Zone of inhibition

CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

Periodontitis is a type of severe gum infection that can damage bone and soft tissue supporting the teeth. Plaque is bacteria that accumulates on the teeth and leads to staining. Infected by gum disease bacteria, the periodontal ligament and supporting alveolar bone will progressively get destroyed over time (Jain et al., 2008). Clinically visible attachment loss represents the main clinical characteristic that distinguishes periodontitis from gingivitis. This means that the connective tissue fibers that bind the tooth to the bone have been lost, together with the gum pushing off the teeth to reveal the root's surface. In 2022, the WHO revealed that about 1 billion people suffers from periodontitis. This accounts nearly 19% of the global adult population (Baldi et al., 2023). However, approximately 75% of Australians aged 15 years and over suffer from moderate to severe periodontitis according to the recent National Study of Adult Oral Health (NSAOH) 2017-2018 (Ju et al., 2023, Ellershaw et al., 2020).

However, periodontitis should also be noted as a type of bacterial infection despite such factors as alcoholism, diabetes mellitus, obesity, and smoking all being risks for it (Nascimento et al., 2017). The main reason for periodontitis is the presence of calculus tartar and dangerous bacteria in the gums because of the accumulation of plaque on the teeth surface. Smoking, uncontrolled diabetes and obesity are the possible risk factors for the aggravation and progression of periodontitis. Smoking which is one of the major risk factors that affect the immune response, reduce blood supply to the gums and inhibit healing. Another complication of uncontrolled diabetes is having high glucose in the saliva, and this leads to an increase in the multiplication of bacteria and aggravated periodontitis. Obesity, leading to chronic inflammation and compromised immune function, increases the chances of developing periodontitis (Xu and Duan, 2020).

1.2. Pathogenic mechanism of periodontitis

Pathogenesis is a complex process that occurs in various stages leading to destruction of periodontal tissues and the tooth support structures (Tonetti et al., 2018). Periodontal disease starts with the formation of bacteria plaque on the tooth's surface. Bacteria colonize the mouth lining and stick onto the tooth surface, resulting in the formation of this plaque. Plaque is considered a biofilm, and the residing bacteria are embedded in an extracellular polymeric

substance (EPS) (Cardoso et al., 2018, Graves, 2008). The bacteria attach themselves to teeth surface through this matrix in a way that makes it difficult for removal using the mechanical forces of brushing or rinsing. Dental plaque matrix contains polysaccharides, protein, lipids, nucleic acids, and the EPS matrix (Graves, 2008). Bacteria produce polysaccharides, which are polymers of glucose-based molecules necessary for maintaining a biofilm's structural integrity. The matrix also includes some of the host-derived compounds from saliva and gingival crevicular fluids that provide nutrients to the bacteria as well as contribute to the EPS structure. The host initiates an immune response after the bacteria plaque comes to exist in it. This response involves the release of cytokines such as IL-1, and TNF the alpha that come from the immune system for the site of infection leading to the formation of the immune cells (Graves, 2008). These include neutrophils and macrophage immune cells that attempt to get rid of the bacteria in the infection site of attack. This results into chronic inflammatory process due to continued formation of bacterial plaques (Cardoso et al., 2018). Chronic inflammation, which is a prolonged immune activity, may result in damage to the surrounding periodontal tissues. In their attempts to eliminate bacteria, immune cells can result into production of enzymes and ROS that damage the surrounding tissues (Bartold et al., 2020). Moreover, the immune response can also increase osteoclast activity, causing a breakdown of the alveolar bone to support the tooth. The gingival tissues gradually break down, allowing formation of pockets that enable bacteria to spread further into such tissues. In severe cases, this disease could lead to loosing of teeth supports structure due to lack of enough alveolar bone supports. The link between periodontitis with other systemic diseases such as rheumatoid arthritis, heart disease, and adverse pregnancy outcomes is attributed to the underlying inflammatory component of periodontal disease (Balta et al., 2017, Zhang et al., 2021, Liccardo et al., 2019). This can worsen inflammation in other parts.

1.3. Stages of Periodontitis

Periodontitis is a condition that is divided into several stages depending on its severity and extent of spread. In initial periodontitis radiographic bone loss less than 15% of the root length, max. probing depth less than 4 mm and clinical attachment loss of maximum. 2 mm. It could also present bleeding on probing as well as inflammation. Moderate Periodontitis has radiographic bone loss between 15% and 33% of the root length, a maximum probing depth

between 4 and 6 mm, clinical attachment loss (AL) of a minimum of 3 mm. There will also be deepening of periodontal pockets and recession of gingiva (table 1).

Severe Periodontitis is considered a state where radiographic bone loss of more than 33% of the root, probing depth of 6 mm maximum and 5 mm or more of clinical attachment loss occurs. Mobility of tooth and furcation involvement might also be noted. At the last stage, radiographic bone loss goes to the middle or apical one-third and tooth loss is highly probable and there is approximately 5 mm of clinical attachment loss. Due to the huge amount of tissue and bone loss, the probing depth cannot be determined. This understanding is very important in the diagnosis and treatment of periodontitis (Tonetti et al., 2018). This shows how important it is to detect the periodontitis as early as possible so that they do not advance with their consequent complications.

Table 1: Stages of periodontitis (Tonetti et al., 2018).

Stage i	Stage ii	Stage iii	Stage iv
Initial periodontitis	Moderate periodontitis	Severe periodontitis.	Advanced periodontitis.
Maximum probing depth is ≤4mm	Maximum probing depth is ≤5mm.	Maximum probing depth is ≥6mm.	----
Clinical attachment loss is 1-2mm	Clinical attachment loss is 3-4mm.	Clinical attachment loss is ≥5mm.	Clinical attachment loss is ≥5mm.
No tooth loss	No tooth loss.	Tooth loss	Tooth loss

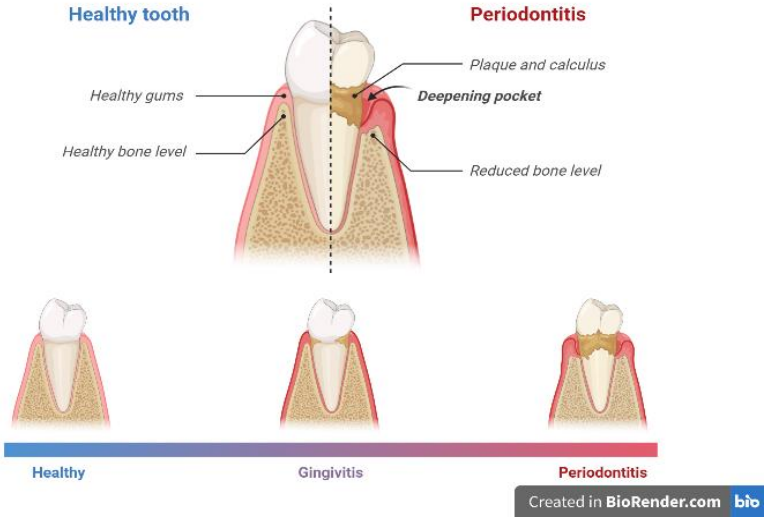


Figure 2: Comparison of diseased and healthy periodontal tissue, showing the periodontal pocket (Image made with BioRender).

The early detection and treatment of periodontitis are very important since it is a severe gum disease, which has serious consequences. Inflammation and infection of the tissues that support teeth can result in tooth loss, impaired oral function, and systemic health complications such as heart disease and diabetes. Early intervention is not only helpful in terms of preventing tooth loss, maintaining oral function, and reducing discomfort; it also proves to be more cost effective than using complex and expensive treatments (Ramenzoni et al., 2021).

1.4. Microbiota associated with periodontal diseases.

The microbiotas that are characteristic for periodontal disease consists of a decrease in Gram positive bacterial profile with a growth in pathogenic species like *Treponema denticola, Aggregatibacter actinomycetemcomitans*. Due to their capability to attack the periodontal tissues, produce virulence factors and trigger inflammation, these pathogens are regarded as some of the major contributors to the development of periodontitis (Jiang et al., 2021a). The "red complex" bacteria include among other bacteria *P. gingivalis, Tannerella forsythia, and T. denticola* – all highly linked with periodontal disease (Nickles et al., 2017, Curtis et al., 2020). The presence of these bacteria is adapted to the low-oxygen environment in subgingival pockets and are prevalent in subgingival plaque. This phenomenon gave rise to the term "red complex" as these bacteria appear red when cultured together. These periodontal pathogens

are classified as “keystone pathogens” (Thurnheer et al., 2014). This means that they act as triggers for dysbiosis within subgingival microbiota, which is an massive growth of pathogenic bacteria followed by a breakdown of the periodontal tissues *P. gingivalis* possesses virulence factors which avoid destruction by the immune system to break down periodontal tissue. The presence of *T. forsythia* is associated with production of enzymes destructive to the periodontal tissues while *T. denticola* is known to be very stable and has the capability to penetrate various kinds of tissues (Chigasaki et al., 2021).

Staphylococcus aureus often occurs in skin and soft tissue infections but also can be identified in plaque of people having periodontitis (Fritschi et al., 2008). The presence of *S. aureus* in periodontal pockets does not occur so often compared to the anaerobic bacteria and poses interesting questions regarding *S. aureus* contribution to the disease’s development. Its potential in being pathogenic is the ability to express several virulence factors that include toxins, and cell wall components. Such an inflammatory response could be involved in the destruction of periodontal tissues (Kim and Lee, 2015). Moreover, *S. aureus* can secrete collagenase enzymes which can bind anaerobic periodontal pathogens thus compromising the integrity of periodontal connective tissues and the general microbial equilibrium in the subgingival biofilm (dos Santos et al., 2014).

1.5. Conventional Treatment Approaches

Common treatment methods for periodontitis include scaling and root planing (SRP), antibiotic therapy, and surgical interventions like flap surgery. Scaling is the removing of plaque and tartar above and below the gumline using special dental instruments. Following scaling, root planing is done to discourage the accumulation of bacteria and debris by smoothing the rough surfaces of the tooth roots. In its beginnings and up to moderate stage, periodontitis is successfully managed using SRP (Liu et al., 2022). It prevents the progression of the periodontal disease. However, sometimes it works only if there are no severe bone loss. SRP is a very vital process and once all this is done, patients need to do their best to preserve healthy habits. Another way of treatment approach is antibiotic therapy**.** These antibiotics serve to supplement other periodontal treatments. They attack and clear the bacteria in the oral cavity hence controlling infection and inflammation. These antibiotics may be administered orally as a pill or capsule, or it can be applied externally. Antibiotics like doxycycline, metronidazole, amoxicillin and many others are commonly used in treatment of periodontitis (Heitz-Mayfield, 2009). The antibiotic choice depends on the bacteria type and

the condition of the patient. Though, there are some concerns with the use of antibiotics like possible negative effects, possibility of bacteria resistance development and carefulness required while applying(Aline Vicentini et al., 2015). They will generally constitute a short-term regimen and often perform well in combination with other therapies.

Flap surgery is a procedure that comes into consideration when the non-surgical measures become inefficient for managing periodontitis. Primarily, this device is designed to deepen the pocket and develop a suitable atmosphere for healing and maintenance. The gum incision is done by the dentist or periodontist during flap surgery (Palaiologou et al., 2019). This make easier getting into the tooth roots thus giving a chance to get rid of tartar, reshape underlying bone, and thoroughly cleanse the teeth. Following this, the gum is shaped again and secured with stitches. Good aftercare which requires maintaining oral hygiene and attending follow-up sessions is necessary for proper healing and recovery. Meanwhile, flap surgery is a surgical procedure and can lead to some level of pain after it, as well as swelling. Flap surgery is not a suitable procedure for all patients and, its success depends on the general health, compliance, and the skills of the surgeon (Nibali et al., 2020)**.** Osseous surgery is the surgical procedure used for managing advanced periodontitis with severe bone loss. The procedure seeks to minimize the gap between teeth and reduce pockets, remove the tartar, and restore diseased or missing bones. In osseous surgery, the dentist or periodontist gains access to the tooth roots and adjacent bone through cuts in the gum tissue. They clean up the tartar and irregular surfaces of bone and sometimes insert bone grafts to induce bone regeneration (Palaiologou et al., 2019). Osseous surgery is helpful in promoting the long-term viability of teeth and improving periodontal health by replacing lost bone (Tang et al., 2021, Huang et al., 2020, Alqahtani, 2023, Swami et al., 2021). It is particularly effective when other conservative methods have not produced any success as possible.

1.6. Advanced materials in the intervention of early-stage periodontitis

The reduction of bacterial load within periodontal pockets is achieved with locally administered antimicrobials such as gels and nanoparticles. These bring precise antibacterial agents straight to the infected site. This is achieved through sustained release of anti-microbials, bacterial cell membrane disruption and bacterial growth inhibition mechanisms. The benefits are precise drug delivery, decreased systemic exposure and improved microbial control which increases efficacies of non-surgical treatment (Nakajima et al., 2021). EMD (Enamel matrix derivatives) as well as growth factors serve to stimulate periodontal

regeneration and tissue healing through the utilization of bioactive materials (Esposito et al., 2009, Seshima et al., 2017). These promote the healing of injured periodontal tissues. By promoting the formation of a new cementum, periodontal ligament, and alveolar bone, EMD plays a important role. Cellular growth is induced, and tissue repair stimulated by growth factors. They encourage periodontal tissue regeneration that led to enhanced treatment outcomes.

Electro spun nanofibrous membranes are artificially made up of nanoscale fibres. For the periodontal applications, they act like scaffolds to encourage regeneration and repair of the tissue (Zhao et al., 2022). These membranes serve as three-dimensional scaffolds, which mimic the extracellular matrix (Xue et al., 2019, Zhuang et al., 2019). These promote cell adhesion, proliferation, and differentiation resulting in new periodontal tissues formation. Regenerates are organized in electro spun nanofibrous membrane. Then it serves to create a space to promote growth and integrations. They are flexible enough to be made deliverers of biologically active substances for improved repairing.

Scaffolds are three dimensional constructs which helps in supporting tissue repair or growth in periodontal defects. They replaces the natural extracellular matrix that serve as a support for cell adhesion, proliferation, and differentiation (Abedi et al., 2022). They encourage the development of cementum, periodontal ligament, as well as the alveolar bone. Scaffolds are essential for periodontal tissue regeneration as they hold space required for regrowth and promote ingrowth of the regenerated tissue into existing structure.

Water-soluble polymers are the hydrogels that serve as drug delivery vehicles for periodontal therapy aimed at tissue regeneration. They have been engineered to carry bioactive agents for targeted therapy. Hydrogels allow for controlled release of therapeutic agents making it possible for prolonged exposure to a given site. They form a humid environment that promotes cell proliferation, migration, and tissue repair. Hydrogels are biocompatible and can be engineered to deliver target growth factors/antimicrobials (Mensah et al., 2023, Bottino et al., 2012, Lee et al., 2023, Roldan et al., 2023). It helps in tissue regeneration and control of infection in the promotion of non-surgical periodontal treatments.

Periodontal films are a new kind of biodegradable, flexible sheets meant for drugs delivery and tissue renewal on periodontium (Victor, 2019, Khan et al., 2016). They act as vectors for various therapeutic agents such as the antimicrobials and growth factors. Such drugs are released slowly into the system, keeping therapy concentrations within periodontal pockets

over time. They serve as protective barriers over the treatment area and help promote tissue regrowth in a suitable environment (Sah et al., 2019). They are minimally invasive so that delivery of drug is done directly onto the affected area accurately (Ghavami-Lahiji et al., 2019, Constantin et al., 2022). They helps in microbial control and wound healing when used alongside non-invasive interventions.

The use of the above advanced materials as part of periodontal care methods is an interesting technique that could improve the treatment results in initial stages of periodontitis. Targeted drug delivery, structural support, and regenerative abilities improve the efficacy of non-surgical treatments towards periodontal health restoration.

1.7. Polycaprolactone (PCL) and its purpose of drug delivery

Several works have been conducted on the biodegradable polymer, PCL, and it can be a drug delivery system for periodontal diseases. In addition, PCL allows the direct injection of antibiotics into the periodontal pocket which is prone to infection and inflammation. The objective here is to deliver a sustained and more targeted therapy with a reduced frequency of administration which might improve patient care (Wei et al., 2009, Bhadran et al., 2023).

PCL can be fabricated into a thin film or a scaffold that will gradually supply antibiotics to the involved site during the periodontal pocket. The therapeutic effects of such antibiotics can be optimized while reducing possible adverse effects associated with systemic administration when this controlled release system is taken into consideration. The dimensions of the PCL film or scaffold can be fit to the size and shape of the periodontal pocket, thereby promoting targeted drug delivery (Wei et al., 2009, Mondal et al., 2016)

Using several fabrication methods such as tape casting, solvent casting, electrospinning, or hot pressing are some of the ways that a porous structure within the PCL film or scaffold could be created. Such porous structure allows insertion of antibiotics with a slow release, to prolong therapeutic effect. Furthermore, the polymer undergoes degradation to be eliminated from the body naturally with time eliminating the possibilities of long-term toxicities or other adverse effects.

The use of antibiotics in PCL film constitutes a novel technique to promote localized intervention for periodontal diseases. This technique improves the accuracy of medication delivery and limits the frequency of topical antibiotics administration thereby making it convenient to the patient. It also minimizes the systemic antibiotic exposure that may constitute antibiotic stewardship.

PCL is a polyester polymer that exhibits ester linkages in its chemical structure and lipases are specialized an enzyme that cleaves off ester bonds. The interplay between lipases and PCL forms a key element in the mechanism of periodontal diseases drug delivery systems (Khan et al., 2019).

Upon introduction of the PCL films into the oral environment, especially at the periodontal pocket where oral pathogens reside, the lipases secreted by these bacteria. Molecular scissors termed lipases secreted by oral pathogens targeted the ester bonds of PCL polymer chain. The gradual breakdown of PCL is brought about by this enzymatic process known as hydrolysis.

