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# **Mucoadhesive Thin Films For The Simultaneous Delivery Of Microbicide And Anti-Inflammatory Drugs In The Treatment Of Periodontal Diseases**

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## ABSTRACT

There is an unmet clinical need for new products to address the high percentage of the populous who present with periodontal diseases. Drug dose retention at the point of application would facilitate sustained release and more efficacious treatments. The aim of this study was to evaluate mucoadhesive polymeric thin films for simultaneous *in situ* delivery chlorhexidine and anti-inflammatory and analgesic drugs. Mucoadhesive thin films were prepared using a polymer mixture containing chlorhexidine (25 mg) ± diclofenac sodium (10 and 50 mg), and lidocaine hydrochloride (10 mg) or betamethasone dipropionate (10 and 50 mg). The films were assessed for *in vitro* drug release and localised tissue delivery, followed by determination of modulated prostaglandin E2 (PGE<sub>2</sub>) levels in *ex vivo* tissue and cytotoxicity using a HaCaT keratinocyte cell line. Antibacterial activity of the chlorhexidine/diclofenac film was determined against planktonic and biofilm bacteria associated with periodontal disease and dental plaque. Chlorhexidine release was consistently low (up to 10 % of initial loading) from all films, whereas the release of diclofenac, betamethasone and lidocaine exceeded 50 % within 30 min. The 50 mg betamethasone film released up to 4-fold more than the 10 mg film. Statistically significant reduction of PGE<sub>2</sub> was observed in *ex vivo* porcine gingival tissue for films containing chlorhexidine with or without diclofenac, and betamethasone. No cytotoxicity was observed for any film, apart from 50 mg betamethasone at 24 h. Films loaded with chlorhexidine and diclofenac were inhibitory against relevant test bacteria. Between 3-6 log<sub>10</sub> reductions in bacterial cell recovery was observed after biofilm exposure to the chlorhexidine films irrespective of the presence of the anti-inflammatory or anaesthetic. This work demonstrated that thin film formulations have the potential to simultaneously counter key causative factors in periodontal diseases, namely associated bacteria biofilm and chronic local inflammation.

Gingivitis, periodontitis, chlorhexidine, anti-inflammatory, COX-2, cytotoxicity, polymeric thin film, biofilm

## 1. Introduction

Periodontal diseases are a series of pathological conditions affecting the supporting tissues of teeth. Periodontal disease typically commences with relatively mild gingivitis followed by chronic and aggressive periodontitis and ultimately necrotising periodontitis. Periodontal disease has a very high prevalence and globally affects 50-90% of the adult population with up to 15% experiencing advanced periodontitis (Petersen and Ogawa 2005). The underlying cause of periodontal disease is biofilm formed by opportunistic pathogens (*e.g. Porphyromonas gingivalis*) on the subgingival tooth surface (Schwach-Abdellaoui et al. 2000; Pihlstrom et al. 2005; Johnston et al. 2013). A subtle shift in microbial composition of this biofilm occurs with the presence of increasing numbers of Gram-negative proteolytic bacteria (Marsh 1989; Schwach-Abdellaoui et al. 2000; Jain et al. 2008). These pathogens produce an array of inflammatory factors that initiate host immune responses, resulting in high levels of proinflammatory cytokines. Tissue destruction occurs from the combined effect of bacterial derived proteolytic enzymes and ensuing host immune responses. Without timely intervention, the underlying gingival supporting structures for the tooth are affected leading to the progressive destruction of connective tissues and periodontal ligament (Jain et al. 2008; Johnston et al. 2013). Continued production of prostaglandins means that periodontitis ultimately results in destruction of alveolar bone and tooth supporting connective tissues, loosening of teeth and eventual tooth loss (Pihlstrom et al. 2005; Page 1991). Importantly, periodontal disease is also associated with a number of systemic diseases including cardiovascular disease and diabetes (Page 1998; Teeuw et al. 2016).

Current treatment of gingivitis involves improved oral hygiene through mechanical cleaning such as toothbrushing and flossing, together with use of dentifrices and antibacterial mouthwashes. Non-surgical treatment methods for periodontitis include mechanical scaling and root planing as well as root debridement, with clinical trials showing associated reductions in total microbial load (Winkel et al. 1998; Johnston et al. 2013). Studies have shown that combining mechanical approaches with optimal oral hygiene reduces gingival inflammation and clinical probing depth and improved periodontal attachment levels. Locally administered antibiotics, local antiseptic agents and systemic antibiotics have an additional benefit in reducing probing depth and attachment levels compared with scaling and root planing alone (Schwach-Abdellaoui et al. 2000; Hung and Douglass 2002) and pharmaceutical products have been used in combination with mechanical approaches (Pihlstrom et al. 2005; Ryan 2005). According to expert opinion (Pihlstrom et al. 2005) and NICE guidelines (2012), it is suggested that systemic antimicrobial therapy should be reserved in conjunction with mechanical approaches for patients

with advanced, chronic and aggressive periodontitis that cannot be managed with mechanical therapy alone. This is also the situation for true refractory cases that are unresponsive to conventional treatments or who have fever and lymphadenopathy (Slot 2004; Ryan 2005).