The hydrolysis of PCL is an extremely localized and tightly regulated phenomenon. It occurs in the periodontal pocket as the oral pathogens are most concentrated and active. The gradual dissolution of antibiotics in this polycaprolactone films allows for a gradual release, thereby targeting the site of the infected area. Lipases are enzymes that cleave the ester bonds in PCL that results to controlled release of antibiotic directly into the periodontal pocket, which prevents the bacterial infection and inflammation (Khan et al., 2019).

Such interaction between PCL and lipases represents the smart engineering of PCL-based drug delivery systems against periodontal disease. This technology uses the inherent enzymatic activity of oral pathogens to provide a localized and sustained antibiotic delivery system to optimize treatment accuracy and effectiveness while reducing the frequency of antibiotic applications (Lysik et al., 2022, Blackwell et al., 2018).

1.8. Silk sericin and its properties

Sericin is the silk sericin that is derived naturally from silk fibres. They show excellent biocompatibility, therefore making them ideal for the delicate environment of the oral cavity. It becomes a composite material with PCL as a biodegradable synthetic polymer, providing structural support and safety to patients (Cao and Wang, 2009). Biocompatibility of silk sericin is mainly proteinaceous nature which closely match with components such as proteins of the extracellular matrices of different tissues (Ersel et al., 2016). The wound-healing property of silk sericin makes it suitable for the damages and inflammation resulting from periodontal diseases. Silk sericin promotes the cell proliferation, angiogenesis, and collagen synthesis which are crucial for healing when there is a defect or injury in periodontal tissues.

Periodontal disease is mainly caused by oxidative stress. This oxidative stress has a valuable opposition of silk sericin as strong antioxidant. Silk sericin-PCL films serve as a shield against free radical scavenging and oxidative damage of periodontal tissues. This maintains the integrity of these tissues, allowing them to be regenerated for better periodontal health (Suzuki et al., 2022). Anti-inflammatory properties of silk sericin are responsible for controlling the inflammatory response in the oral cavity. Its anti-inflammatory action can decrease the production of pro-inflammatory cytokines and inhibit immune cell's activation leading to the decrease in inflammation (Aramwit et al., 2013).

Silk sericin-PCL films are novel controlled release platforms for bioactive compounds and growth factors. The films can carry types of known therapeutics for stimulating the tissue regrowth, especially PDGF and TGF-β or even antibacterial agents. These aspects are gradually released from the films thus giving continuous stimuli of cell growth and tissue repair for an extended period to optimize regeneration (Yavuz et al., 2019).

Cell-substrate interaction is crucial during tissue regeneration. The presence of sericin in silk silk-sericin- PCL films improve its bioactivity to cell viability and adhesion to their surface. This forms an extremely important bond which ensures that the cells are held in place at the injury or defect site. Silk sericin also assists in bringing necessary cell types to the wound site by enhancing cell migration. It is this coordinated cellular response that defines the successful tissue regeneration (Kaewkorn et al., 2012).

The protein material, silk sericin, has almost similar composition with the natural extracellular matrix present in various tissues like those in oral cavity (Griffanti et al., 2023). The significance here comes in as a perfect environment for the cells to adhere, migrate and proliferate. Silk sericin-PCL films can serve as scaffolds for cell proliferation.

1.9. Metronidazole and its effect in periodontitis

The effectiveness of metronidazole to the anaerobic oral pathogens in the periodontal disease is attributed to the specific anti-microbial properties and unique mode of action. Anaerobic bacteria, such as *P. gingivalis, Prevotella*, and *Fusobacterium* often contribute to the formation of periodontal infections by surviving in anaerobic environments prevalent within a periodontal pocket (2014).

Therefore, metronidazole is preferred for its strong effects on such anaerobic pathogens. It is a broad-spectrum activity of bactericidal nature, especially directed at these microbes, without harming majorly aerobic bacteria. It is important that this selective antimicrobial action removes pathogens which cause periodontal diseases as it minimizes damaging the equilibrium that exists in the oral microbiome.

In the case of metronidazole, its journey begins penetrating through the bacterial cell, including those that are responsible for the periodontal infections (Dione et al., 2015). This is one of the most crucial steps in the antimicrobial action of metronidazole. Remarkably, metronidazole is notably inactive when it enters these cells. However, this initial state is extremely important given that it contrasts greatly with its active and antimicrobial form in the anaerobic stage. Transformation of metronidazole is one of the incredible processes that takes place within the bacterial cell. This is the main reason this agent acts so effectively on anaerobic bacteria, making it one of a kind antibiotic for various periodontal infections (Murgia et al., 2019).

Metronidazole is inactive and has no antimicrobial effects in aerobic environments. Its dependency on anaerobic environment further shows its selectiveness since it mainly focuses on the anaerobic bacteria while avoiding the aerobic bacteria (Dione et al., 2015).

Selective toxicity is what defines metronidazole, which is the key ingredient that makes it effective. This is because it targets the anaerobic bacteria which can activate such a drug. It is worth noting that this selectivity ensures that they do not harm the aerobic bacteria as their balance maintains the balance of the whole oral microbiome. Therefore, these complex mechanisms ultimately make metronidazole ideal antimicrobial agent. The key merit of using metronidazole is their ability to selectively destroy anaerobic bacteria that provide the foundation for periodontal infections. At the same time, it protects the oral microbiome balance, making it an effective against periodontal diseases (Dione et al., 2015).

1.10. Vancomycin and its effect in a periodontitis

Its efficacy against *S. aureus*, in particular Methicillin resistant *S. aureus* (MRSA) is attributed to its targeted action and mechanism of action. Its effectiveness in fighting Gram-positive bacteria is attributed to its unique binding capability with their structural elements of cell walls. The bacterial killing mechanism of vancomycin is based on its ability to inhibit cell wall

synthesis. Gram-positive bacteria such as *S. aureus* have essential bacterial cell walls which maintain cell shape and act against osmotic pressure changes. In this case, the cell wall has peptidoglycan as a supporting structure. The way in which vancomycin acts is by specific binding on the peptidoglycan precursor molecule's D-alanine-D-alanine segment (Gardner et al., 2018). The binding of the former to the precursor inhibit its incorporation into the extending peptidoglycan chain, which does not permit cross-linking. This leads to disruption of the normal cell wall structure and weakening of the cellular structure, thus making the cells more susceptible to external forces.

Bacterial cell death is the result of vancomycin's actions. This sets in motion a sequence of events when the bacterial cell wall starts to breach by the compromised peptidoglycan synthesis, and by increased membrane permeability and leaking out of the cellular contents. Osmotic pressure imbalances due to disruption of the cell membrane and loss of structural integrity. These unbalances together with the damage of cell membrane culminate in cell lysis. Bacterial cell lysis occurs when the cell splits and releases its content into the environment. The total collapse of the bacterial cell is fatal to *S. aureus* as it can no longer keep intact or perform essential metabolic functions (Walsh and Howe, 2002).

1.11. Hypothesis

(a) The incorporation of metronidazole and silk sericin in PCL thin films will result in antimicrobial activity with slow release and tissue regeneration against various sub-gingival anaerobic periodontal pathogens commonly isolated from closed-flap periodontal pockets compared to PCL thin films.

(b) The incorporation of vancomycin and silk sericin in PCL thin films will result in antimicrobial activity with slow release and tissue regeneration against various sub-gingival aerobic periodontal pathogens commonly isolated from open-flap periodontal pockets compared to PCL thin films.

1.12. Aims

Aim 1: Fabrication of enhanced PCL periodontal films

To develop PCL periodontal films incorporating an emulsifying agent, metronidazole (to target anaerobic bacteria *P. gingivalis*), vancomycin (to target aerobic bacteria *S. aureus*), and silk sericin using the tape cast technique.

Aim 2: Assessment of antimicrobial activity

To evaluate the antimicrobial activity of the enhanced PCL periodontal films with the emulsifying agent using Zone of Inhibition (ZOI) assay, CFU analysis and Live/dead assay.

Aim 3: Characterization of drug release

To study the release profiles of antibiotics (metronidazole and vancomycin) from the films using UV-vis spectroscopy to assess sustained drug release.

Aim 4: Evaluation of cytocompatibility

Perform cytocompatibility tests involving Human Gingival Fibroblasts (HGFs) to determine the films' biocompatibility and their potential for promoting cell proliferation.

Aim 5: Physicochemical characterization

To conduct water contact angle measurements to assess surface properties and perform mass loss studies to evaluate film degradation behaviour.

CHAPTER 2: METHODOLOGY

2.1. Materials

Polycaprolactone [PCL] (medium molecular weight of 48 kDa and low molecular weight of 14000 kDa), phosphate buffer saline (PBS) tablets, Columbia sheep blood agar, lipase from pseudomonas cepacian, MTT and common solvents were purchased from Sigma-Aldrich Australia, Tween 20, DMEM, Foetal bovine serum, penicillin/ streptomycin from ThermoFisher, silk sericin powder (99.9% purity) was purchased from Nano Silk Medtech, Vietnam, Ultrahigh purity water gained from a Milli-Q system (Millipore Milli-Q Academic, USA), with a resistivity greater than 18 MΩ·cm, was used for all experiments. *P. gingivalis* (strain W50- ATCC 53978), and *S. aureus* (strain: ATCC 25923) were the bacterias for the antimicrobial determination. Human gingival fibroblast (ATCC CRL-406, hTERT gingival fibroblast used for cytocompatibility determination.

2.2. Fabrication of PCL, PCL/antibiotics/Silk sericin/antibiotics film with and without emulsifying agent

2.2.1. Dissolution of PCL in ethyl acetate

Careful preparation of the PCL/ethyl acetate (PCL/EA) solution was done to attain equal uniformity, with regard to the strength requirements for the polymer film and the appropriate viscosity for tape casting technique (Tang et al., 2004). Initially, 2g of carefully weighed and transferred poly(ε-caprolactone) (PCL) polymer pellets in a clean and dry 20mL vial was precisely measured. To start the dissolution process 20 ml ethyl acetate (EA) solvent were added in fume hood due to volatile nature of EA and the dissolving started by magnetic stirrer which had a speed of 500 rpm.

A critical stage of this process involved exposing samples to ambient temperature under well-ventilated fume hood, for five hours. It was taken to an oven heated to 40ºC following the first mixing period. In this case, the solution was heated for another 10 minutes at a low temperature. Controlled heating process was crucial because all PCL had to dissolve completely in the solvent and be mixed evenly so that one could obtain a transparent solution with the right concentration which was 0.1 g/ml.

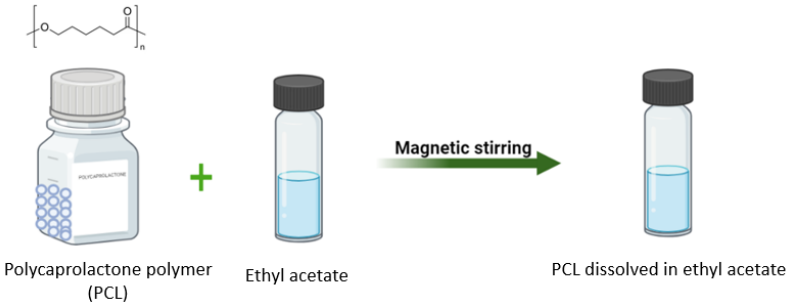


Figure 3: Dissolution of PCL in ethyl acetate (Image made with BioRender).

2.2.2. Preparation of antibiotic solutions

2.2.2.1. Preparation of vancomycin Solution for targeting aerobic bacteria (*S. aureus*):

Prepared vancomycin solutions for preparation of films at 20µg/film, 50µg/film, and 100µg/film concentrations. For example, 1 mg milligram of vancomycin is added into 10 ml of Milli-Q water creating a 1 mg/10 ml solution of vancomycin. This also involves the preparation of 2.5mg vancomycin in 10 ml of Milli-Q water to attain a total concentration of 50 µg/film. Five milligrams of vancomycin are again weighed and dissolved by vortexing for 15 minutes in 10 millilitres of Milli-Q water for the highest concentration of 100 µg/film. Such correctly calculated solutions help achieve the required Vancomycin concentrations as the basis of the further film manufacturing.

2.2.2.2. Preparation of metronidazole solution for targeting anaerobic bacteria (*P. gingivalis*)

To target anaerobic bacteria like *P. gingivalis*, metronidazole solutions are prepared at concentrations of 5 µg/film, 12.5 µg/film, and 25 µg/film; the amounts of metronidazole required for each solution are calculated such that the resultant concentrations per solution will be as required. Therefore, for instance, 250 ug of metronidazole is measured accurately before mixing it with 10ml of Milli-Q water by vortexing for 15 minutes for a 5µg/film solution. For the other concentrations, the same procedure is applied by adding the proper amount of metronidazole in 10 ml of Milli-Q water.

2.2.3. Preparation of Silk sericin/antibiotic solution

Preparation of a 0.05% (w/w) solution of Bombyx mori silk sericin powder was done in this procedure. The production of this solution involves mixing 9.8 mg of silk sericin power in a hot Milli-Q water (80ºC) of 19.6 millilitres in a magnetic stirrer with hot plate until completely dissolving. Moreover, this process includes the addition of antibiotics vancomycin and metronidazole solution to achieve bactericidal films against aerobic bacteria (*S. aureus*) and anaerobic bacteria (*P. gingivalis*) at different concentrations. vancomycin solutions are prepared at concentrations of 20 µg/film, 50 µg/film, and 100 µg/film for targeting aerobic bacteria. The right amount of Vancomycin was mixed with the silk sericin solution at a constant concentration 0.05% w/w and vortexed for 15 minutes (figure 4).

Again, for anaerobic bacteria, metronidazole solutions are prepared in strength 5, 12.5, and 25 µg/film. The amount of metronidazole necessary for the preparation of a solution with a constant silk sericin concentration and the required antibiotic concentration are calculated.

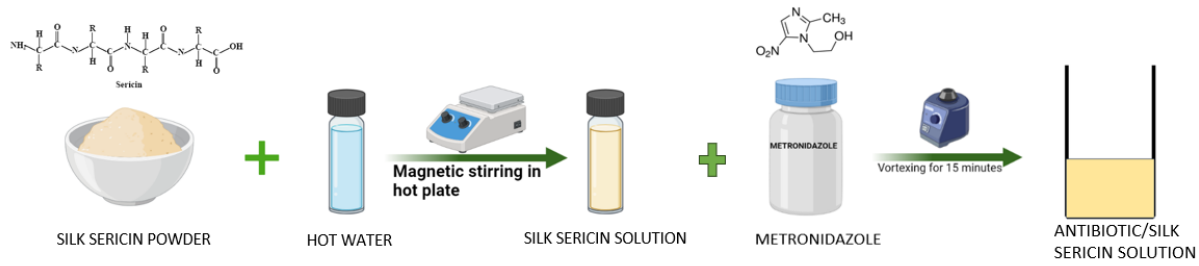


Figure 4: Preparation of Silk sericin/antibiotic solution (Image made with BioRender).

2.2.4. Tape cast technique

2.2.4.1. Principal of techniques

A thin film with uniform thickness are commonly obtained by using tape casting method. It requires the placement of a suspension onto a smooth glass slide and then the removal of a solvent to result in a thin film. The film thickness was depend on the gap between a glass rod coated with tapes and another glass slide. This method was widely employed in the fabrication of periodontal films (Almazrouei et al., 2019).

A suspension comprising of the active ingredients would have to be prepared to fabricate a periodontal film using the tape casting method. The suspension might include different

polymers, emulsifier, and solvents that will ensure that the formed film will stick together. The active ingredient(s) may include an antimicrobial agent or a therapeutic measure against periodontal disease. A glass rod coated on both edges with tape would be used to suspend the liquid above a flat reference surface such as that of a glass plate (figure 5). The solvent would be removed from the solution by exposing it to air in a controlled temperature and humidity environment to leave behind a solid film. The film would be removed from the suspension and cut into the desired shape and size (Almazrouei et al., 2019).

2.2.4.2. Experimental details

Fabrication process of PCL film using tape casting requires some stages in order to obtain homogeneous and controlled thickness film. The technique starts by placing a glass slide on a flat surface (a horizontal plane), which is then followed by a glass rod with tape attached on its respective ends. This flexible approach, however, provides for the adjustment of film dimension to certain requirements.

Thereafter, a 1ml of the already prepared PCL/ethyl acetate solution is applied at the centre of the glass slide. Finally, the solution is spread evenly on the surface with a glass rod covered in tape, so that the film will have uniform consistency overall.

The casting process is over, but the drying process is important for the solvent evaporation and stabilization of the surface layers. They take the glass slide with the freshly cast PCL film together with the 1 mL-solution to a controlled environment inside a fume hood. In this regard, the film is exposed to drying process for 24 hours to ensure complete solvent extraction. During this period, it is important to maintain constant temperature and reduced air movement to get desired results.

After drying, the PCL film is tightly stuck to the glass slide. Using tweezers, the film is carefully removed without compromising its integrity. The fabricated film is then transferred to a clean Petri dish and carefully covered.

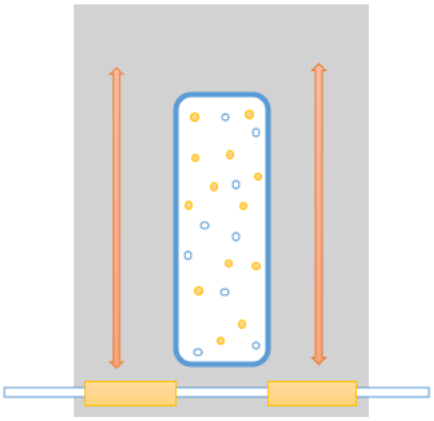


Figure 5: Preparation of films using tape cast technique

2.2.4.3. Fabrication of PCL films incorporating antibiotics or antibiotics/silk sericin without an emulsifying agent.

Firstly, prepared antibiotic solutions (Vancomycin or Metronidazole) or Silk sericin/antibiotic solution in Milli-Q water with appropriate concentrations (refer 2.2.2 and 2.2.3). Mix 800 µL of a PCL-ethyl acetate blend with 200 µL prepared antibiotic in a fume hood. The formed antibiotic globule maintained its distinct form when mixed with PCL/EA solution. To make a uniform mixture, conducted the probe sonication at 20% amplitude, under ice bath conditions. It carried out for one minute, interrupted by 10 seconds after every 20 seconds, until antibiotic solution was dissolved into the PCL polymers solution. The subsequent steps for tape casting, drying remain the same as in the protocol (refer 2.2.4.2).