Local drug delivery is site-specific and eliminates or reduces systemic absorption (Ryan 2005; Ahmed et al. 2009). In recent years, the use of mucoadhesive polymers in buccal drug delivery has received much interest (Salamat-Miller et al. 2005; Smart 2005; Khutoryanskiy 2011; Shaikh et al. 2011). As the gingiva is a moist, mucosal surface, mucoadhesive polymeric materials can interact with mucosal glycoproteins via hydrogen bonding, Van der Waals forces, and electrostatic attraction. Once mucoadhesion has been achieved, loaded drug experiences greater retention at the site of application and facilitating sustained release (Andrews et al. 2009; Khutoryanskiy 2011). Such films also have the potential for self-administration. As periodontal disease is caused by a combined effect of biofilm and chronic inflammation it follows that addressing both aspects, simultaneously will provide a more efficacious treatment. Chlorhexidine has broad-spectrum activity (Leikin and Paloucek, 2008) with a prolonged bacteriostatic action in the oral cavity through its adsorption onto tooth enamel (Bonesvoll et al. 1974; Jenkins et al. 1988; Schwach-Abdellaoui et al. 2000). As pro-inflammatory responses to persistent microbial challenge in periodontitis also lead to local tissue destruction (Jain et al. 2008; Johnston et al. 2013), inclusion of an anti-inflammatory agent e.g. betamethasone dipropionate (Williams et al. 1989; Ahmed et al. 2009) would also be beneficial.

In this study, we hypothesised that a dual action mucoadhesive topical drug delivery system could be developed to facilitate drug dose retention at the point of application on the gingiva and provide the slow release of chlorhexidine with diclofenac, lidocaine or betamethasone. The aims of this study were to develop a range of thin films and evaluate their dissolution, drug release, anti-inflammatory activity, cytotoxicity and antimicrobial activity.

## **2. Materials and methods**

### *2.1. Materials*

Diclofenac sodium, betamethasone dipropionate, chlorhexidine digluconate (20% solution), polyethylene glycol (PEG) 400, trifluoroacetic acid ( $\geq 99.0\%$ ), Hanks buffer, protease peptone, trypticase peptone, yeast extract, KCl, haemin, vitamin K<sub>1</sub>, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), L-cysteine, HCl, glucose, Mueller Hinton agar and Mueller Hinton broth were all obtained from Sigma-Aldrich, Gillingham, UK. Phosphate buffered saline (PBS) tablets, Dulbecco's modified Eagle's medium, fetal calf serum (FCS), L-

glutamine, amphotericin B, penicillin G, streptomycin sulfate, HPLC-grade water, acetonitrile and ethanol were obtained from Fisher Scientific (Loughborough, UK). Carbopol 917 (CP) was a gift from Noveon Inc., Cleveland, U.S.A. Hydroxypropylmethyl cellulose was a gift from Shin-Etsu Chemical Co. (Tokyo, Japan). Primary human gingival fibroblasts were purchased from LGC standards, UK. Pig heads were obtained from a local abattoir and used within 2 h of slaughter.

## *2.2. Preparation of drug-loaded thin films*

Unless otherwise stated all experiments were undertaken in triplicate. A series of thin films were prepared containing binary combinations of chlorhexidine with diclofenac, lidocaine or betamethasone. A 1% (w/v) polymeric solution of HPMC, PEG400 and Carbopol 917 was prepared in deionised water (Peh and Wong 1999; Chan et al 2016). This was then divided into 4 x 50 mL portions, prior to drug incorporation as detailed in Table 1. Mixtures were stirred overnight at room temperature to obtain a homogenous mixture, before being placed in an ultrasonic bath for 2 h. Once fully dispersed and degassed, 50 mL of solution was poured into a Petri dish and placed in an oven at 60°C until completely dry (approximately 24 h). The film was carefully removed from the Petri dish, inspected for any imperfections and cut according to the size required for subsequent testing.

## *2.3. Determination of dissolution time*

Thin films were prepared with different levels of PEG400: 40 – 88.8 % (w/w) of total polymer content. Once dry, 1 cm diameter film samples were excised using a cork-borer, immersed in 1 mL PBS buffer in microcentrifuge tubes and left in a 37°C water bath for 10 min. The time taken for film samples to visually dissolve was recorded.

## *2.4. Determination of drug release*

Aliquots of 1 mL pH 7.4 PBS buffer were added to microcentrifuge tubes containing 1 cm thin film samples. After a predetermined time, the film was removed and the solution transferred to a 2 mL autosampler sample vial for HPLC analysis. Simultaneous drug release profiles were plotted as mass ( $\mu\text{g}$ ) as a function of time; for chlorhexidine/diclofenac the percentage release data were also determined, along with release from single drug films.

### *2.5. Drug localisation in freshly excised porcine gingiva, in vitro*

Samples (1 cm diameter) of mucoadhesive films containing diclofenac and chlorhexidine were pressed onto porcine gingiva at 37°C. After 2 h or 6 h the residue of the film was removed using a wetted cotton bud and the diffused area excised by blunt dissection using a scalpel. The tissue was homogenised and extracted into 2 mL water then centrifuged prior to HPLC analysis.

### *2.6. Quantitative analysis by HPLC*

HPLC was performed using an Agilent 1100 instrument fitted with a Phenomenex Kinetex 5 µm C18 150x4.6 mm column, with flow rate on 1.5 mL/min, injection volume of 20 µL and detection by UV. Other conditions are described in Table 2.

### *2.7. Modulation of prostaglandin E<sub>2</sub>, PGE<sub>2</sub>, levels in porcine gingiva, ex vivo*

Gingival tissue was excised from the jaw of a freshly slaughtered pig by blunt dissection under constant irrigation in Hanks balanced salt buffer. Tissue was cut into small pieces and placed in tubes containing 1 mL of Hanks balanced salt previously incubated overnight containing a 1 cm<sup>2</sup> film section or with test drugs directly added into the buffer. These were then incubated for 3 h in a water bath at 32°C and upon removal, tissues were immediately frozen in liquid nitrogen and ground to a fine powder with a pestle and mortar. To this was added 20 mL of acetone. The tube was thoroughly vortex mixed prior to being centrifuged. The supernatant was evaporated at 50°C, and the residue was taken up in 350 µL of ELISA assay kit buffer before being thoroughly vortex mixed and analysed for PGE<sub>2</sub> content using an ELISA kit (ADI-900-001, Enzo Life Sciences, supplied by Fisher Scientific, Loughborough, UK), with n = 2 or 3.