2.2.4.4. Experimental Details of fabrication of Poly(ε-caprolactone) (PCL) Films with antibiotics or antibiotics/silk sericin with Emulsifying Agent

The protocol for films with an emulsifying agent follows the same steps as described above (section 2.2.4.3) for films without an emulsifying agent, with the only difference being the addition of 50 µL of 0.01% Tween 20 to the PCL-ethyl acetate solution after the probe sonication step. This addition facilitates emulsification and ensures uniform distribution and stability of antibiotics in the PCL polymer solution, thus forming an emulsion. The subsequent steps for tape casting, drying remain the same as in the protocol without an emulsifying agent (refer 2.2.4.2).

2.3. Antimicrobial assays

2.3.1. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

For the determination of MIC, as the first step, the solution of antibiotic was made in multiples of ten greater than the highest concentration. Likewise, a 640 µg/mL stock solution was prepared for a concentration range of 64 µg/mL. Sterile culture media was aseptically poured into the wells of a 96-well plate with 170 uL in the first column and 95 uL in the remaining columns. Then, 20 µL of the antibiotic stock solution was added to the first column to dilute it 1:10 times the starting concentration (64 µg/mL). A multichannel pipette was used to serially dilute the antibiotic at a ratio of 1:10 by transferring 95 µL across the plate. Additionally, 95 microliters of media were added in three wells for the no-antibiotic control, while in three other wells, 100 µL of media were added and used for the media-only control.

A bacterial suspension (10^7 CFU/mL) was prepared from the culture that had been incubated overnight in the media. Then, 5 µL of this suspension was put into each well to ensure a final concentration of approximately 5 x 10^5 CFU/mL. The plate was incubated at 37°C for one night. After incubation, a plate reader (SPECTROstar Nano Microplate Reader, BMG Lab Tech, AU0 was used to shake the plate for 5 seconds and read the absorbance at 600nm. The mean absorbance for the medium was deducted from all others. The minimum inhibitory concentration (MIC) was identified as the lowest antibiotic concentration that lacked visible bacterial growth (Hancock et al., 2008).

After determining the MIC, the MIC and three concentrations below it was selected. Added 10ul of each sample to relevant agar plates (duplicated it). (Columbia sheep blood agar plates are used for *P. gingivalis* and incubated at 37ºC in an anaerobic jar; and anaerogen sachet place to remove oxygen from the jar. Meanwhile, TSA plates are used for *S. aureus* and incubated at 37ºC). The MBC is checked after 24 hours for *S. aureus* and 5 days for *P. gingivalis*.

2.3.2. Zone of inhibition assay

The initial step in the experimental procedure was to prepare a culture of *S. aureus* and *P. gingivalis* that had been incubated overnight. This was possible through placing 5ml of TSB media into a sterile tube and adding a loopful of *S. aureus* from a pure culture or stock. Thereafter, the tube was maintained at a temperature of around 37 degrees Celsius for about 18 – 24 hours, meanwhile using HIB (ThermoFisher, Massachusetts, USA) supplemented with vitamin K (1 µg/ mL) and hemin (5 µg/mL) as a medium for *P. gingivalis*. The *S. aureus* multiplied and built up one of the strongest overnight cultures by that incubation stage, and *P. gingivalis* took 5 days. The bacterial density was an important parameter after incubation. At 600nm, spectrophotometric measurements were taken to determine optical density (OD). The bacterial cell density was inferred from the OD measurement, based on a previously established understanding that an OD value of 1 is equivalent to 10^9 CFU/mL.

A dilution step was carried out to ensure a uniform bacterial concentration for subsequent experiments. For this, the appropriate dilution factor was calculated to achieve a target bacterial count of 10^8 CFU/mL. The fresh culture that was diluted or concentrated in new media was mixed thoroughly to get that desired bacterial density. The prepared bacterial culture was then spread aseptically on the surface of the plate (TSA for *S. aureus* and Columbia sheep blood agar plates were used for *P. gingivalis*) forming a uniform bacterial lawn. The bacterial culture was readjusted and then 200 µL was evenly spread on the agar plate using a sterile spreader. The methodical nature of this spreading led to a uniform spread bacterial lawn on the agar surface.

Afterwards, the PCL films made with or without the antibiotic depending on the experimental design were placed into the bacterial lawn. It was important to let the agar plate dry for a while before placing it so that the excess moisture in the agar could be absorbed. The process came to an end when the TSA dish was incubated along with the PCL films. The 24-hour incubation was done at 37°C. And the blood agar plate was incubated along with the PCL films in an anaerobic jar with anaerogen sachet place to remove oxygen from the jar and incubated for 5 days. After the specific period of incubation, inhibition was determined by measuring clear zones around the PCL films. The diameters of the ZOIs were very accurately measured using a ruler.

2.3.3. Colony forming unit assay

The colony forming unit assay starts with the preparing culture of bacteria (refer 2.3.2). The bacterial density is then determined by measuring the absorbance of a sample at a set wavelength of 600 nm using spectrophotometer with optical density (OD) providing an indication of the culture's density. The dilution factor required to achieve a desired bacterial density of 10^8 CFU/mL is determined. Next, the overnight culture is diluted with fresh media to the desired density with proper mixing. Place the films to diluted culture. Thereafter, the treated bacterial suspension is subjected to several serial dilutions. In each subsequent dilution, 450 µL of media is transferred to a new well and mixed with 50 µL of original sample culture resulting in dilution. After these dilutions, the samples are plated onto agar plates which have six segments. The last six dilutions are aseptically transferred 10 µL each to the respective sections on agar plates, thus creating duplicate samples.

After the agar plates are dried, they incubated at 37°C (about 24 hours for *S. aureus* and 5 days for *P. gingivalis*; *P. gingivalis* in an anaerobic jar with and anaerogen sachet place to remove oxygen from the jar). The plates incubate viable bacterial colonies over this period. After incubation, the number of colonies on each segment of the agar plates is determined by counting using ImageJ software.

The CFU/mL of bacteria in the original culture is determined from zero dilution and the zero dilution CFU/mL used to determine the concentration of bacteria in the original culture. This value considers the number of colonies, the dilution factor and amounts from the serial dilution.

2.3.4. Live/dead viability analysis of films

The procedure for live/dead analysis started with the preparation of bacterial cultures. Bacterial cultures were grown to late exponential phase, and, prior to viability analysis, their cell concentrations were assessed using an optical density measurement of 600 nm wavelength in a spectrophotometer (OD600nm). Then cultures were diluted to a final cell density of 10^8 CFU/mL. The cell suspension for *S. aureus* was prepared in TSB (ThermoFisher, Massachusetts, USA) and for *P. gingivalis* in HIB (ThermoFisher, Massachusetts, USA) supplemented with vitamin K (1 µg/ mL) and hemin (5 µg/mL). Then, wells in a 24-well plate were loaded with antibiotic-loaded PCL films, and each immersed into respective 500 µL of

diluted cell suspension. The bacterial samples were incubated at 37°C for 24 h, while the staining related to Bac Light Live/Dead (according to Invitrogen, Massachusetts, USA) Syto9 (1.5 µL/mL of PBS) and propidium iodide was incubated with the sample at room temperature in darkness for 15 min. Zeiss LSM80 confocal laser scanning microscope (CLSM; Carl Zeiss AG, Oberkochen, Germany) was immediately used to image samples. Three images were recorded at random spots in each of the samples. ImageJ was used to quantify red and green stained cells. They did this by separating the different colour channels into red, green, and blue. For the red and green channels, 'find maxima' feature was used to count the individual cells. A measure of viability was calculated with the following formula:

Viability = (green cells/total cells) * 100.

2.4. Mass loss study

2.4.1. Principles

The principle of the mass loss study of PCL films (polycaprolactone) with a lipase enzyme in PBS (phosphate-buffered saline) solution was to observe the degradation behaviour of PCL film presence with an enzyme (Ma et al., 2020). The main principle was the evaluation of the interaction between a biodegradable polymer, PCL, and an enzyme called lipase that helped to break down of fats. The hydrolysis of the ester bonds, which were found in PCL, by lipase could cleave PCL polymer chains (Khan et al., 2019). The main parameter that was studied was the weight loss of PCL films over time. The lipase enzyme in the PBS solution hydrolysed PCL chains as the PCL films got exposed to it. This consequently caused the PCL films to slowly degrade and reduced their mass. Collecting the data at different times enabled formulating the degradation profile and PCL film degradation rate by the lipase enzyme. To keep the research valid, the experiment was carried out in a controlled environment. PBS supported stable and biologically meaningful conditions. The temperature of 37°C for incubation was selected to keep the enzyme active because most enzymes show optimal aerobicities within specific ranges of temperature. The PBS solution maintained a pH of 6, thereby mimicking biological systems. This made it possible for the enzyme to be stable and active.

2.4.2. Experimental details

One PBS tablet was dissolved in 200 ml of Milli-Q water to create the PBS solution in a clean container. It was shaken mildly, until the tablet dissolved entirely, ensuring no solid particles were visible in the solution. After dissolving, the PBS solution was ready to use. The lipase enzyme was accurately weighed for the preparation of 0.1 mg/mL. The weighed lipase was mixed with the already prepared PBS solution slowly with stirring to dissolve completely. A pH meter was used to adjust the PBS solution to a pH of 6. The lipase solution could be stored at 4°C until it was required for the study. The PCL periodontal films were submerged in Milli-Q water, PBS solution, and lastly, PBS with the lipase solution in centrifuge tubes. The samples were incubated at 37°C to keep the enzyme active during the study. The solutions were retrieved at predetermined time points after fitting sets of samples. The rinsed samples were washed with deionized water to ensure all the enzymes or solutions were completely cleared off. The films were dried cautiously in an incubator at 40°C until they were dry. The dried films were weighed, and the weight (Wt) was recorded. It was important to ensure the films were completely dry to obtain correct weight readings.

The weight loss percentage of each sample was calculated using the formula:

Weight Loss Percentage = [(W0 – Wt)/W0] × 100%,

where W0 is the initial weight of the film before incubation and Wt is the weight of the film after incubation.

2.5. Release study

The first procedure involved a calibration curve that was obtained by serially diluting the vancomycin and metronidazole stock solutions by a factor of two. Using this linear equation, antibiotic concentration was determined for a dissolution study performed in a PBS solution, with 0.1 mg/ml lipase simulated periodontal pockets condition. Samples underwent a 72-hour thermal shake incubation (37°C, 200 rpm). Periodically, a sample of 2ml was withdrawn from each of the tubes for the determination of the placebo releases. For every measurement, the withdrawn dissolution medium was replaced with fresh dissolution medium to ensure that volume remained at a final value of 15 ml per tube. A UV-Vis spectrophotometer (PerkinElmer Lambda 365) with a 1cm quartz cuvette was taken and the measurement taken included the

wavelengths, 220 – 400nm. For vancomycin-loaded samples it was measured using 280 nm under while, 320 nm was used for metronidazole-loaded samples. Release profiles were created using zero-order and Higuchi models, based on the data obtained. The model with the highest R2-value was deemed as the most applicable for the data analysis.

ZERO ORDER MODEL	HIGUCHI MODEL
Qt=Q0 + K0t Q0 = Initial amount of drug Qt = Cumulative amount of drug release at specific time. K0 = Zero order release constant t = time in hours	**Mt/M∞ = Kh√t** Mt/M∞ = Fraction of drug released at each time point Mt = The amount of drug released at specific time. M∞ = The amount of drug released after time ∞ Kh = Higuchi constant t = time in hours
The above equations are used calculate and plot the release profiles.	

Loading capacity (LC) was determined by dissolving a known mass of the film in 2 ml acetonitrile, and then centrifuged it at 1000 rpm for thirty minutes. The tubes were then vortexed and ultrasonicated for four cycles of 15 min each. The samples were then allowed to settle for another hour before analysis and their quantity measured using a UV-Vis spectrophotometer. Re-measurement following 24 hours was used to assess the full disintegration of the film.

Yield (%) is calculated using the following equation:

Yield (%) = (Actual weight/Theoretical weight) x 100

Loading capacity (LC %) is calculated using the following equation:

Loading capacity (LC %) = (Total entrapped antibiotics/Total weight of the film) x 100

2.6. Wettability measurement

The determination of wettability or hydrophobicity/hydrophilicity of surfaces, including that of thin films, involved describing how a liquid, in this case, water, contacted with a solid surface. The contact angle was the angle at which a tangent to the liquid surface at the point of contact made with the solid surface (Namen et al., 2008). PCL films, PCL with antibiotics, and PCL with silk sericin and antibiotics were kept clean and dry to avoid interfering with the experiment due to any contamination. The contact angle goniometer was prepared following the manufacturer's guidelines. The calibration of the goniometer was verified on a standard reference material with contact angle information. One small water was dispensed on the surface of the PCL film using a micro syringe, ensuring they were dispensed without force to prevent deformation of the droplet. Pictures of the drops at the film surface were taken, using a camera mounted on the goniometer. The camera was focused well, and the room had sufficient light to capture clear images. It was ensured that the images were extremely visual and that the outline of all droplets was clearly visible. The water contact angle was measured using ImageJ software.

2.7. Cytocompatibility study

Samples were examined and tested using MTT assay in the case of the cells. Foetal bovine serum as supplement to DMEM with penicillin and streptomycin (1% v/v) comprised the growth medium for this study. 96 well plates were seeded with human gingival fibroblasts (20K cells/well). And incubated at 37°C with 5% CO2 for 24 hours. After 24 hours, the film samples were then sterilized under ultraviolet light for 30 minutes and placed into the appropriate wells after checking the confluency of HGF, which reached 80-90%. They were kept again in an incubator at 37°C with 5% CO2 for a day. Then after 24 hrs, 10µl of MTT solution (5 mg/ml, Sigma Aldrich, Australia) is added in all the wells. Subsequently, put the well plate in incubator at 37°C and 5% CO2 for 2 hours. Two hours later, 150 µl of DMSO is placed on each well. Absorbance is then measured at 570 nm using a microplate reader after shaking for 10 minutes.

Cell viability (%) = (Mean absorbance of the sample/Mean absorbance of the control) x 100

CHAPTER 3 RESULTS AND DISCUSSIONS

3.1. Fabrication of PCL, PCL/antibiotics/Silk sericin/antibiotics films with and without emulsifying agent

3.1.1. Fabrication of PCL films

Films with different PCL concentrations (0.05 g/mL, 0.15 g/mL, 0.2 g/mL – (mass of PCL per ml of ethyl acetate) were prepared using medium Mw 48 kDa and low Mw 14 kDa PCL in ethyl acetate. Unfortunately, these attempts were unsuccessful as they are not suitable for the fabrication through the tape casting technique. Meanwhile, the films prepared using the 0.1g/ml PCL solution (Mw 14 kDa) showed low strength and significant brittleness.

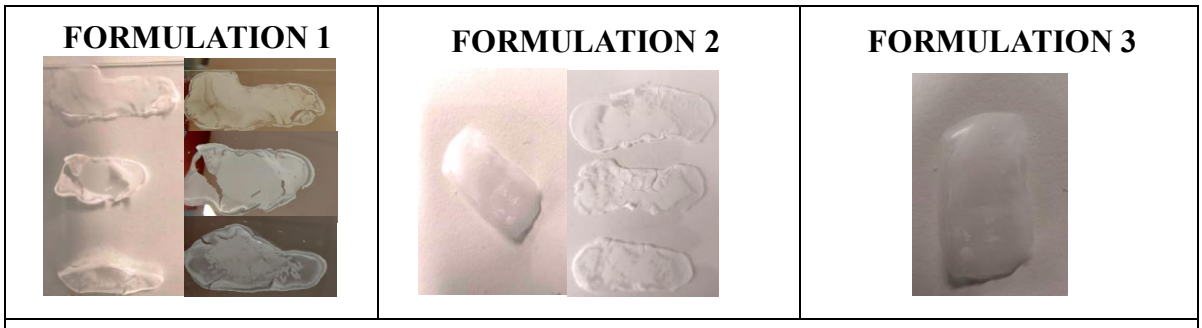


Figure 6: PCL films Formulation 1: Low molecular weight 80mg PCL film, Formulation 2: Low-medium molecular weight PCL film (8mg-low, 72mg-medium), Formulation 3: Medium molecular weight 80mg PCL film.

In practical terms, formulation 1 is a PCL (polycaprolactone) film with a relatively low molecular weight. This produces a film whose appearance was white and granular. One significant disadvantage is that the films were too brittle. This material cannot be bent and stretched without breaking hence they are inappropriate for use to where such properties are required. It also belongs to formulation 2 of low to medium molecular weight PCL films. The molecular weight of the polymer was slightly higher in comparison with formulation 1 and

thus shorter polymer chains. The texture of the film was smooth compared with the roughness of formulation 1. This results into better surface qualities but formulation 2 still inherits the brittleness experienced in the low molecular weight PCL films.

On the other hand, formulation 3 constitutes a move away from the brittleness of the prior formulations. It belongs to a medium molecular weight PCL film having a large length of polymer chains among all the three solutions. Nevertheless, it is the nature and a bit of flexibility that make formulation 3 stand out from its peers. It bends when subjected to a finger elasticity test and returns to its initial shape. The mixture of hardness and adaptability in formulation 3 makes it a perfect selection for situations that require hardness as well as the ability to bend.