### *2.8. Cell proliferation/cytotoxicity*

An MTT assay was used to determine cytotoxicity of the released test drugs to primary human gingival fibroblasts by measuring cellular metabolism (Riss et al. 2013). Cells were seeded at  $7.3 \times 10^3$  per well in 100 µL of DMEM supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, and antibiotics/antimycotics (100 U/mL penicillin G, 100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B), and incubated overnight at 37°C in an atmosphere of 5% CO<sub>2</sub> to allow the cells to adhere. The medium was removed, and the cells washed with PBS. Aliquots (100 µL) of drug medium, based on the maximal concentrations of drug released, were

added to three wells and incubated at 37°C in 5 % CO<sub>2</sub> for 1, 6 and 24 h. The drug medium was then removed and 100 µL of DMEM and 50 µL of MTT solution added to the cells. The MTT solution was added to a further three cell-free wells as a negative control, then incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for 2 h. The medium was gently removed and 100 µL of dimethyl sulfoxide added and incubated for 30 min. Absorbance of the wells was read at 540 nm and 'No Drug' values were set to 100 % cell viability.

### *2.9. Microbiological evaluation of the chlorhexidine and diclofenac thin film: zone of inhibition (ZOI) and anti-biofilm*

Test microorganisms were cultured on Mueller Hinton agar at 37°C for 24 h prior to sub culture in 10 mL of MHB, which was incubated at 37°C for a further 24 h. Test bacteria included those species associated with dental plaque and also of relevance to periodontal disease and included both Gram-positive facultative anaerobes and Gram-negative anaerobes. *Streptococcus* species, including *S. mutans* were used in all microbiological testing and Gram-negative anaerobes were included in agar diffusion assay and biofilm assays. A standardised inoculum of each test microbe in MHB was obtained by measuring optical density at 600 nm with broth addition until an OD range of 0.08-0.13 was obtained (equivalent to a 0.5 McFarland standard). Standardised cultures were directly used for ZOI assays where the bacterial preparation was spread evenly over an MHA plate. Medicated and drug-free films were cut to standard size and placed on the inoculated agar surface. Agars were incubated at 37°C for no less than 24 h. The ZOI was then measured from the film to the edge where microbial growth was noticeable. The combination films were tested to determine if the antimicrobial activity of chlorhexidine was affected or potentiated by diclofenac sodium.

The antibiofilm activity of test films was assessed against mixed species biofilms prepared in a constant depth film fermenter, with biofilm medium (BM) prepared as described previously (McKee et al. 1985; Hill et al. 2010). Briefly, mixed species biofilms were cultured anaerobically (80% N<sub>2</sub>, 10% CO<sub>2</sub>, 10% H<sub>2</sub>) at 37°C for 5 days on a 4.75 mm diameter polytetrafluoroethylene plug insert at a constant depth of 400 µm. Prior to inoculation, culture medium was recirculated for 30 min with a turntable speed of 20 rpm to mimic the enamel pellicle formation. A 5-µL volume of each microorganism culture was added to biofilm medium (Hill et al. 2010) and, in separate experiments, recirculated for 24 h to seed the system. After this period, the inoculum was discarded and fresh un-inoculated culture medium was fed into the system (McKee et al. 1985; Hill et al. 2010) at a rate of 20 mL/h for 5 days. Mucoadhesive film samples were applied to the cultured biofilms for 1, 2, 6 and 18 h, followed by addition of 5 mL



fastidious anaerobe broth and incubation at 37°C. The suspensions were then mixed and serially diluted, before 20 µL aliquots were plated onto blood agar and Fastidious Anaerobe Agar and incubated for 24 to 48 h, after which the resulting colonies were enumerated (Miles and Misra 1938).

### *2.10. Statistical analysis*

GraphPad InStat 3 statistical software was used to analyse all data (GraphPad, San Diego, CA, USA). Statistical significance was determined using ANOVA test with appropriate post-test. In the case of PGE<sub>2</sub> determination, the Dunnet post-test was used with blank film set as control. A p <0.05 was considered statistically significant.

## **3. Results**

### *3.1. Preparation of drug-loaded films*

Polymer based mucoadhesive films incorporating chlorhexidine alone, or with either diclofenac, betamethasone or lidocaine were successfully produced as shown in Figure 1. The films were transparent (at the drug loadings used), flexible and soft. Films were also tacky when wetted, confirming mucoadhesive properties (data not presented). The films, as produced in standard Petri dishes, were 8.8 mm in diameter, with an average mass of 0.5 g and a mean thickness of 0.1 mm; previous work has demonstrated consistency in drug distribution across the film (unpublished data).

### *3.2. Effect of PEG400 on dissolution*

Figure 2 shows the effect of PEG400 content on the time taken for a 1 cm diameter film sample to dissolve and revealed a clear relationship between increasing percentage of PEG400 and more rapid dissolution. Films containing the highest amount of PEG400 (88.8%w/v) dissolved by 30 min, whereas the lowest content evaluated, 40%, took ~140 min to dissolve. However, 40% PEG400 was found to be the least pliable, and 50% PEG400 with a dissolution time of 120 min or 2 h was deemed suitable to progress to further testing.