3.1.4. PCL films having antibiotics without tween 20

FORMULATION 4	FORMULATION 5

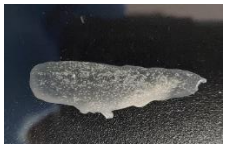



Figure 7: The PCL/vancomycin films prepared without the addition of tween 20. Formulation 4: Medium molecular weight 80 mg PCL having 1.25 % of vancomycin, Formulation 5: Medium molecular weight 80 mg PCL with 0.025 % of Vancomycin.	

Formulation 4 containing 1.25% vancomycin was strong enough and it shows that vancomycin did not compromise film's structure. Formulations 5 containing extremely low quantity of vancomycin at 0.025%, demonstrate good strength showing that the strength reduces are not due to the quantity of antibiotic.

3.1.5. PCL films having antibiotics with tween 20

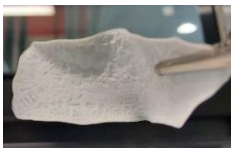
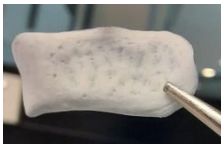
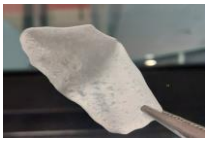
FORMULATION 6	FORMULATION 7
	
FORMULATION 8	**FORMULATION 9**
	

Figure 8: Antibiotic loaded PCL films prepared with the addition of tween 20. Formulation 6: Medium molecular weight 80 mg PCL having 0.125 % of vancomycin in the presence of 0.01% Tween 20, Formulation 7: Medium molecular weight 80 mg PCL having 0.125 % of vancomycin and 0.01% of silk sericin in the presence of 0.01% Tween 20, Formulation 8: Medium molecular weight 80 mg PCL having 0.03125 % of metronidazole in the presence of 0.01% Tween 20, Formulation 9: Medium molecular weight 80 mg PCL having 0.03125 % of metronidazole and 0.01% of silk sericin in the presence of 0.01% Tween 20.

The above results refer to the different formulations of films made from medium molecular weight PCL containing varying concentrations, vancomycin (an antibiotics), and sometimes silk sericin, combined with Tween 20, Alternatively, silk sericin was incorporated as a means of determining its impact. Consistently observed good strength and uniform thickness in all films show that the preparation method was successful in producing stable films characterized by homogenous distribution of ingredients.

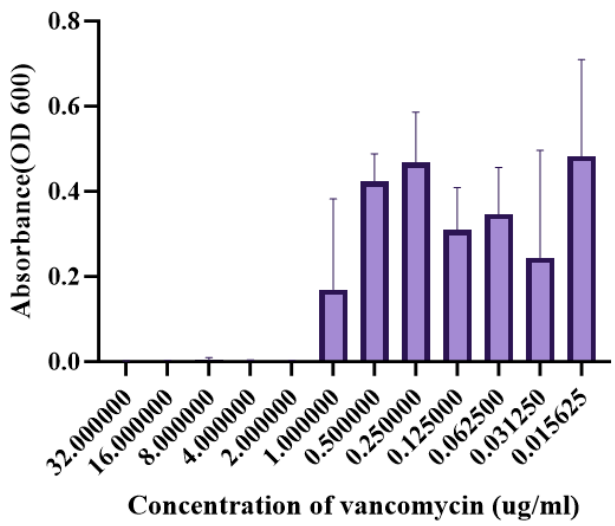
Formulations 8 represent films made from medium molecular weight PCL, with 0.03% metronidazole, an antibiotic, and, in some instances, the inclusion of silk sericin, all emulsified with Tween 20 to enhance component miscibility (formulation 9). The differing levels of Metronidazole across intended to investigate its impact on the film's properties. The consistent observation of good strength and uniform thickness in all films indicates the

effectiveness of the preparation method in producing stable films with even distribution of components.

3.2. Antimicrobial study

3.2.1. MIC and MBC of Vancomycin on *S. aureus.*

A



B

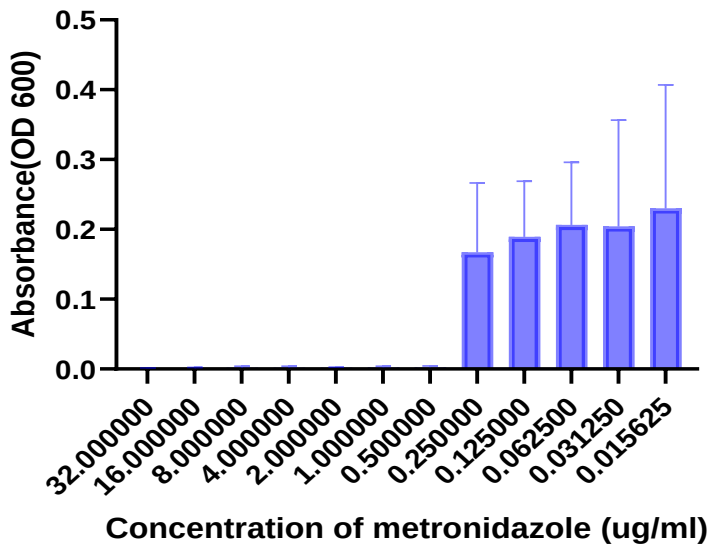
Vancomycin		
MIC (µg/ml)	MBC (µg/ml)	MBC:MIC
2	2	1

Figure 9A: The minimum inhibitory concentration (MIC) of vancomycin against *Staphylococcus aureus*. **Figure 9B**: MBC: MIC ratio of vancomycin against *Staphylococcus aureus.* (Graph made with the help of Graph pad prism).

MIC is defined as the lowest concentration of an antibiotic required to stop visual microbial growth after overnight incubation. The values for MIC of vancomycin on *S. aureus* have been established in samples of clinical analysis, and they show different results. MBC is defined as the lowest concentration of an antibacterial drug that eliminates 99.9% of a primary inoculum. It is evident from the above result that Vancomycin inhibitory to *S. aureus* at the concentration of 2µg/ml. This ratio equals 1, hence it has the bactericidal activity of vancomycin against staphylococcus aureus MBC = MIC. The result shows similar observations to the previous research articles (Honda et al., 2011, Moses et al., 2020).

3.2.2. MIC and MBC of Metronidazole against *P. gingivalis.*

A



B

Metronidazole		
MIC (µg/ml)	MBC (µg/ml)	MBC: MIC
0.5	0.5	1

Figure 10A: The minimum inhibitory concentration (MIC) of metronidazole against *P. gingivalis*. **Figure 10B**: MBC: MIC ratio of metronidazole against *P. gingivalis.* (Graph made with the help of Graph pad prism).

Several studies on the MBC of metronidazole against *P. gingivalis* have also been documented, yielding varying results. The bactericidal effect of an anti-microbial agent may be determined using the MBC and MIC relative ratio. The obtained findings demonstrate similar results to previous research that metronidazole has bacteriostatic and bactericidal against *P. gingivalis* at the concentration of 0.5 µg/m (Moses et al., 2020, Larsen, 2002). The ratio MBC to MIC is 1 and shows that metronidazole possesses good bactericidal action for *P. gingivalis.*

3.2.3. Zone of inhibition of films without tween 20

3.2.3.1. Zone of Inhibition of medium molecular weight 80mg PCL films having high concentration of vancomycin (1.25%) against *Staphylococcus aureus* to check the releasing efficiency of the film.

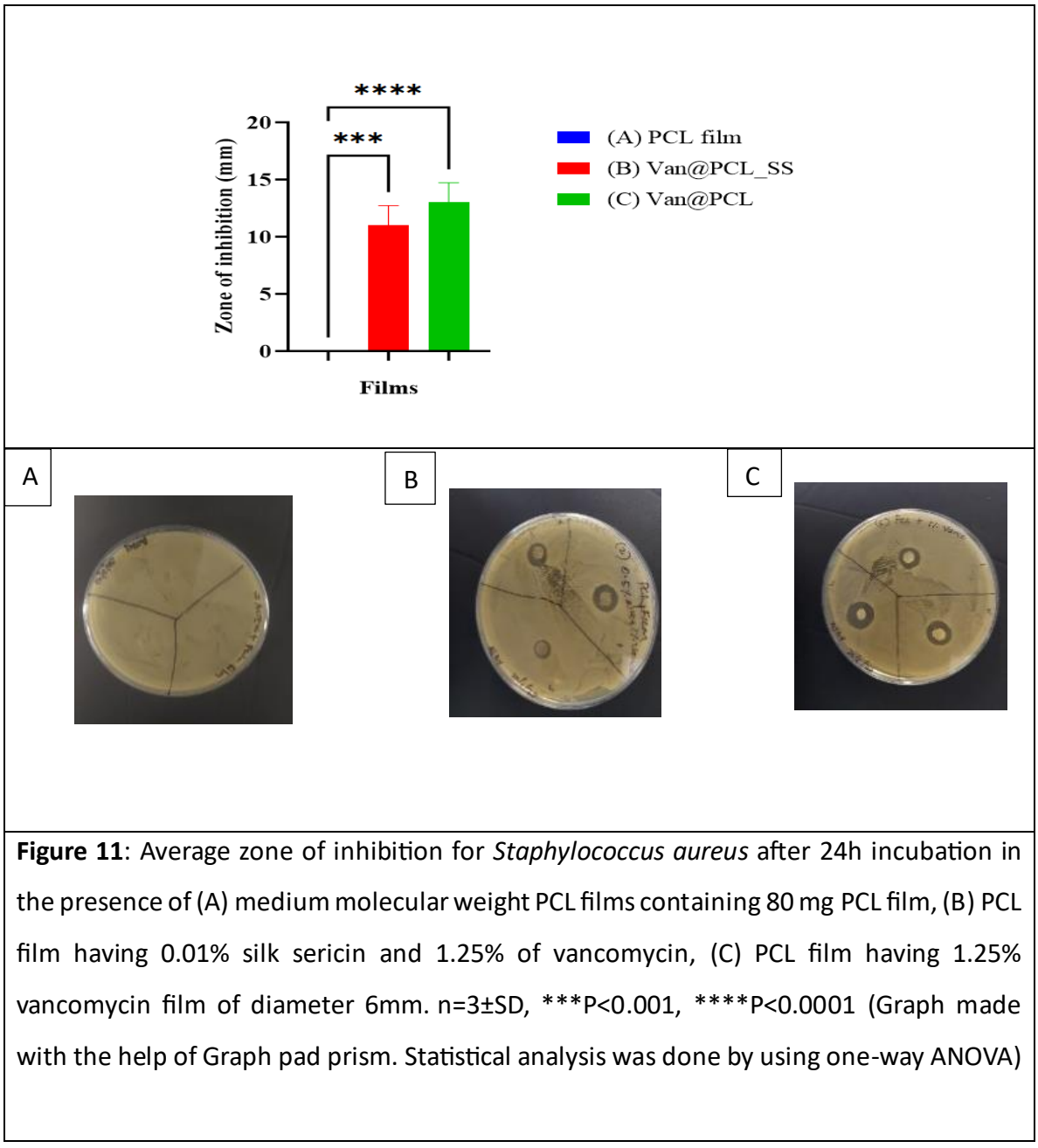


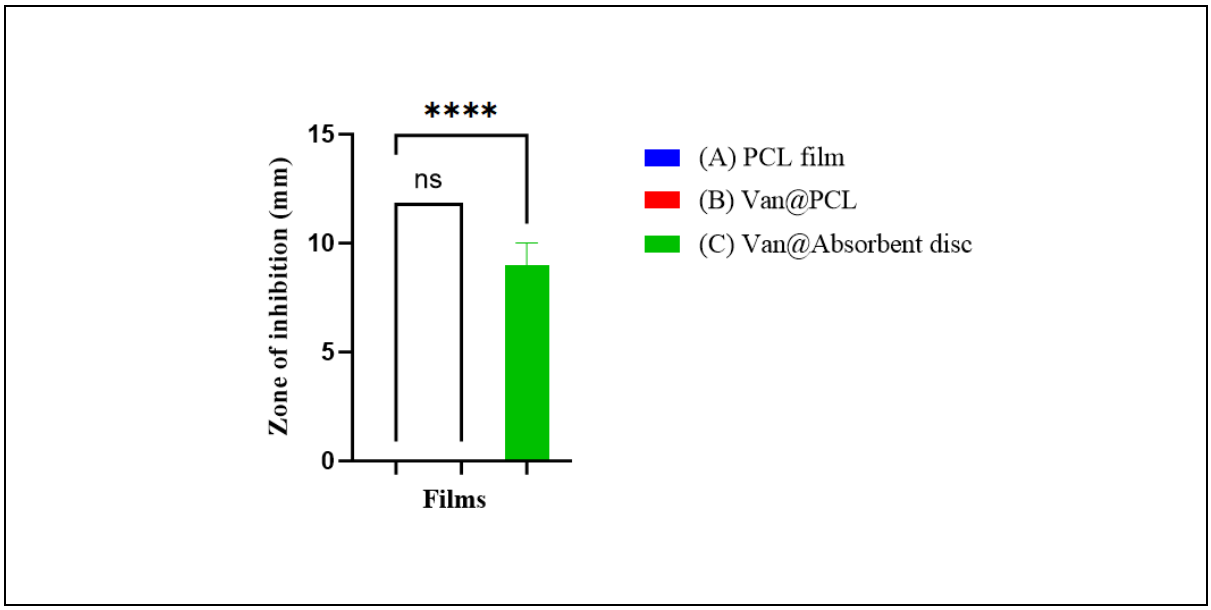
Figure 11: Average zone of inhibition for *Staphylococcus aureus* after 24h incubation in the presence of (A) medium molecular weight PCL films containing 80 mg PCL film, (B) PCL film having 0.01% silk sericin and 1.25% of vancomycin, (C) PCL film having 1.25% vancomycin film of diameter 6mm. n=3±SD, ***P<0.001, ****P<0.0001 (Graph made with the help of Graph pad prism. Statistical analysis was done by using one-way ANOVA)

The bar graph illustrates the average zone of inhibition (ZOI) for Staphylococcus aureus when exposed to different types of films: one negative control (PCL film); one positive control (PCL

film loaded with 1.25% of vancomycin); and the test material (PCL film containing 0.01% silk sericin in combination with 1.25 % vancomycin). Notably, the results display distinct ZOI measurements: The PCL film 0 mm (control), 11 mm, and 13.5mm for the PCL film with 1.25% vancomycin, the PCL film loaded silk sericin & vancomycin. In addition, films with the presence of Silk sericin show a more controlled release of vancomycin.

These results show that 1.25% PCL film with vancomycin can inhibits the growths of *staphylococcus aureus* efficiently. Moreover, a wider zone-of-inhibition (ZOI) suggests the antimicrobial activity for the film impregnated with both silk sericin and vancomycin. Interestingly, some of the past researches shows antibacterial effectiveness of silk Fibroin microspheres loaded with vancomycin against *Escherichia coli* and S. *aureus* (Zhang et al., 2023). Another study using biodegradable poly (vinyl alcohol)-microspheres in silk sericin-based hydrogel found a synergistic effect of combining gentamicin with vancomycin which improved bacterial killing rates as well as biofilm inhibition against methicillin-resistant staphylococcus aureus (Bakadia et al., 2022). The sum of all these individual findings indicates toward the possibility of synergetic effect on antimicrobial action when using vancomycin together with different components derived from silk directed against *staphylococcus aureus* strains.

3.2.3.2. Zone of Inhibition of medium molecular weight 80mg PCL films having 0.025% vancomycin against *staphylococcus aureus.*



A B C

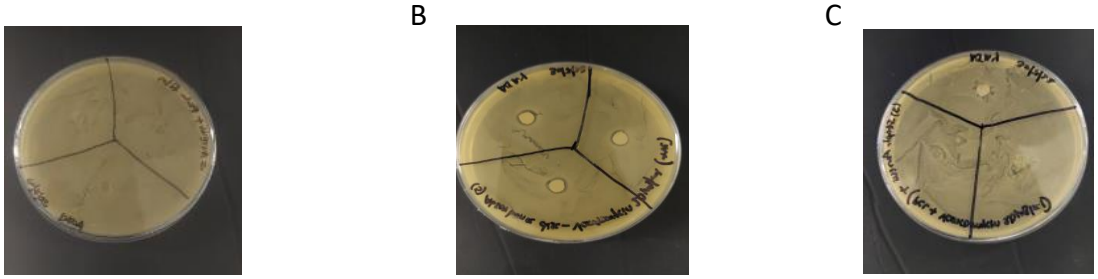


Figure 12: Average zone of inhibition for s*taphylococcus aureus* after 24h incubation in the presence of (A) medium molecular weight PCL films containing 80 mg PCL film, (B) PCL film having 0.025% vancomycin film of diameter 6mm and (C) Absorbent disc having 20ul aliquot of vancomycin at a concentration of 1mg/ml. n=3±SD, **** P<0.0001 (Graph made with the help of Graph pad prism. Statistical analysis was done by using one-way ANOVA)

The bar graph indicates that the ZOI value of negative control film was on average 0 mm, meaning no antimicrobial effect was observed. A positive control disc made up of 20ul aliquot of vancomycin at a concentration of 1mg/ml gave an impression average of 9 mm of inhibition zone, showing antibacterial strength from vancomycin. Similarly, the average ZOI obtained for the PCL film containing 0.025% vancomycin was zero, which indicates that there is no release of vancomycin by the PCL films. The implications of these findings indicate that the PCL films even when loaded up with vancomycin, show no antimicrobial activity. Perhaps, this can occur because of the non-release of the vancomycin from PCL films.

The lack of antibiotic release from the PCL films may be because of several reasons which could include the molecular weight of your PCL film; the percentage by which you loaded the antibiotics in it or how the antibiotics are incorporated into your polymer matrix. Therefore, in the following stage of research, it is determined to increase the percentage of vancomycin loaded.

3.2.3.3. Zone of Inhibition by 80mg PCL films having 0.125% of vancomycin and in presence 0.01% of silk sericin

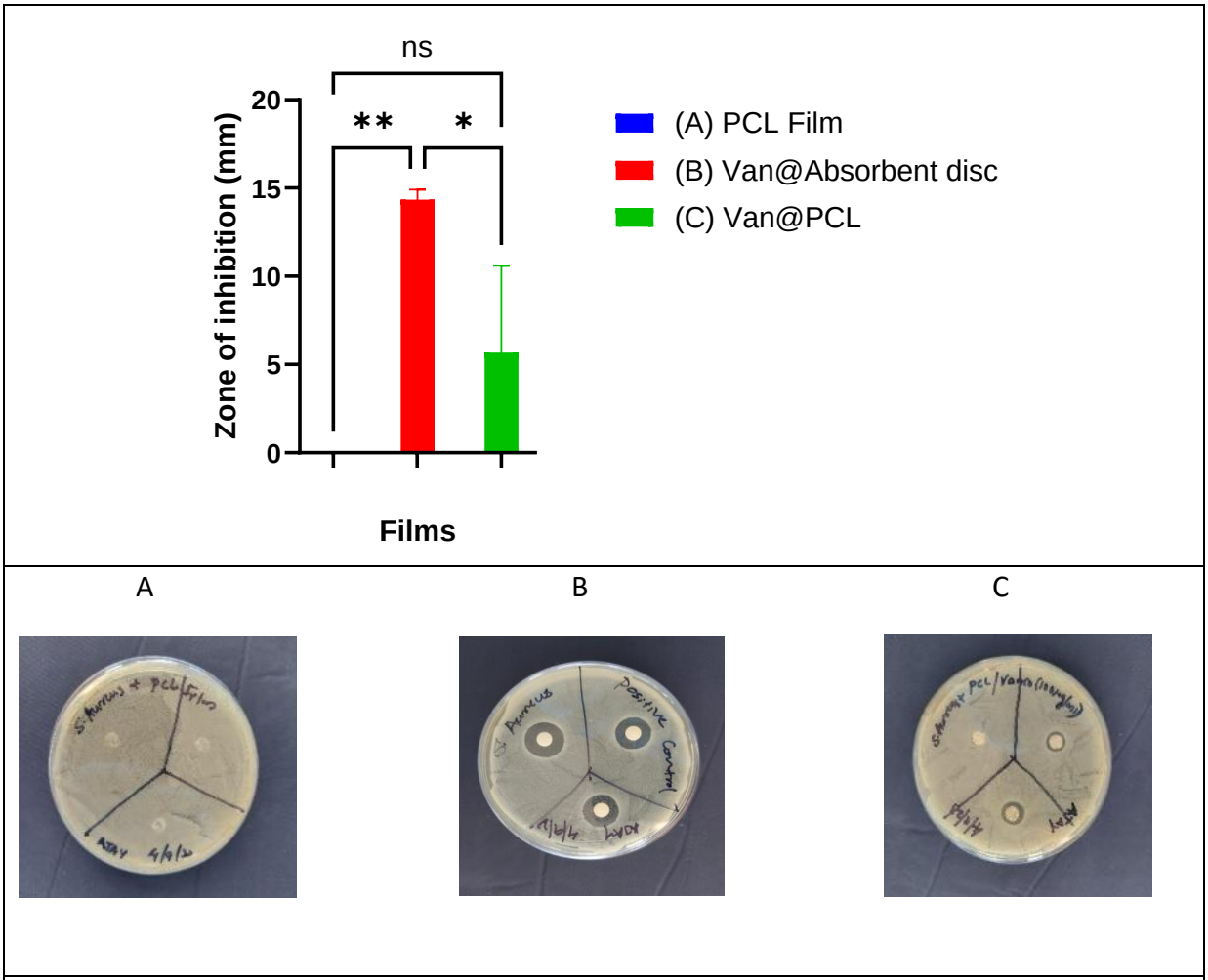


Figure 13: Average zone of inhibition for *staphylococcus aureus* after 24h incubation in the presence of (A) medium molecular weight PCL films containing 80 mg PCL film, (B) PCL film having 0.125% vancomycin film of diameter 6mm and (C) absorbent disc having 50 ul aliquot of vancomycin at a concentration of 2mg/mL. n=3±SD, *P<0.05, **P<0.01 (Graph made with the help of Graph pad prism. Statistical analysis was done by using one-way ANOVA)

Figure shows average ZOI of *staphylococcus aureus* after 24 hours incubation in presence of medium molecular weight PCL films containing 80 mg PCL film, PCL film having 0.125% vancomycin film of diameter 6mm. The findings showed that the negative control medium molecular weight PCL films with 80 mg PCL film had average ZOI of zero, demonstrating absence of *staphylococcus aureus* suppression. There was an average ZOI of 14 mm for the absorbent disc containing 50 ul aliquot of vancomycin at a concentration of 2mg/mL (positive

control), implying substantial inhibition of bacterial growth. This PCL film containing 0.125% vancomycin film of diameter 6mm exhibited an average ZOI of 5.5mm that is smaller than positive control and greater than the negative control thus indicating some level of bacterial growth inhibition.

The release was not observable on all medium molecular weight 80mg PCL films with PCL film 0.125% vancomycin film of thickness 6mm. This may mean that the vancomycin in the PCL film may not have been released effectively enough to prevent bacterial growth. The uneven distribution of vancomycin in the poly-caprolactone film may also be the reason for the lack of its release. A change of the fabrication process was therefore considered as the most appropriate way to address this. A change was made by including tween 20 to aid uniform distribution of antibiotic for greater release.

3.2.4. Zone of inhibition of films made in the presence of tween 20

3.2.4.1. Zone of Inhibition by 80mg PCL films having 0.125% of vancomycin and in presence 0.01% of silk sericin with 0.01% of tween 20.

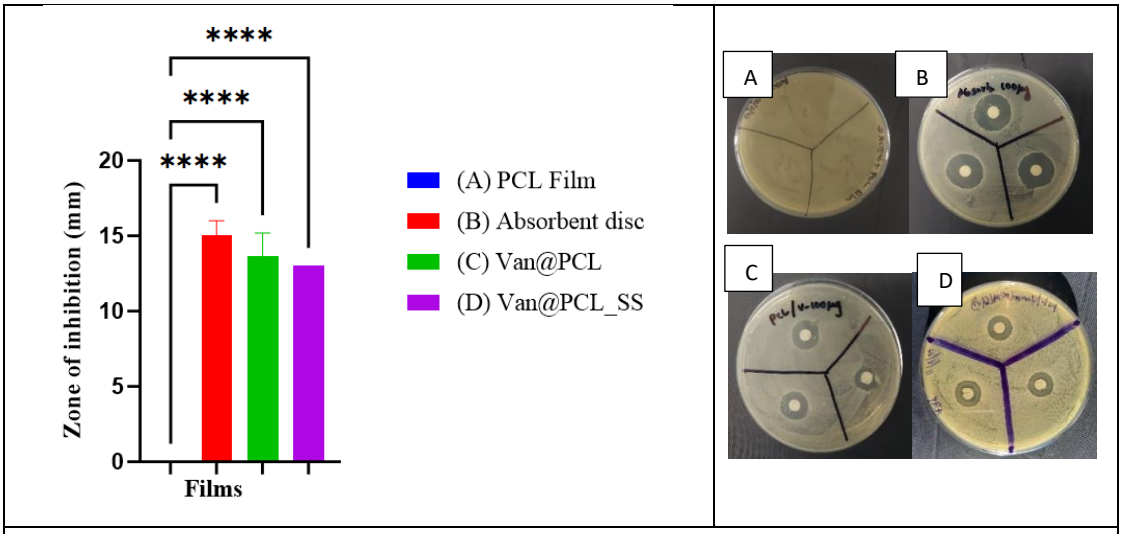


Figure 14: Average zone of inhibition for *staphylococcus aureus* after 24h incubation in the presence of (A) medium molecular weight 80 mg PCL films of diameter 6mm, (B) Absorbent disc having 50 ul aliquot of vancomycin at a concentration of 2mg/mL, (C) vancomycin medium molecular weight 80 mg PCL films having 0.125% vancomycin with 0.01% of tween 20, (D) medium molecular weight 80 mg PCL films having 0.125% vancomycin and 0.01% silk sericin with 0.01% of tween 20. n=3±SD, ****P<0.0001. (Graph made with the help of Graph pad prism. Statistical analysis was done by using one-way ANOVA).

The bar graph shows the average ZOI on *staphylococcus aureus* after 24 hours incubation in different films. The objective of this testing was to determine the antimicrobial properties of numerous film compositions. ZOI was absent in the control one, the baseline (negative control) film including medium molecular weight, PCL 80 mg. The absence of a ZOI indicates that this film variant does not demonstrate any antibacterial activity in this case.

By contrast, the positive control in the experiment was an absorbent disc with 50 ul aliquot of vancomycin at a concentration of 2mg/mL that showed a mean zone of inhibition of 15 mm. This result demonstrates that vancomycin is a strong antibacterial against *staphylococcus aureus*. Additional film varieties were used to investigate the effects of additives on antimicrobial capabilities. The PCL film (80 mg) with 0.125% vancomycin and 0.01% tween 20 showed a mean ZOI of 13.5mm for antimicrobial action. This shows that the incorporation of these additives is a factor for the film's antibacterial properties.

As expected, another film type with 0.125% vancomycin, 0.01% silk sericin, and 0.01% Tween 20 had a mean ZOI of 12.5 mm. However, this means that the inclusion of silk sericin in the films may affect vancomycin release kinetics. Combined use of vancomycin silk sericin, which would have led to a slow release of vancomycin possibly reduced the ZOI.

Moreover, the choice of 0.125% vancomycin for films is justified by the results from Live/dead staining analysis-confocal laser microscopy.

3.2.4.2. Zone of Inhibition by 80mg PCL films having 0.03% of metronidazole and in presence 0.01% of silk sericin with 0.01% of tween 20.

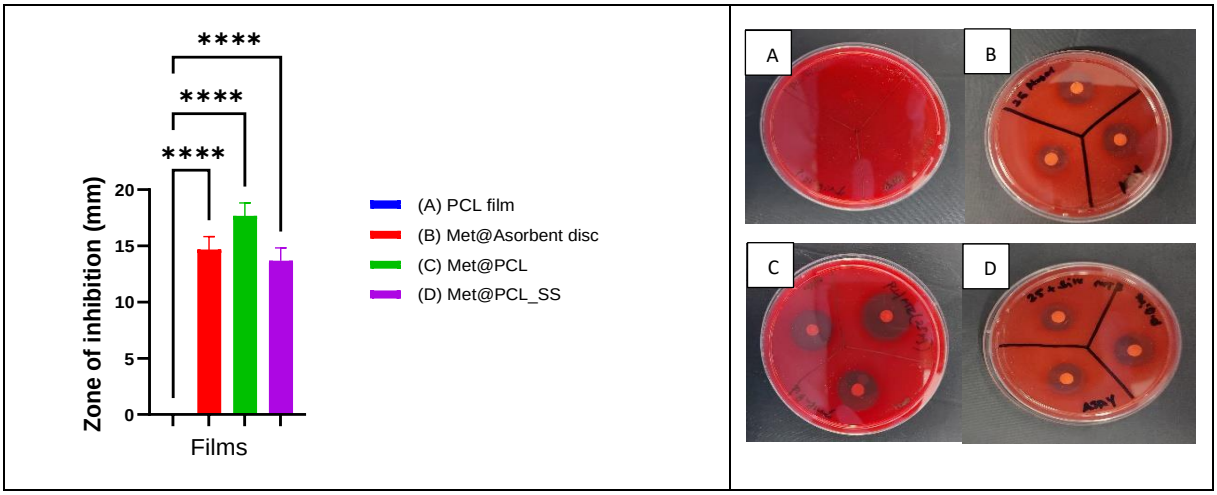


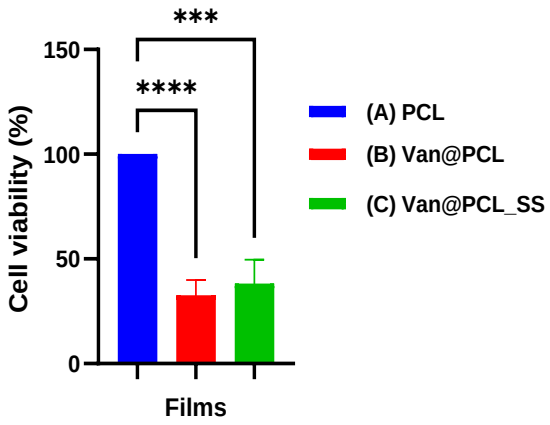
Figure 15: Average zone of inhibition for *P. gingivalis* after 5 days incubation at 37°C in an anaerobic jar in the (A) presence of medium molecular weight 80 mg PCL films of diameter 6mm, (B) Absorbent disc having 50ul aliquot of vancomycin at a concentration of 1.25 mg/ml, (C) medium molecular weight 80 mg PCL films having 0.03% metronidazole with 0.01% of tween 20, (D) medium molecular weight 80 mg PCL films having 0.03125% vancomycin and 0.01% silk sericin with 0.01% of tween 20. n=3±SD, ****P<0.0001 (Graph made with the help of Graph pad prism. Statistical analysis was done by using one-way ANOVA)

The aim of this experiment was to check if medium molecular weight polycaprolactone (PCL) films loaded with metronidazole could inhibit the growth of *P. gingivalis*. Average of ZOI was recorded after 5 days incubation at 37°C in anaerobic condition. It highlighted some specific ZOI measurements in relation to various film compositions. Significantly, negative control (80 mg medium molecular weight PCL film without metronidazole) did not show any inhibition, whereas the positive control (absorbent disc containing metronidazole) had an average ZOI of 14.5 mm. In addition, adding 0.03% metronidazole and 0.01% Tween 20 to the PCL films yielded an average ZOI of 17.5mm. However, the mean ZOI reduced to 13.5mm when metronidazole was combined with 0.01% silk sericin and 0.01% Tween 20 in PCL films at 0.03%. The reduction implies that metronidazole may be slower released in presence of silk sericin than the film containing no silk sericin.

The metronidazole concentration used in this study was determined from a confocal laser microscope live/dead staining analysis showing efficacy at 0.03%. However, comparison with other studies where inhibition happened at higher concentrations (5 mg/ml and 2.5 mg/ml) is complicated because of the deficiency in exact description of the formulations used in this study (Serbanescu et al., 2022). The study reveals the effect of different compositions on the release of metronidazole from PCL films and their potential in preventing *P. gingivalis* growth.

3.2.5. Live/dead bacterial viability using confocal laser scanning microscopy.

3.2.5.1. Live/dead Bacterial Viability of *S. aureus* after treating with Vancomycin films made without the presence of tween 20



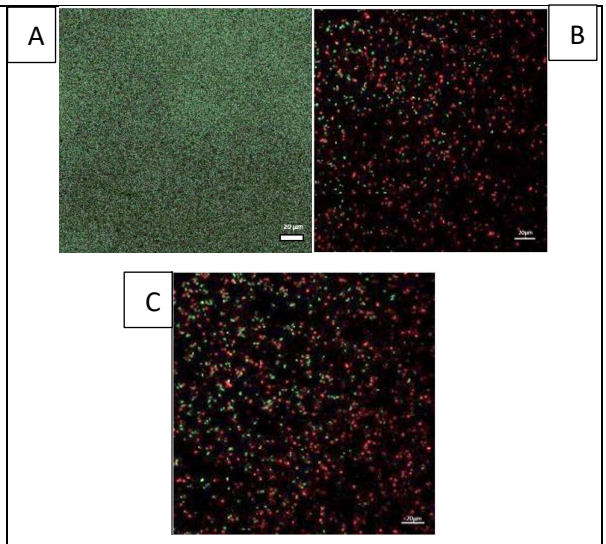


Figure 16: Average cell viability of *staphylococcus aureus* after 24h incubation at 37°C in the presence of (A) medium molecular weight 80mg PCL films, (B) medium molecular weight 80mg PCL films having 0.125% vancomycin without the presence of tween 20 and (C) medium molecular weight 80mg PCL films having 0.125% vancomycin and 0.01% of silk sericin without the presence of tween 20. n=3±SD, ***P<0.001, ****P<0.0001 (Graph made with the help of Graph pad prism. Statistical analysis was done by using two-way ANOVA)