### *3.3. Drug release: diclofenac and chlorhexidine*

Figure 3a shows the mass of diclofenac released as a function of time from a film containing either diclofenac (blue) or chlorhexidine (red). The amount of diclofenac release reached plateau phase at 30 min. There was no significant difference in the release profile after 30 min ( $p > 0.05$ ,  $n=3$ ) with a maximum amount of 110.7  $\mu\text{g}$  of diclofenac released from a 1 cm diameter film disc. Chlorhexidine was released from a 1 cm diameter ( $0.785 \text{ cm}^2$ ) disc at a significantly lower level and after 6 h a mean amount of 14.1  $\mu\text{g}$  chlorhexidine was released. Figure 3b shows the *simultaneous* release of diclofenac and chlorhexidine as a function of time from a 1 cm diameter,  $0.785 \text{ cm}^2$  sample of the combination film. Diclofenac release was more sustained than from the single drug film, with the amount released reaching plateau phase at 2 h. The amount of diclofenac released at 6 h from the combination film (43.7  $\mu\text{g}$ ) was less than half that released from the diclofenac-only film at (110.7  $\mu\text{g}$ ). The amount of chlorhexidine released from the same combination film sample reached a plateau phase at 1 h. The amount of chlorhexidine released from the combination film was slightly lower (11.3  $\mu\text{g}$ ) than from the chlorhexidine-only film (14.4  $\mu\text{g}$ ). Figure 3c shows the data as percentage simultaneous release of diclofenac and chlorhexidine as a function of time from the 10 mg diclofenac/25 mg chlorhexidine combination film. The release of diclofenac reached a plateau after 2 h, with a mean maximum of 40.9 % of the amount in the film sample. The release of chlorhexidine from film reached a plateau phase after 1 h, with a mean maximum of only 3.9 % release.

#### 3.4. Drug release: betamethasone and chlorhexidine

Two levels of betamethasone were used, 10 mg and 50 mg. Betamethasone is a Class 2 potency steroid and the purpose of including the higher level was to maximise the likelihood of a positive result in the anti-inflammatory work. Figure 4a shows the simultaneous release of diclofenac and chlorhexidine as a function of time from the 10 mg betamethasone/25 mg chlorhexidine combination film (1 cm diameter,  $0.785 \text{ cm}^2$  sample). Diclofenac release was sustained until reaching plateau phase at the 1 h time point. After 6 h, the amount of betamethasone released was 68  $\mu\text{g}$ . Chlorhexidine release from the same combination film sample reached a plateau phase within 1 h reflecting findings of the diclofenac/chlorhexidine combination film. The amount of chlorhexidine released from this combination film was 12.8  $\mu\text{g}$ , which is the same as found in the diclofenac films ( $p < 0.05$ ). Figure 4b presents the simultaneous release of betamethasone and chlorhexidine from a 1 cm diameter,  $0.785 \text{ cm}^2$  sample of the 50 mg betamethasone/25 mg chlorhexidine combination film as a function of time. Betamethasone release was sustained until reaching plateau phase at the 1 h time point. At 6 h, the amount of

betamethasone released was 220 µg, some 3.2x the amount released from the 10 mg betamethasone/25 mg chlorhexidine combination film. Chlorhexidine release from the same combination film sample reached a plateau phase at approximately 1 h with the amount of chlorhexidine released from being 15 µg, with no significant difference in the release profile after 1 h. The release profile for chlorhexidine was very similar to that observed in diclofenac/chlorhexidine and 10 mg betamethasone/25 mg chlorhexidine combination films.

### 3.5. Drug release: lidocaine and chlorhexidine

Figure 4c shows the simultaneous release of lidocaine and chlorhexidine from a 1 cm diameter, 0.785 cm<sup>2</sup> sample of the 10 mg lidocaine/25 mg chlorhexidine combination film as a function of time. Lidocaine release was sustained until reaching plateau phase between the 2 and 4 h time points; unlike the other films described above. At 6 h, the amount of lidocaine released was 102 µg. The amount of chlorhexidine released from the same combination film sample again reached plateau phase at approximately 1 h with the amount of chlorhexidine released from being 10 µg, with no significant difference in the release profile after 1 h. Again, the release profile for chlorhexidine was very similar to that observed in diclofenac/chlorhexidine and both betamethasone/chlorhexidine combination films.

### 3.6. Drug localisation in porcine gingiva, *in vitro*

The localisation of diclofenac and chlorhexidine in gingival tissue after film application for 2 and 6 h of is shown in Figure 5. For diclofenac,  $5.84 \pm 1.11$  µg/cm<sup>2</sup> penetrated into the tissue after 2h, which increased to 12.00 µg/cm<sup>2</sup> at 6 h. For chlorhexidine the respective amounts localised were significantly less than diclofenac ( $p < 0.001$ ), with 0.252 and 0.301 µg/cm<sup>2</sup> at after 2 and 6 h. The mucoadhesive nature of the films was reaffirmed by a high level of grab between the film sample and moist gingiva.

### 3.7. Modulation of PGE<sub>2</sub> levels in porcine gingiva, *ex vivo*

The amount of PGE<sub>2</sub> extracted from *ex vivo* porcine gingival tissue after application of test films was determined by ELISA. Figure 6 shows the levels of PGE<sub>2</sub> in units of ng/g in decreasing order. Control (blank) film showed a level of approximately 30 ng/g in the gingival tissue. The 25mg chlorhexidine film produced a higher amount, although this was not significant. Films containing 10 mg diclofenac+25 mg chlorhexidine and 10 mg lidocaine+25 mg chlorhexidine,

were both lower than the control, but not significantly ( $p > 0.5$ ). The remaining treatments all showed significantly reduced PGE<sub>2</sub> levels compared to the control. Diclofenac (50 mg)/chlorhexidine (25mg), and diclofenac alone gave similar results, with both being significantly lower than controls ( $p < 0.5$ ). The following treatments all demonstrated even greater statistical significance relative to the control ( $p < 0.1$ ), and in the order of increasing significance (decreasing PGE<sub>2</sub> level) were: 10 mg betamethasone+25mg chlorhexidine > 50mg diclofenac > 50mg betamethasone+25mg chlorhexidine > 10mg betamethasone > 50mg betamethasone > beamethasone 5mg/mL solution (positive control).