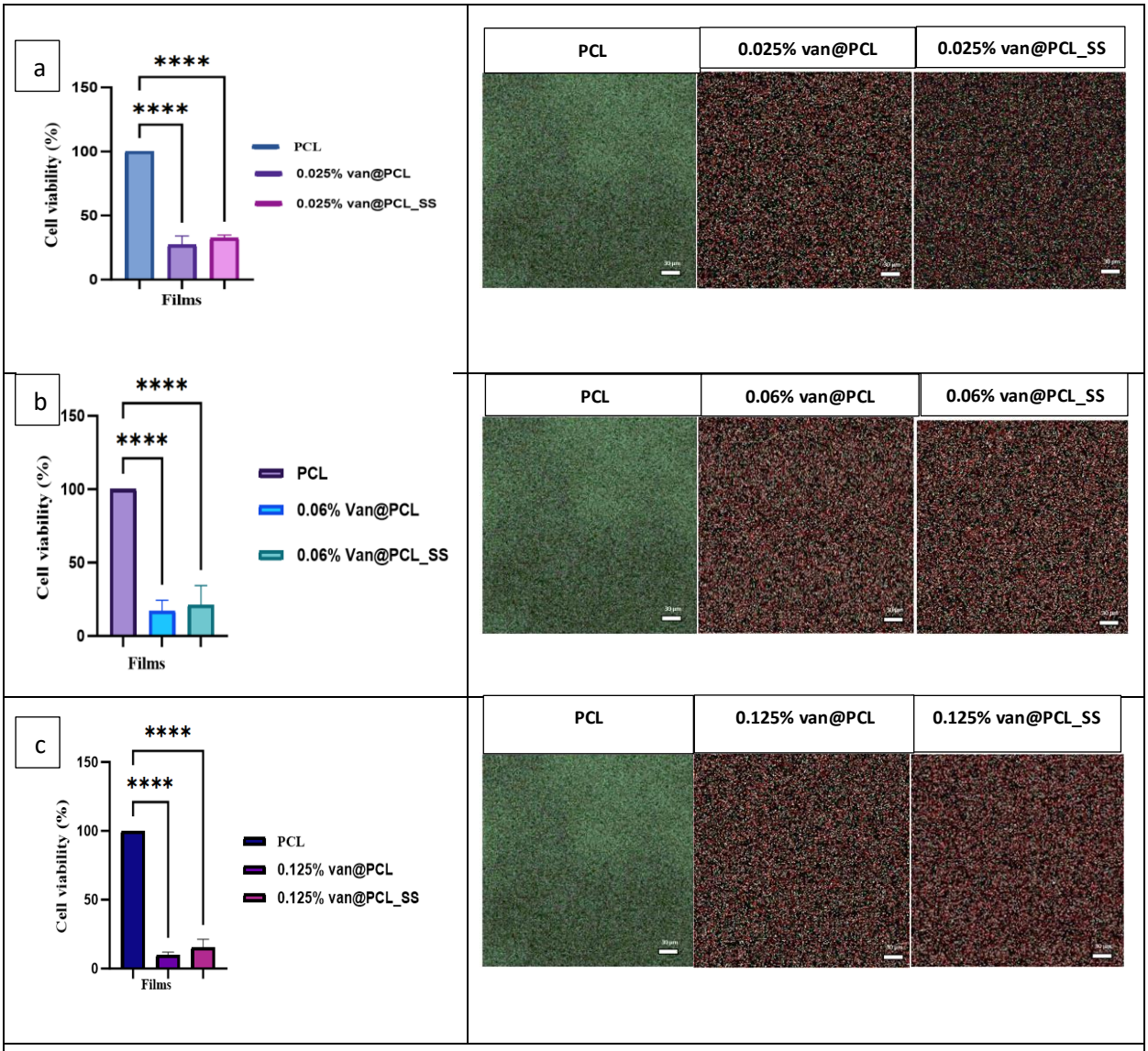
This research tested various medium molecular weight PCL films. These included a control film containing 80 mg PCL, a second film containing 0.125% vancomycin with no tween 20, and a third film with 0.125% vancomycin and 0.01% silk sericin. Addition of silk sericin increase the number of surviving cells compared to films, which did not contain sericin. In particular, the film with vancomycin and silk sericin showed 38.15% cell viability compared to 32.51% viability observed in the film with sole vancomycin. In addition, silk sericin might have led to a slow release of vancomycin.

The reason for going for 0.125% vancomycin may be tied to its effectiveness during the zone of inhibition test.

The examination of zone of inhibition and staining of film without tween 20 showed that the antibiotics were not equally distributed. Therefore, films incorporated with tween 20 were

used as control films in remaining experiments to obtain more uniform distribution of antibiotics in the films which improved the accuracy of the experiment.

3.2.5.2. Live/dead bacterial viability of *S. aureus* after treating with vancomycin films made with the presence of tween 20



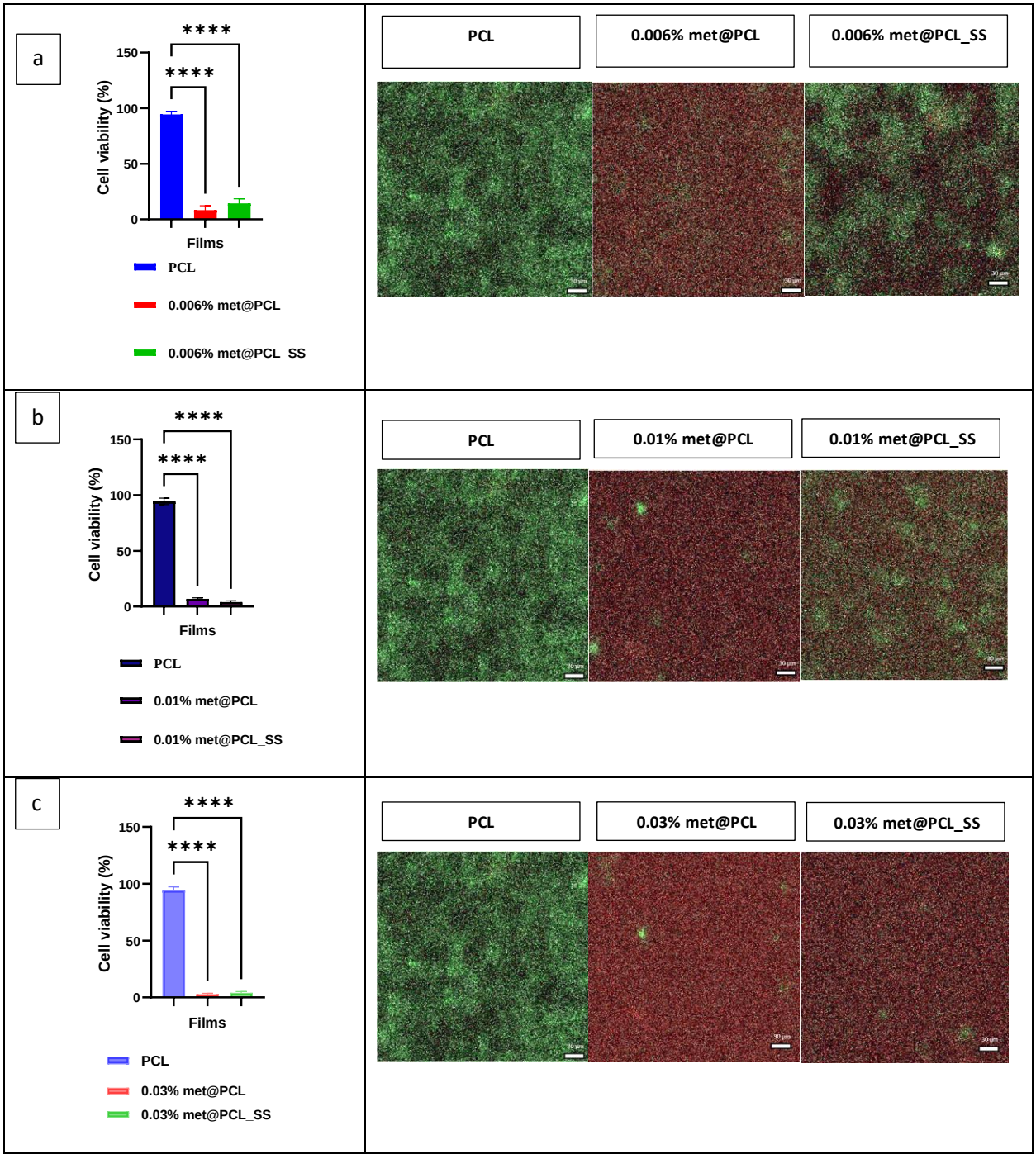
Live/dead bacterial viability of *S. aureus* after treating with vancomycin films made with the presence of tween 20. **Figure 17a**: Medium molecular weight PCL films containing 80 mg PCL, medium molecular weight 80mg PCL films with 0.025% vancomycin in the presence of tween 20, and medium molecular weight 80mg PCL films with 0.025% vancomycin and 0.01% silk sericin in the presence of tween 20. **Figure 17b:** Medium molecular weight PCL films containing 80 mg PCL, medium molecular weight 80mg PCL films with 0.0625% vancomycin in the presence of tween 20, and medium molecular weight 80mg PCL films with 0.0625% vancomycin and 0.01% silk sericin in the presence of tween 20. **Figure 17c**: Medium molecular

weight PCL films containing 80 mg PCL, medium molecular weight 80mg PCL films with 0.125% vancomycin in the presence of tween 20, and medium molecular weight 80mg PCL films with 0.125% vancomycin and 0.01% silk sericin in the presence of tween 20. n=3±SD, ****P<0.0001 (Graph made with the help of Graph pad prism. Statistical analysis was done by using two-way ANOVA).

Above are the bar graphs representing the mean cell viability of staphylococcus aureus exposed in different conditions after 24 h incubation at 37°C. The figures shows a comparative analysis: In Figure 17a samples with medium molecular weight 80mg PCL films with 0.025% vancomycin and 0.01% silk sericin, with tween20 depict an improved cell viability (32.57%) than without silk sericin (27.23%). Likewise, in Figure 17b cell viability is increased, 21.07% in case of presence of 0.01% silk sericin with 0.0625% vancomycin and tween 20 compared to without silk sericin (16.94%). This trend is continued in Figure 17c, where more significant cell viability is observed in samples with silk sericin and vancomycin than those without. Therefore, the improved cell viability (15.19%) is demonstrated from samples comprising medium molecular weight 80mg PCL films with 0.125% vancomycin and not with silk sericin, compared to those without the component (9.74%).

The presence of silk sericin in medium molecular weight PCL films notably increases cell viability as compared to films that do not include any silk sericin as provided in the obtained results. This may result from possible reduction in the release of vancomycin by silk sericin. This could be a viable method to slow down the release of antibiotic and enhance its effectiveness in due course. Therefore, the samples medium molecular weight 80mg PCL films having 0.125% vancomycin with presence of tween 20 and medium molecular weight 80mg PCL films having 0.125% vancomycin and silk sericin with presence of tween 20 are subjected to further experiments.

3.2.5.3. Live/dead bacterial viability of *P. gingivalis* after treating with metronidazole films made with the presence of tween 20



Live/dead bacterial viability of *P. gingivalis* after treating with metronidazole films made with the presence of tween 20. **Figure 18a**: Medium molecular weight PCL films containing 80 mg PCL, medium molecular weight 80mg PCL films with 0.006% metronidazole in the presence of tween 20, and medium molecular weight 80mg PCL films with 0.006% metronidazole and 0.01% silk sericin in the presence of tween 20. **Figure 18b**: Medium molecular weight PCL films

containing 80 mg PCL, medium molecular weight 80mg PCL films with 0.015% metronidazole in the presence of tween 20, and medium molecular weight 80mg PCL films with 0.015% metronidazole and 0.01% silk sericin in the presence of tween 20. **Figure 18c**: Medium molecular weight PCL films containing 80 mg PCL, medium molecular weight 80mg PCL films with 0.03% metronidazole in the presence of tween 20, and medium molecular weight 80mg PCL films with 0.03% metronidazole and 0.01% silk sericin in the presence of tween 20. n=3±SD, ****P<0.0001 (Graph made with the help of Graph pad prism. Statistical analysis was done by using two-way ANOVA).

This was a study to determine the bactericidal effect of different compositions of PCL films, under anaerobic conditions at 37 °C for 24 hours. A notable finding was that negative controls (medium molecular weight PCL films with 80 mg of PCL) had an average cell viability of 94.37%.

The three figures illustrated different compositions of PCL films and their influence on the cell viability of cells. The incorporation of 0.006% metronidazole and 0.01% silk sericin with tween 20 presented an average cell viability of 11.18% (Figure 18a) compared to the sample without silk sericin (8.35%). Also, in figure 18b, the sample containing 0.015% metronidazole and 0.01% silk sericin showed a cell viability of 9.62% compared to the sample without silk sericin (6.87%). In figure 18c, showed that the 0.03% metronidazole with 0.01% silk sericin increased value of cell viability of 9.5487% versus no silk sericin (2.82%).

It was established that when there was silk sericin, cell viability was higher than when it was absent. It may be due to the slow release of metronidazole in the presence of the silk sericin. Therefore, the samples with 0.03% of metronidazole with 80 mg/ml PCL (medium molecular weight) with and without containing 0.01% silk sericin in the presence of tween 20 will be considered for further experiments. This was done since they showed lesser cell viability as compared to the others.

3.2.5.4.Bacterial viability assessed by colony forming unit (CFU)

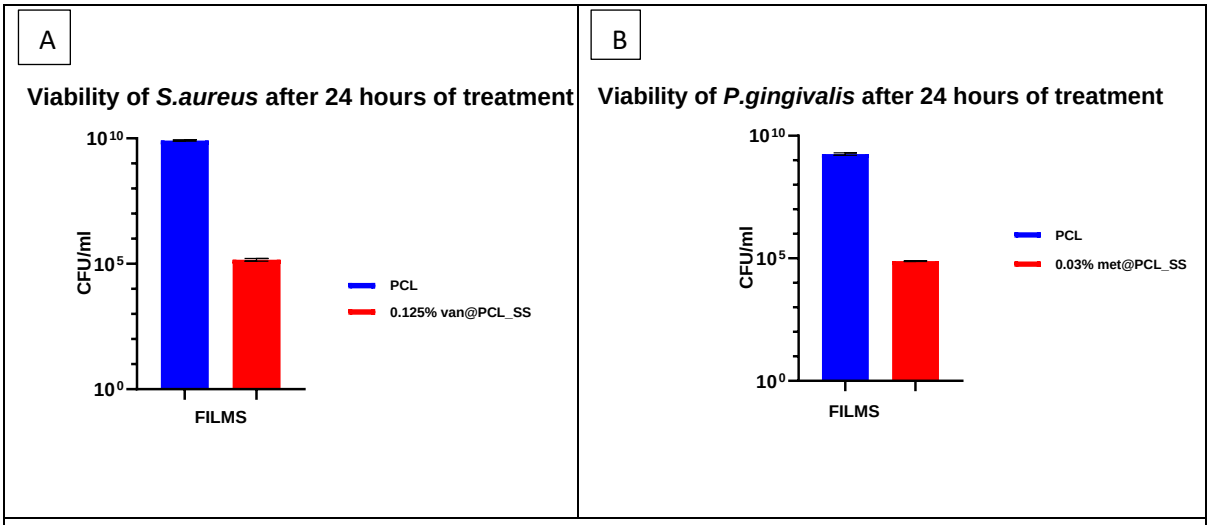


Figure 19A: *S. aureus* bacteria viability assessed by colony forming unit (CFU) when treated by medium molecular weight 80 mg PCL, medium molecular weight 80 mg PCL having 0.125 % of vancomycin in the presence of 0.01% Tween 2, medium molecular weight 80 mg PCL having 0.125 % of vancomycin and 0.01% of silk sericin in the presence of 0.01% Tween 20.

Figure 19B: *P. gingivalis* viability assessed by colony forming unit (CFU) when treated by medium molecular weight 80 mg PCL, medium molecular weight 80mg PCL film having 0.03125% metronidazole in the presence of 0.01% tween 20, medium molecular weight 80mg PCL film having 0.03125% metronidazole with 0.01% silk sericin in the presence of 0.01% tween 20.

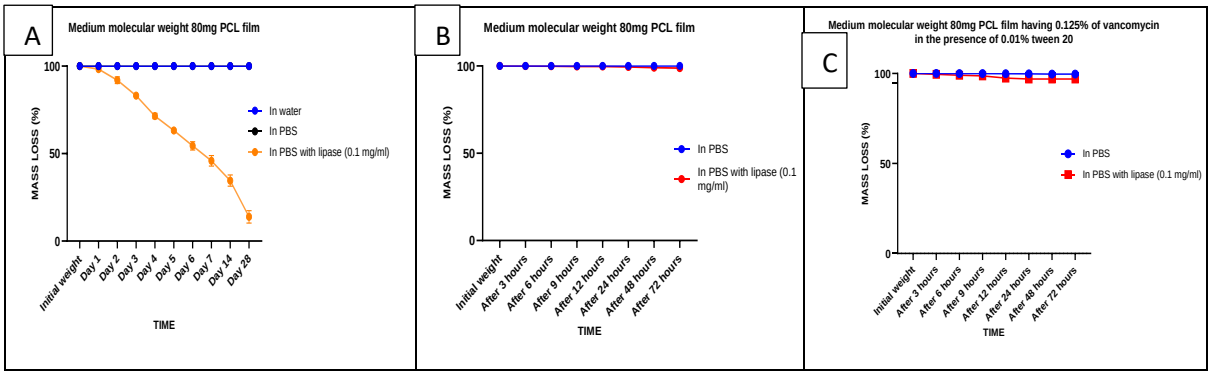
(Graph made with the help of Graph pad prism).

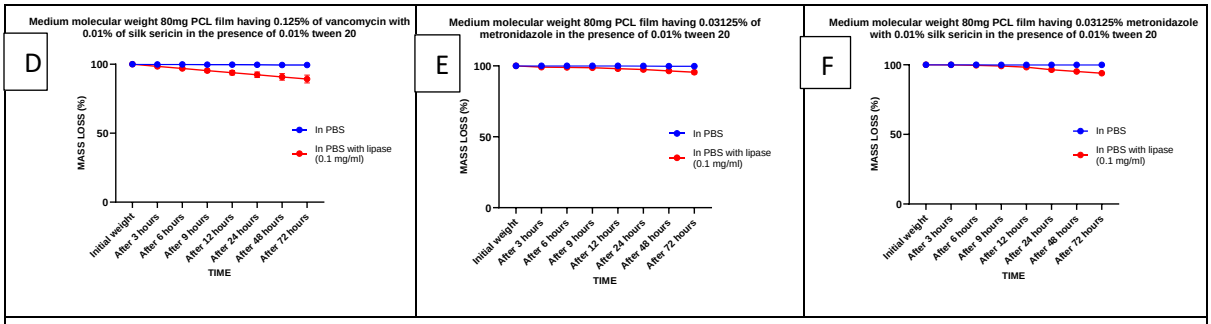
Figure 19A is relatively comparing bacterial concentrations in two distinct film samples represented as colony forming units per millilitres (CFU/ml). In this graph, the x-axis refers to various film samples and the y-axis demonstrates mean CFU/ml from replicate determinations against each of these. The graph-based evaluation of *S. aureus* concentration in CFU/ml allows comparing the efficacy between two PCL film formulations. The first film is just a sheet made of plain medium molecular weight PCL at 80mg. The second film is also at 80mg PCL, but combined with vancomycin, silk sericin and tween 20. For plain PCL film, the average CFU/ml is 1.33 x10^8, while for the PCL with silk sericin it changes to 4.52 $x10^5$.