### 3.8. Effect of released drug concentrations on HaCaT cell line proliferation/cytotoxicity

Given that the film dissolution/drug release generally occurs within 2 h, a 6 h exposure time was deemed appropriate for determining potential cytotoxic effects of the released levels of drug on human gingival fibroblast proliferation. With the exception of the 50 mg betamethasone/chlorhexidine film, no significant reductions in cell viability across all films ( $n=3$ ), using the stringent Dunnet post-test (Figure 7). This was true for all time intervals, demonstrating that prolonged use of the films would not be harmful to patients. Furthermore, it is intended that the films would be applied to the gingiva *in vivo* would dissolve within 1-2 h, hence the effects seen at 24 h may not an appropriate scenario.

### 3.9. Zone of inhibition (ZOI) assay

Blank (drug-free) film and films containing diclofenac, lidocaine and betamethasone alone (10 mg/500 mg of film; standard size of 1 cm diameter/0.785 cm<sup>2</sup>) demonstrated no antimicrobial activity against test bacteria (*P. gingivalis*, *F. nucleatum* (508-06), *F. nucleatum* (515-14), *A. actinomycetemcomitans*, *S. mutans*, *S. pyogenes* and *S. gordonii*). Films containing chlorhexidine alone at concentration of 25 mg/ 500 mg of film and films containing diclofenac and chlorhexidine in combination at concentration of 10 mg/500 mg of film and 25 mg/500 mg of film, respectively, showed significant inhibitory activities against all test bacteria compared to control ( $p < 0.05$ ) (Figure 8). In combination, no potentiation of antimicrobial activity of chlorhexidine by any antiinflammatory agent was observed ( $p > 0.05$ ), meaning that the observed antimicrobial activity was entirely attributable to chlorhexidine and that the presence of the antiinflammatory has no effect (retarding or accelerating) on diffusion of chlorhexidine from films. The average ZOI for Gram-positive facultative anaerobes was 20 mm, while for Gram-negative anaerobes was slightly lower at 15 mm. This suggests that the antimicrobial activity of

thin films containing chlorhexidine against Gram-positive facultative anaerobes (*S. mutans*, *S. pyogenes*, and *S. gordonii*) was slightly greater than against Gram-negative anaerobes (*P. gingivalis*, *F. nucleatum* (508-06), *F. nucleatum* (515-14) and *A. actinomycetemcomitans*) ( $p < 0.05$ ).

### 3.10. Biofilm cell recovery after exposure to mucoadhesive film

Figure 9 shows the mean log cell recovery (CFU/mL) of Gram-positive bacteria in biofilms after exposure to all test films and controls for desired period of time. The greatest CFU/mL of approximately 11-log in average was recovered as expected from positive control (biofilm only) and blank film (drug free) meaning that there was an actual biofilm recovery from the PTFE peg and this was not a polymer related result. Chlorhexidine/diclofenac solution was the most effective, with the lowest cell recovery and approximately a 6-log<sub>10</sub> reduction in comparison to positive control. Chlorhexidine/diclofenac solution was used as a comparison because it is not dependent on release of drug from film and represents the maximum achievable effect in the event of 100 % drug release. It was compared against positive control (biofilm only) because the positive control was not in contact with any antibacterial agent, which gave the highest cell recovery.

For films containing chlorhexidine alone and in combination with diclofenac, there was a significant reduction in bacterial viability after 1 h contact time compared to positive control. Significant reductions in bacteria viability range from 3 to 6-log<sub>10</sub> reductions were seen at 1, 2, 6 and 15 h (overnight) contact time, in comparison to the cell recovery after exposure to films containing diclofenac alone, blank film and positive control ( $p < 0.05$ ,  $n=3$ ). However, the relationship between chlorhexidine's antibacterial activity and contact time is less clear. There was no significant difference in log reduction for all contact times ( $p > 0.05$ ). Addition of diclofenac in the combination film had no influence on or potentiation of chlorhexidine's antibacterial effect for all contact times ( $p > 0.05$ ,  $n=3$ ). In contrast to the formulation containing chlorhexidine, there was no significant reduction ( $p > 0.05$ ,  $n=3$ ) in bacterial counts after 1 and 2 h exposure to formulation containing diclofenac alone, when compared to the controls. Interestingly, there were significant reductions ( $p < 0.05$ ,  $n=3$ ) in bacterial counts for formulation containing diclofenac alone at 6 and 15 h contact time.

The mean log cell recovery (CFU/mL) of Gram-negative anaerobes in biofilms after exposure to all test films and controls for desired period of time is shown in Figure 10. The anaerobes cell (CFU/ml) recovered from positive control (biofilm only) and blank film (drug free) was unexpected as it was lower than the cell recovery after 1 h exposure for all medicated

films. Both mucoadhesive films containing chlorhexidine alone and in combination worked best with the lowest recovery observed after 15 h exposure, followed by the chlorhexidine/diclofenac standard solution, with only 1- $\log_{10}$  reduction difference in between them. The results clearly showed a trend of greater reduction in anaerobes viability as the time of exposure to formulations containing chlorhexidine increased from 2 to 15 h as opposed to the trend observed for aerobes.

Comparisons were made between films containing chlorhexidine alone and in combination with diclofenac. The addition of diclofenac in the combination film was found to have no synergy with /potentiation of the antibacterial effect of chlorhexidine at 1 and 15 h contact times ( $p >0.05$ ,  $n=3$ ), and with only 1  $\log_{10}$  reduction difference at 2 and 6 h contact time. However, there were also significant reductions ( $p <0.05$ ,  $n=3$ ) in bacterial counts for formulation containing diclofenac alone at 15 h contact time.

## 4. Discussion

### 4.1. Polymer based mucoadhesive films

Drug dose retention at the point of application followed by sustained release *in situ* has recognised benefits in periodontal diseases (Fiorellini and Paquette 1992). In this work HPMC, PEG 400 and CP were used to provide film drug retention, mucoadhesion, flexibility and elasticity (Peh and Wong 1999). The carboxylic acid groups of Carbopol are able to form hydrogen bonding with mucins thereby providing good mucoadhesion and PEG 400 was added to make the films pliable based on the evidence of improvement in chain interpenetration and mucoadhesion upon PEG addition in previous literatures (Miller et al. 2005; Khutoryanskiy 2011). The hydrophilic polymers incorporated in this novel film are capable of forming hydrogen bonding with the mucosal membrane, thereby providing a good mucoadhesion and control of PEG400 can be used to control the rate of film dissolution (Khutoryanskiy 2011). The thin films have appropriate physical properties - they are soft and flexible, readily affix to the gum, whilst at 100  $\mu\text{m}$  in thickness, they are also sufficiently thin to be unobtrusive once affixed to the gum.

Local drug delivery systems have been explored in an effort to treat or control periodontal diseases (Kalsi et al. 2011; Khutoryanskiy 2011). Polymeric fibre (Johnston et al. 2013) and chitosan materials (Ahmed et al. 2009), both medicated with ciprofloxacin and diclofenac have been reported. The mucoadhesive polymeric film developed in the current study

is a neater solution to the issue of gingival drug delivery compared to the thread fibre devices (Johnston et al. 2013) which lacks mucoadhesive, hence drug retention, properties of the film developed in this research. Moreover, ciprofloxacin is being phased out due to its increasing resistance issue and its anti-microbial mode of action (Neuhauser et al. 2003; Jacoby 2005). On the other hand chlorhexidine is microbicidal and also widely established in a range of formulations and products, such as Corsodyl at 0.2 % vol/vol.

#### *4.2. Thin film dissolution*

The dissolvability of thin films is an important factor in optimal drug release and is strongly associated with types of polymer and plasticiser used in film formulation (Fadda et al. 2008). In the current work, dissolvable thin films were successfully attained with a combination of water-soluble film forming polymers, HPMC, Carbopol 971 and varying amount of plasticizer, PEG400 (Peh and Wong 1999). The hydrophilic nature of HPMC and Carbopol 971 can form hydrogen bonds with water molecules and enhance the dissolvability of thin films, allowing drug release through diffusion. The addition of plasticizer also showed evidence in improved film flexibility and promoting dissolution of films; the effect of PEG400 concentration in dissolvability of fast-dissolving films was reported previously based on polymers HPMC (Jadhav et al. 2018) and pullulan (Bala and Sharma 2018). This could be explained by the ability of polar oxygen atom of PEG monomers forming hydrogen bonds with water molecules which cause the films to dissolve completely in the presence of water (Lüsse and Arnold 1996).

#### *4.3. Drug release from thin films*

Simultaneous release of both drugs from each of the combination mucoadhesive films evaluated. A maximum of 3.9 % chlorhexidine was released from chlorhexidine/diclofenac film, which is approximately ten times lower than that of diclofenac released from chlorhexidine/diclofenac film (40.9 %). Both drugs were released in a micromolar ratio of diclofenac : chlorhexidine, 11.67:1 over a 6 h time period. The difference in the percentage release could be attributed to the presence of several bonding mechanisms of interaction between the functional groups of polymer chains and chlorhexidine, thereby limiting the extent of drug release. HPLC analysis of samples from drug release over 6h time period showed that chlorhexidine release appeared to plateau at 1 h time point with no further significant increase afterwards, which explains why the samples from drug release over 2, 4, and 6 h time period were consistently acting at an inhibitory level, as a higher concentration is needed to kill the bacteria. The low release of chlorhexidine

suggested that chlorhexidine was retained in the mucoadhesive film in a greater degree compared to diclofenac.

Drug release is a result of the combination of many factors, one of which is the association with the polymers, as stated in a review (Kamel et al. 2008). Fini et al. (2011) reported a slower release of chlorhexidine (1% w/v) where complete release was only achieved after a 8h time period upon combination with HPMC and other mucoadhesive polymers in a gel formulation. The viscosity nature of HPMC and its capability to form hydrogen bonds and interact with other components in controlling drug release are detailed in previous studies (Kamel et al. 2008; Fini et al. 2011; Khutoryanskiy 2011). In addition to the above, Khutoryanskiy (2011) further described the roles of several structural characteristics such as hydrogen bonding group, high molecular weight, cationic or anionic charges and flexibility of chain in forming mucoadhesive interactions. Chlorhexidine digluconate is released as a cation after dissociation at physiological pH with a high molecular weight (505.4 Daltons) and formula weight (897.8 Dalton) compared to diclofenac (318.1 Dalton), is therefore able to form greater degree of interaction through hydrogen bonding with the functional groups of mucoadhesive polymer chains and this explains the retention of chlorhexidine seen in drug release studies (Fini et al. 2011; Khutoryanskiy 2011).

Although a low release (up to 4 %) of chlorhexidine was observed in this study, its effectiveness as an antiseptic and antibacterial agent at a low concentration (0.1-0.2 % w/v) has been proved in marketed product such as mouthwashes (Löe and Rindom Schiøtt 1970; Ryan 2005). Thus, chlorhexidine released from films should demonstrate efficacy as an antibacterial agent even at a level of 4% release. A study on human dermal fibroblast cells has reported that chlorhexidine can inhibit host DNA synthesis through depletion of cell ATP from concentration as low as 0.0001% (0.1 µg/mL) (Hidalgo and Dominguez 2001) meaning that a higher amount of release could potentially lead to overdosing and cytotoxicity. In this study, the concentration of chlorhexidine released from film, ranging from 1.6-2.7 µg/mL is higher compared to the concentration (0.1 µg/mL) shown to inhibit DNA synthesis in previous study (Hidalgo and Dominguez 2001). Despite its low release profile, microbiological evaluation proved the antibacterial activity of chlorhexidine against all test bacteria in this research. This suggests that a low chlorhexidine release profile, yet shown to be active against microbes could be potentially more beneficial than a high release of chlorhexidine in terms of safety, considering the fact that inhibition on DNA synthesis was reported at a low concentration of chlorhexidine in previous study (Hidalgo and Dominguez 2001).