The modified PCL film with vancomycin, silk sericin and tween 20 has a decrease in bacterial concentration of almost 3-log compared to the unmodified film. This shows that, with these compounds incorporated, they have significantly more effective antibacterial properties. Vancomycin might have conferred the antibacterial activity, whereas silk sericin and tween 20 would have aided in the dispersion of vancomycin uniformly throughout the film and to improve its release rate.

Figure 19B is about comparison of two PCL film formulations in terms of *P. gingivalis* concentration, CFU/ml. The first film is simple medium molecular weight PCL at 80mg. The second film is 80mg medium molecular-weight PCL with 0.03125% metronidazole, 0.01% silk sericin and 0.01% tween 20. The count of films produced with plain PCL was 1.85x10^8 CFU/ml and 5.18x10^5 CFU/ml for those made with PCL that were modified with metronidazole, silk sericin and tween 20. From the over 3-log reduction, the additive greatly improved the antibacterial properties of PCL film. The enhanced antibacterial activity might have arisen from an increase in effect of metronidazole, silk sericin and tween 20. As an antibiotic, the main antibacterial action was by way of metronidazole. Silk sericin and tween 20 may have provided for homogenous distribution and controlled release of metronidazole within PCL matrix. The active inhibition of bacteria was thus achievable for an extended period.

3.3. MASS LOSS STUDY





Mass loss study. **Figure 20A**: Medium molecular weight 80mg PCL film, **Figure 20B:** Medium molecular weight 80mg PCL film under different conditions: in the presence of lipase and in PBS. **Figure 20C**: Medium molecular weight 80mg PCL film having 0.125% vancomycin in the presence of 0.01% tween 20. **Figure 20D**: Medium molecular weight 80mg PCL film having 0.125% vancomycin with 0.01% silk sericin in the presence of 0.01% tween 20. **Figure 20E**: Medium molecular weight 80mg PCL film having 0.03125% metronidazole in the presence of 0.01% tween 20. **Figure 20F**: PCL film was loaded with 0.03125% of metronidazole and silk sericin with 0.01% tween 20. (Graph made with the help of Graph pad prism).

Figure 20A shows the pattern of increasing mass loss percentages per day in lipase-containing PBS solutions indicated a time dependent destruction of the PCL film. This data clearly shows a trend, it begins at 1.67% average mass loss on Day 1, indicating an initial and the slow degradation. Nevertheless, the degradation rate increases significantly from day 2. Mass loss percentages continue to increase and have averages of 5.53%, 13.51%, 27.13% and 86.67% on Days 2, 3, 4 and 28 respectively. The fact that these percentages of mass loss exhibit this pattern very strongly indicates an exponential increase of the PCL film's degradation that occurs because of its exposure to the lipase containing PBS solution. Starting from day 4 the mass loss increases very sharply to above 20 per cent every following day. This shows the catalytic influence of the enzyme on the breakdown of the polymer. However, the sharp increase to the degradation rates beyond Day 4 means that the lipase acts more efficiently to cause more damage with time. Such an acceleration corresponds to the normal behaviour for enzymatic activity that increases along with the reaction.

Figure 20B illustrates the degradation of medium molecular weight 80mg PCL (polycaprolactone) film under different conditions: in the presence of lipase and in PBS. However, no mass loss was seen in PBS which demonstrated the PCL film checked stable under

this environment. The above lack of degradation implies that the PCL material does not degrade in the PBS during this experiment period. But in PBS with lipase (0.1mg lipase in 1 ml PBS), the results showed that PCL films was degraded. The mass loss data observed shows that PCL material has broken down slowly near the presence of the enzyme. The recorded percentages of mass loss are the measure of degradation at various time intervals. In the beginning, 0.05% was lost within three hours. It rose steadily and by the 72nd hour, it reached 1.18%. The increase in the mass loss reflects the ability of lipase enzymes to act on PCL film thereby causing its degradation within the duration of the experiments. However, the data proves that in combination with PBS, the presence of enzyme triggers the degradation of PCL film. The variations in the percentage of mass loss in figure 20A and 20B may due to the difference in the mass of the films.

The **Figure 20C** details the degradation behaviour of a medium molecular weight 80mg PCL (Polycaprolactone) film having 0.125% vancomycin in the presence of 0.01% tween 20 in two distinct environments: PBS and in PBS containing lipase, an enzyme that hydrolyses lipids such as PCL. The PCL film in PBS environment proved stability over the measured period through no observable mass loss in PBS condition without lipase introduction. The absence of degradation in the presence of a specific enzyme suggests that PCL film did not structurally change or alter over the given time frame in PBS Only. On the other hand, evidence showed that when the PCL film is immersed in PBS containing lipase, there was stepwise degradation. The PCL film was gradually lost over time, indicating its degradation by the enzymatic action of lipase according to the initial measurements. The degradation followed an increasing trend from minor mass loss in early hours to a higher degradation rate with subsequent time intervals, but after the 24th hour, it seemed to have reached a plateau at 3.06%, where it stagnated until the end of the 72 hours.

Figure 20D represents the mass loss of medium molecular weight 80mg PCL film having 0.125% vancomycin with 0.01% silk sericin in the presence of 0.01% tween 20 in two different environments: PBS and PBS containing lipase enzyme (0.1mg lipase/ml PBS). The differences in mass loss at particular time intervals between the two conditions lend insight into the impact of lipase on the degradation of the PCL film. The mass loss of the PCL film is relatively low with time in PBS. At 3 hours to 24 hours, this is as low as 0.09% and up to only 0.35% degradation, respectively. A rapid rise is observed at 48 hours to 0.52% and by the 72-hour

mark; a substantial increase to 1.52% is noticed. The fact that this degradation is gradually describes the breakdown of the PCL film in PBS slowly, with a more predominant shift after the first 48 hours. Conversely, PCL film kept in PBS containing lipase shows accelerated degradation. With the presence of lipase enzyme, the breakdown rate is much faster compared to PBS alone environment. The mass loss is much more marked, whereby a notable increases is visible in the first 24 hours. The degradation started at 1.53% by 3 hours, to a higher value by much over five folds reaching the deterioration peak of degrading was 7.65% at 24 hours and finally ending up with the highest peak after 72 hours which is equal to 10.71%. This data suggests that the PCL film experiences a remarkably quicker release upon the introduction of the lipase enzyme; meaning that the presence of lipase catalyses a faster degradation process.

The degradation profile of a medium molecular weight 80mg PCL film having 0.03125% metronidazole in the presence of 0.01% tween 20 in different environments at a specific time period is presented in **Figure 20E**. Without lipase, there is noticeable mass decrease as time progresses, in the phosphate-buffered saline (PBS) solution. Degradation also tends to increase over the next 72 hours. The film shows virtually no degradation for the first 12 hours with a mass loss of just 0%. Indeed, it is a fact that the trend of degradation visible is minimal as time goes on. Only 0.05% of the film is degraded after 24 hours, which later increases to 1.18% at the end of 72 hours. The slow and noticeable degradation in the case of PBS confirms the fact that metronidazole film on PCL is stable in this environment. On the other hand, the degradation rate of PCL film increases dramatically when it is immersed in lipase PBS. Lipases are enzymes, that decompose lipids and in this case, they appear to assist in the degradation of PCL film. This however is not the case in the PBS only where small degradation occurs. The first noticeable increased loss in mass is in the 12^{th} hour, almost 2%. After 72 hours the film has experienced mass loss of approximately 4.5% in comparison to its degradation under PBS alone.

Figure 20F represents the mass loss of a specific PCL film under two distinct conditions: in PBS (Phosphate Buffered Saline) and in PBS containing lipase; a group of enzymes that breakdown lipids. PCL film was loaded with 0.03125% of metronidazole and silk sericin as well as added with 0.01% tween 20. The loss of mass was consistently low throughout the experiment in PBS that lacks lipase enzyme. The weight, which was already in minus 0.05% over 72 hours

has not changed. These data indicate that without the lipase enzyme the PCL film formulation suffered minor degradation within the tested period. However, in the PBS solution with lipase, the PCL film's loss mass was relatively higher over time. The degradation progression was noted through increasing percentages of mass loss. After 72 hours, the mass loss was found to be about 6.02%, which was quite high compared to the behaviour of film in the absence of enzyme, after incubation with the lipase.

The degradation of the films was conducted by using Lipase from pseudomonas cepacia, and incubating at 37°C.

Some studies shows that the PCL films with 2 to 8 wt % lipase and studied their enzymatic degradation under static conditions for up to 8 days of incubation (Khan et al., 2019).

3.4. In-vitro release study

3.4.2. Complete degradation study

The first set of experiments revolved around vancomycin-loaded-PCL films (table 1). However, in the absence of silk sericin, the yield of the final product is 75 mg approximately with a percentage yield of about 93.75%. In this instance, the drug loading capacity (LC) is approximately 0.122%. This implies that the production process for vancomycin-loaded PCL without silk sericin is very efficient yielding a relatively high yield and drug-loading capacity.

The yield percentage reduces to about 92.5% when silk sericin is added to the vancomycin-loaded PCL films, giving a yield of approximately 74 mg. Nevertheless, the LC is only affected to a limited extent of around 0.118%. Although the inclusion of silk sericin results in a slight decrease in drug quantity and percentage yield in the final product, it is not significant and a lot of its uniformity in capacity for the drug remains.

Table 2: Comparison of two formulations of vancomycin and metronidazole loaded films with and without silk sericin.

Loaded sample	Yield (mg)	Yield (%)	LC (%)

Medium molecular weight 80 mg PCL having 0.125 % of Vancomycin in the presence of 0.01% Tween 20	75±1.000	93.75±1.750	0.122±0.002
Medium molecular weight 80 mg PCL having 0.125 % of Vancomycin and 0.01% of silk sericin in the presence of 0.01% Tween 20	74±3.034	92.5±3.771	0.118±0.002
Medium molecular weight 80mg PCL film having 0.03125% metronidazole in the presence of 0.01% tween 20	73±2.039	91.25±2.548	0.0273±0.0006
Medium molecular weight 80mg PCL film having 0.03125% metronidazole with 0.01% silk sericin in the presence of 0.01% tween 20	74.6±2.016	93.25±2.520	0.027±0.0005

In the second set of experiments which involved metronidazole-loaded PCL films, without silk sericin, the total yield obtained for the final product was about 73 mg with a yield percentage of 91.25%. In the current metronidazole LC percent is around 0.0273%. The yield and yield percentage of adding metronidazole to PCL films is lower than those of vancomycin, indicating differences in the properties and behavior of these drugs.

The incorporation of silk sericin into the metronidazole-loaded PCL films increases the yield to approximately 74.6mg and the yield percentage up to 93.25 %. The LC is quite stable and at around 0.027%. Interestingly, the incorporation of silk sericin, in this case, seems to increase yield and yield percentage/ which suggests that the association between silk sericin and metronidazole-loaded PCL may be more favourable, which leads to a higher-end-product outcome.

In conclusion, these data demonstrate that the effect of silk sericin on PCL films depends on the incorporated drug. The direction and magnitude of the impact will be determined by the specific drug being used, while the addition of silk sericin seems to have a marginal effect on yield and yield percentage.

3.4.3. Dissolution study

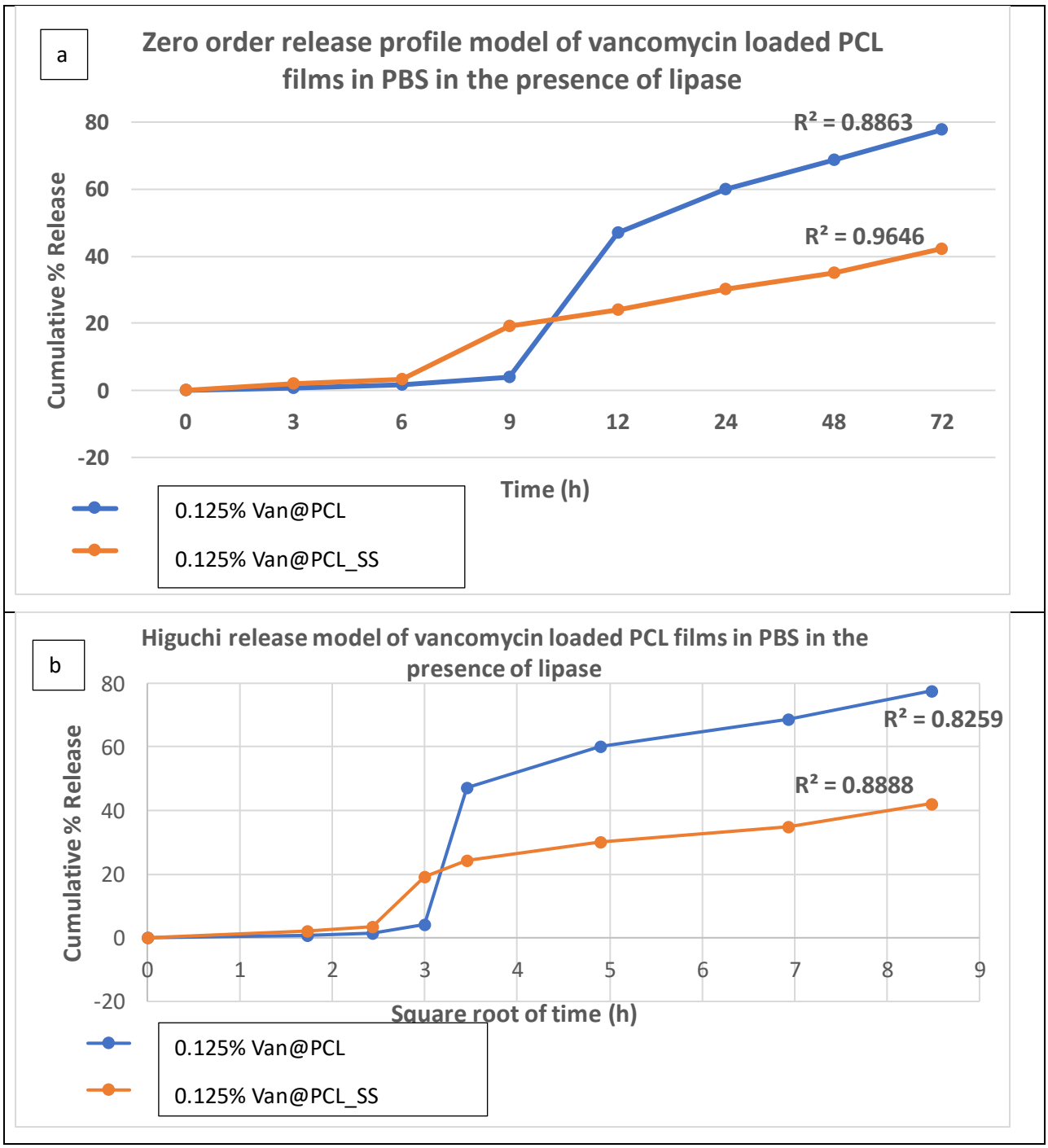
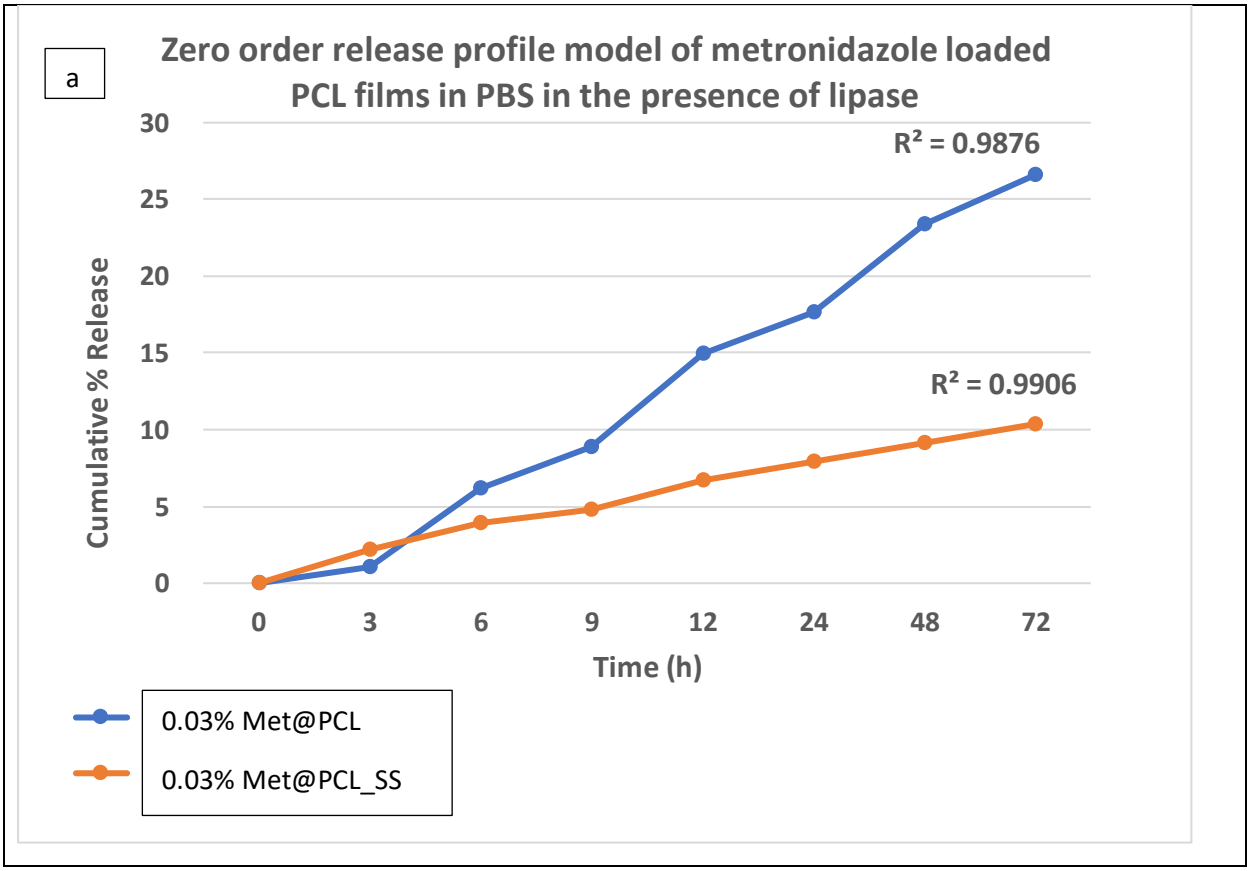


Figure 21: Dissolution studies of 0.125% vancomycin loaded medium molecular weight 80mg PCL with (0.125% Van@PCL_SS) and without silk sericin (0.125% Van@PCL) in PBS with lipase (0.1mg/ml), denoted as two release kinetics- [zero order (a)], [Higuchi model (b)].