Release of chlorhexidine to 10% is relatively low compared to other work, including films containing rizatriptan benzoate where up to 100% release was reported (Salehi and Boddohi 2017).

Although not always a clinical problem, patients may experience pain and tenderness in infected gums particularly when chewing or following the formation of an abscess. A film designed to deliver anaesthetic thus has merit in a slow-release thin film formulation. Lidocaine-loaded mucoadhesive thin films prepared from HPMC, chitosan and xanthan gum were found to release 4 mg/cm<sup>2</sup> after 30 min: considered sufficient to guarantee the anaesthetic effect (Pleguezuelos-Villa et al. 2019). As mentioned previously, the connection between buccal health and systemic conditions are known and the beneficial effect of the gingival application of local anaesthetics including lidocaine on the treatment of periodontal diseases on endothelial function of systemic arteries has been proposed (Saito et al 2017).

Overall, the release of drugs from the films arises due to a combination of diffusional AND disintegrational. The primary process is diffusional release, which is the liberation of drug from its binding sites with the polymer matrix – for polar drugs, this will be facilitated by the ingress of water from the moisture on the gum within the buccal cavity. Ingress of water gives rise to the secondary process of disintegrational release. As the integrity of the film is lost, resulting fragments expose more drug at the interface; as the film continues to break up, this release process continues until complete dissolution is attained. This appears to be the case for diclofenac, betamethasone and lidocaine, although maximum release at ~50% means that the balance (~50%) remains bound to the polymer chains, even though the chains themselves are free of the film matrix. This process is even more pronounced for chlorhexidine, with 85+% remaining bound to the polymers and only some 15% liberated.

#### *4.4. Antiinflammatory activity*

Microbial components, especially lipopolysaccharide (LPS) are able to induce macrophages to secrete cytokines interleukin-1 (IL-1) and tumor-necrosis factor-alpha (TNF-alpha), prostaglandins, especially PGE<sub>2</sub>, and hydrolytic enzymes (Page 1991). Prostaglandin E2 (PGE<sub>2</sub>) is a proinflammatory mediator biosynthesized by the constitutive enzyme cyclooxygenase-2 (COX-2) in response to insult including gingival tissue from subjects with chronic periodontitis (Morton and Dongari-Bagtzoglou 2001). COX-2 has a short lifespan of 1-2 min at V<sub>max</sub> and becomes permanently inactivated after converting several hundred arachidonic acid molecules (Chandrasekharan and Simmons, 2004). As inflammatory mediators such as COX-2 have such

short half-lives, constant stimulation is needed to maintain inflammation. In this way, any unnecessary inflammation is kept to a minimum. Once the injury is repaired, or other stimulus has been removed, the inflammatory cells begin to return to normal status. Similarly, COX-2 levels will diminish if an anti-inflammatory agent is present in sufficient quantity such that it can block the enzyme or interfere with another part of the inflammatory pathway upstream or downstream or the tissue loses viability. COX-2 levels are related to PGE<sub>2</sub>, making it a useful metric of dynamic inflammation states in tissue. Elevated levels are responsible to a range of physiological effects including vasodilation (reddening) and pain; molecules such as PGE<sub>2</sub> also play a major role in the extent and duration of degradative activity within an affected gum (Bage et al 2011).

The anti-inflammatory effects of steroidal agents believed to be due in part to the inhibition of the release of arachidonic acid, a precursor of both lipoxygenase and cyclooxygenase activity – this would have a knock-on effect of downregulating COX expression, hence PGE<sub>2</sub> levels. Betamethasone dipropionate was reported to inhibit PGE<sub>2</sub> production more strongly than betamethasone (Tsuji et al. 1997). Although betamethasone is a potent Class 2 steroid, no statistically significant difference in the rate of resolution of the inflammatory response between betamethasone and diclofenac was observed following strabismus surgery in a human trial (Wright et al. 1997). Overall, downregulation of COX-2 will lead to a reduction the levels of PGE<sub>2</sub> as is known to be the outcome of using the wide range of COX and COX-2 inhibitors in use today. In terms of periodontitis, achieving successful local delivery in the gingival tissue should also result in the rapid arrest of further degradation.

#### *4.5. Cytotoxicity*

MTT assay is a widely used colorimetric assay, and a standard model for measuring toxicity (Riss et al. 2013) by evaluation of the cell metabolic activity. However, MTT toxicity assays probe the effect of a drug challenge on a monolayer of cells which is not a reliable interpretation of the gingiva, but it is useful to assess cell viability as drugs need to penetrate mucosal membrane - in this sense MTT data represents the worst-case scenario. With the exception of the high betamethasone content, the results showed that even 24 h film contact time did not affect cell viability, demonstrating the drug concentration released from films are not at a toxic level for fibroblasts, and therefore can reasonably extrapolate that the films will not be cytotoxic to the gingiva. This is a positive additional outcome as it poses no risk if dissolution is delayed.

Chlorhexidine is known to be toxic to cells (Burstein, 1980; Giannelli et al., 2008) and is why it is used at low concentrations in many formulations. Since standard treatment of

chlorhexidine 0.2% mouthwash is swirled in the mouth for approximately 1min, contact time is short compared with mucoadhesive films, therefore prolonged contact time at this concentration would have potentially been cytotoxic. Burnstein (1980) observed some cell damage after chlorhexidine 0.01 % was placed directly onto cells. However, drugs are never fully released from films, therefore a chlorhexidine 0.01 % concentration in a mucoadhesive film was decided appropriate, as some drug will be retained in the film. Lowering the concentration below 0.01 % came with the risk of ineffective antimicrobial activity, which was essential in a thin film product. Betamethasone was found to be antiproliferative at  $10^{-4}$  M against HaCaT cells using the MTT assay (Guichard et al 2015).