Note: The concentration of antibiotics is calculated by equation received from standard curve. (Graph made up with help of Excel software).



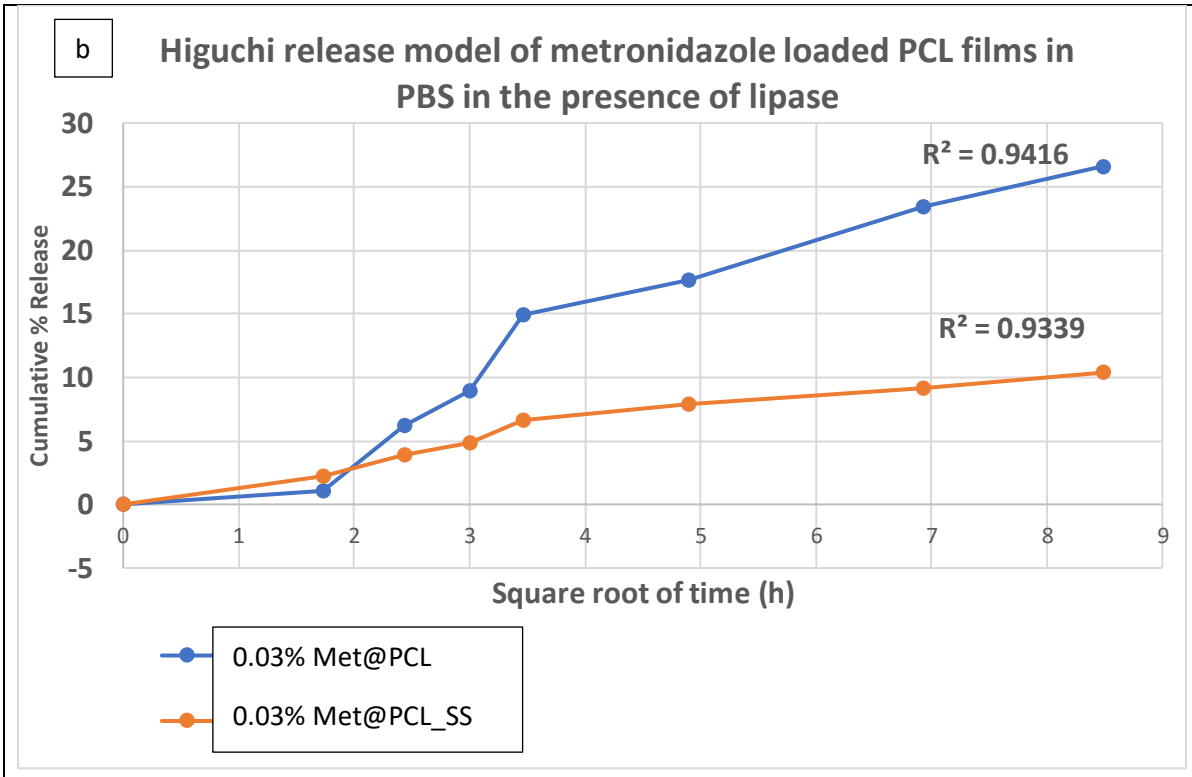


Figure 22: Dissolution studies of metronidazole loaded medium molecular weight 80mg PCL with (0.03% Met@PCL_SS) and without silk sericin (0.03% Met@PC) in PBS with lipase (0.1mg/ml), denoted as two release kinetics- [zero order (a)], [Higuchi model (b)].
Note: The concentration of antibiotics is calculated by equation received from standard curve. (Graph made up with help of Excel software).

The above graphs illustrate the release profiles of two drugs – that is metronidazole and vancomycin – from polycaprolactone (PCL) films in the presence of various additives including lipase enzyme in phosphate-buffered saline (PBS). Different release profiles modelled by zero-order and Higuchi release models used in the study are demonstrated between sericin films and those without.

First, the zero-order release profile represents the percentage cumulative release of drugs as a function of time. Indeed, quite a notable change in the release pattern of vancomycin is observed when 0.01% of silk sericin is added to PCL films with Tween 20. The statistics show a significant rise in R^2 from 0.8863 to 0.9646 upon the introduction of silk sericin. This increase is indicative of a relatively linear and sustained drug release pattern for vancomycin, which may imply that silk sericin controls the release of this drug from the PCL films.

The zero-order model for metronidazole also displays a tendency of this type. The PCL films containing silk sericin whose linearity of the drug release profile increases with the addition of Tween 20 will lead to an increase in R^2 value. Therefore, silk sericin also appears to have a modulating effect on the release rate of metronidazole providing more control and sustained pattern of release.

The diffusion-based Higuchi release model involving the plot of cumulative percentage release versus square root time, was suitable for describing the drugs' release mechanism. This indicates that diffusion from the PCL films is the main controlling mechanism of release kinetics.

These findings indicate the importance of incorporating a small amount of silk sericin into the PCL films for improved controlled release of both drugs. This is observed from an increase in the linearity of the zero-order release and diffusion-based release suggested by the Higuchi model. The zero-order release profiles of vancomycin and metronidazole from various formulations of polycaprolactone (PCL) films with and without silk sericin in the presence of 0.01% Tween 20. The cumulative percentage release of these drugs over fixed time variations is expressed using values.

In the case of vancomycin, the data reveals notable differences between PCL films with and without silk sericin. Then, the lack of silk sericin results in a steady release of vancomycin from the PCL films with 0.125% vancomycin and 0.01% Tween 20 with time. Yet, the presence of 0.01% silk sericin in the films leads to a considerably lower vancomycin release rate. At each time point, the total release of vancomycin is much less in the presence of silk sericin as compared to no silk sericin. Release in 72 hours is 42.238% compared to the 77.67% without silk sericin.

In the same way, metronidazole shows an alteration of its release profile due to the introduction of silk sericin in PCL films. Both formulations also show an increase in the cumulative release of metronidazole over time, and there is again a slower release in the presence of silk sericin. In the presence of silk sericin, the cumulative percentage release at each time point is much less than in its absence. In 72 hours, the release is cumulative 26.6% with sericin silk while that without the sericin silk is 10.36867%.

Therefore, the observation of the release kinetics of vancomycin and metronidazole from PCL films where 0.01% silk sericin was incorporated reveals that there is a significant influence in the presence of this additional protein on drug delivery. The rates of drug release for both drugs are much higher, and the action is perceived to be more controlled even sustained compared to the sericin-less formulations. This implies that silk sericin is crucial in the control of drug release from the PCL films, thus enabling controlled and prolonged therapeutic effects for vancomycin and metronidazole.

3.5. Wettability measurement

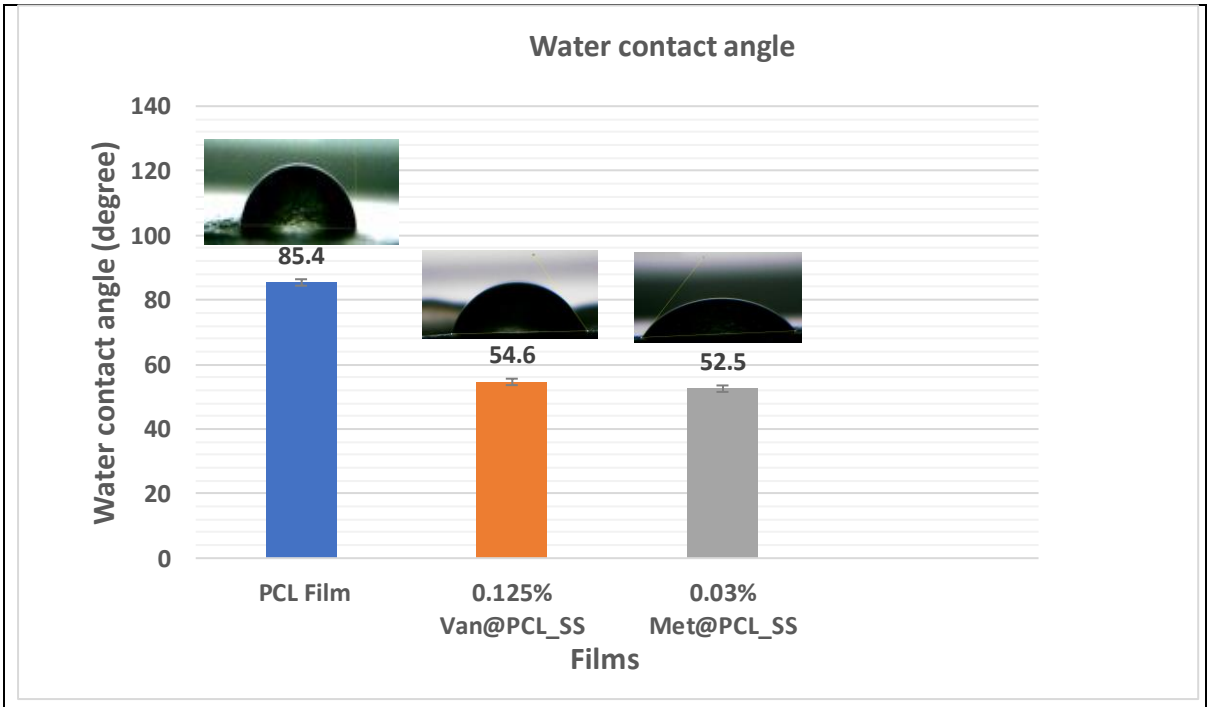


Figure 23: Water contact angle measurements of medium molecular weight 80 mg PCL film, medium molecular weight 80 mg PCL film containing 0.125% vancomycin in the presence of 0.01% tween 20, medium molecular weight 80 mg PCL film having 0.03125% metronidazole and 0.01% silk sericin together with 0.01% Tween 20, vancomycin, and metronidazole as control. (Graph made up with help of Excel software)

The figure depicts water contact angle and helps in determining hydrophilic or hydrophobic characteristic of certain films. This study shows different features for three different categories

of film in this respect. The first film, consisting of low molecular weight PCL of 80mg, has a water contact angle of 85.4 degrees, which is relatively hydrophobic. On the other hand, the second film, made of medium molecular weight PCL (80 mg), combined with vancomycin (0.125%), silk sericin (0.01%), has a water contact angle of 54.6 degree. Likewise, the third movie made up of 0.03125% metronidazole, 0.01% silk sericin, and tween 20 together with 80mg of medium molecular weight PCL shows an angle of contact of These low contact angles indicate more hydrophilicity than the first film.

The increased hydrophilicity in these three films could be as a result of the inclusion of water soluble antibiotics and tween 20. The modification results in a more hydrophilic character of the films which favors their adhesion to the periodontal pocket. This attribute becomes especially beneficial in biomedical applications, which imply greater adhesion to biological tissues.

3.6. In-vitro cytocompatibility study

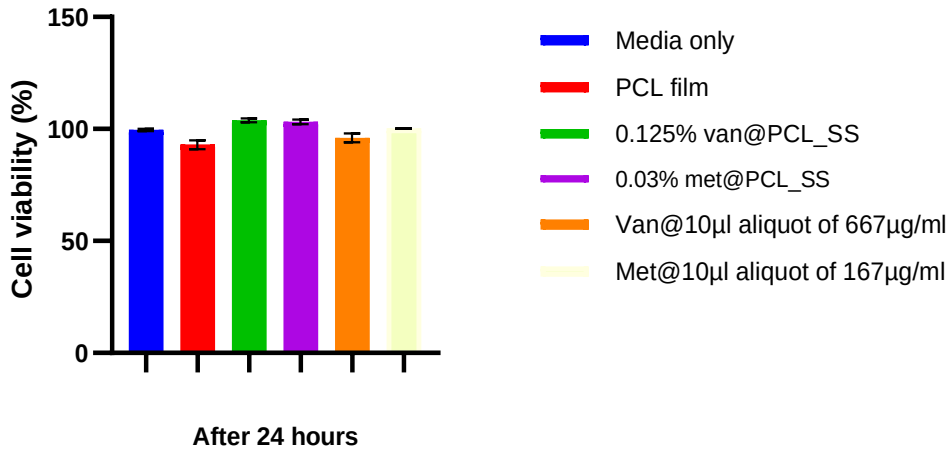


Figure 24: Cytocompatibility study of human gingival fibroblasts after treating with medium molecular weight 80 mg PCL film, medium molecular weight 80 mg PCL film containing 0.125% vancomycin in the presence of 0.01% tween 20, medium molecular weight 80 mg PCL film having 0.03% metronidazole and 0.01% silk sericin together with 0.01% Tween 20, vancomycin (10ul aliquot of 667µg/ml), and metronidazole (10ul aliquot of 167µg/ml as control. (Graph made with the help of Graph pad prism).

Note: The concentration of controls (vancomycin and metronidazole) is determined from the calculation of amount of antibiotic present in the film. 5mg weighed 6mm diameter films are added as treatment

A unique point of view on the impact of different types of the film on cell viability is provided by the MTT assay of HGF (human gingival fibroblasts (HGF) cells. The x and y axes of the bar graph represent different films and cell viabilities of HGF respectively. It is interesting to note that films with silk sericin were found to be especially effective in promoting cell proliferation as reported earlier (Ming et al., 2022). Silk sericin may promote favourable cellular reactions, which, perhaps, is because of the biocompatibility inherent in this material plus its ability to support cell growth. For comparison, a media-only group was taken as the baseline with 100% cell viability. Cell viability for the medium molecular weight 80 mg PCL film slightly decreased to 93.779±2%. The loss of cell viability could be due to polymer degradation, which alters the cellular reaction.

However, the 80 mg PCL film containing 0.125% vancomycin and 0.01% silk sericin together with 0.01% Tween 20 surprised for its cell viability which was at 104±2%. Similarly, the 80 mg PCL film with 0.03% metronidazole and 0.01% silk sericin in the presence of 0.01% tween 20 showed high cell viability (103±2%) implying that silk sericin together with metronidazole may enhance cellular viability.

Moreover, Vancomycin exhibited a cell viability of 96.1±3% at 10ul aliquot of 667µg/ml, while Metronidazole showed a cell viability of 100±1% at 10ul aliquot of 167µg/ml when tested alone. The presence of antibiotics by itself was not enough to substantially suppress cell survival but in some instances may even have increased positive responses. Decreased cell viability observed in the PCL film alone may indicate that material parameters do play a role in cell-cell interaction. Nevertheless, this heightened cellular viability in the presence of both silk sericin and antibiotics within the films suggests that customized film formulations can impact the proliferation of the cells.

SUMMARY AND FUTURE DIRECTION

This research concentrated on the development of periodontal films through utilization of medium molecular weight PCL as a binder and other additives including vancomycin, metronidazole, silk sericin, and tween 20 to find out their influence on films. For all film formulations, good strength and constant thickness were consistently observed in all experiments, thus confirming successful preparation of stable homogeneous films consisting of uniformly distributed constituents. Vancomycin showed bactericidal properties against *Staphylococcus aureus* while metronidazole indicated a bacteriostasis and bactericidal against *P. gingivalis*. Inclusion of silk sericin in some formulations containing antibiotics show a slow release, with the result that the zone of inhibition may be reduced probably due to the delayed release of the antibiotics from the formulation.

Other studies such as cell viability and degradation analysis showed increase in cell viability in the presence of silk sericin. This could be attributed to slower release of antibiotics. Degradation studies from the film showed that lipases break down PCL films and release profiles indicated that drugs have the best controlled release from PCL films especially with addition of silk sericin pointing at sustained therapeutic effects. The water contact angles of the films indicated alteration of hydrophilicity caused by antibiotics and tween 20 leading to enhanced bio adhesiveness. The films were also cytocompatibility with human gingival fibroblasts, with antibiotic compositions showing increased cells survival. However, cell viability in plain PCL films slightly declined, likely resulting from the breakdown of the polymer.

To optimize the films for future directions, it is important to further optimize film compositions to improve antimicrobial properties, understand exactly how silk sericin affects release kinetics of the films, and evaluate the long-term stability of the films in biological environments. Also, analysing the exact methods through which bacteria are blocked, fine-tuning release rate of drugs and studying effects of additives on integration of tissues might lead to creation of the new efficient and biocompatible implant films for periodontal diseases. Comprehending the part played by each additive and the sum of their effects will matter for designing periodontal films for clinical purposes.

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