#### 4.6. Microbiological evaluation

*S. mutans*, *S. pyogenes*, and *S. gordonii* were chosen for microbiological testing based on their relevance to periodontal diseases (Johnston et al. 2013; Dani et al. 2016). The *Streptococcus* species provides a surface for additional bacterial adhesion after their attachment (Darveau et al. 2000). *S. mutans* is a bacterium strongly associated with dental caries, although it has also been linked to periodontal disease (Dani et al. 2016). It is also considered as a major pathogen causing dental caries and regularly exists in dental biofilm. *S. gordonii* plays a vital role in instigating colonization by creating surfaces for other colonizers such as *Fusobacteria* to adhere to, indirectly leading to the formation of a pocket. The mucoadhesive films containing chlorhexidine were active and demonstrated greater antimicrobial activity against Gram-positive facultative anaerobes (*S. mutans*, *S. pyogenes*, and *S. gordonii*) than that against Gram-negative anaerobes as observed in the ZOI assay. This finding was as expected for chlorhexidine and is consistent with evidence from previous reports which had proved that chlorhexidine has the greatest activity against Gram-positive bacteria, though it is also active against Gram-negative bacteria (Denton 1984; Horner et al. 2012). This can be further explained with the inherent difference in cell wall composition between Gram-positive and Gram-negative anaerobes. As ZOI is a test relying mainly on drug diffusion through the agar after release from the film, the antibacterial agent would need to penetrate through an additional barrier, which is the outer membrane of Gram-negative anaerobes before exerting its effect. For *S. pyogenes* and *S. gordonii* the presence of lidocaine appeared to have a slightly negative effect on antimicrobial activity, and such differences may be affected by the presence of lidocaine, but the effect was not significant ( $p > 0.05$ ).

The major role of bacterial biofilm and its interaction with the host response in the etiology of periodontal diseases was recently described (Hasan and Palmer (2014). The oral

biofilm model used in this research contained mixed species of Gram-positive anaerobes (*S. mutans*, *S. pyogenes*, *S. gordonii*) and Gram-negative anaerobes (*P. gingivalis*, *F. nucleatum* and *A. actinomycetemcomitans*) frequently present in dental plaques (Darveau et al. 2000). While human subgingival plaque harbors more than 500 bacterial species, research has shown that the Gram-negative anaerobic *Porphyromonas gingivalis* is a major etiologic agent contributing to chronic periodontitis (How et al. 2016). And although not typically associated with periodontal disease (Socransky et al. 1998), *S. mutans* was included as it has been found in chronic periodontitis subjects, both in saliva and sub-gingival plaque (Dani et al 2016). Following the maturation of biofilm, a stable and complex bacterial community can be established which is generally more resistant to systemic antibiotic therapy. Recent studies had suggested that one of the factors contributing to the resistance issue of biofilm is the limited access to the biofilm and its microcolonies that were sheltered from outer environment (Darveau et al. 2000). The employment of biofilm assay in this research therefore aimed to determine the antimicrobial potential of test thin film formulation and the drugs it contains, against the biofilms through topical delivery.

In this research, a model was employed to produce an oral biofilm mimicking the physiological growth state of the mixed bacteria species present in biofilms in the oral cavity based on its utilisation in previous published works (McKee et al. 1985; Hill et al. 2010). The biofilm assay demonstrated the antimicrobial and antibiofilm potential of mucoadhesive films containing chlorhexidine against oral mixed species in biofilms. Positive control (biofilm only) and blank film were assessed to ensure that there was cell recovery from the PTFE peg and the polymers have no inherent antibacterial activity. Chlorhexidine/diclofenac solution was used for comparison purpose with test films because it is not dependent on the capability of drugs being released from film, as described earlier. In the biofilm assay, reductions of different trends in bacterial viability were observed for Gram-positive anaerobes (3-6 log<sub>10</sub> reductions for all contact times) and Gram-negative anaerobes (reductions increased with contact time) after exposure to mucoadhesive films containing chlorhexidine. Considering the inherent difference in the cell wall composition of Gram-negative anaerobes and as chlorhexidine release appears to plateau at 1h time point, the trend of increasing reduction with contact time seen for Gram-negative anaerobes is suggesting that a longer contact time is needed for chlorhexidine to penetrate through the additional barrier, which is the outer membrane of anaerobes before exerting its effect.

For Gram-positive anaerobes, the relationship between the antibacterial activity of chlorhexidine and contact time is less clear as there was no significant difference in log reduction for all contact times. Possible explanations could be the release of chlorhexidine from

films reached a plateau phase after 1h period, resulting in only a little variation in the reductions of bacterial viability for all contact times. For Gram-negative anaerobes, the recovery from positive control (biofilm only) and blank film (drug free) was lower than expected. Possible explanation includes the loss of cells during the process of being exposed to films in an aerobic condition followed by the recovery procedure, which is unfavourable to anaerobes. The fact that the chlorhexidine/diclofenac thin film was demonstrably active in the biofilm model used indicates, not only that released drug (ie chlorhexidine) was able to destroy the bacteria, but also that it was firstly able to penetrate the self-produced matrix of hydrated extracellular polymeric substances (EPS). It is likely that this penetration was facilitated by physical disruption, ie the physical contact between the thin film and EPS is likely to exert a mechanical disruptive effect on EPS integrity. This will be beneficial when the film is administered *in vivo*.

#### 4.7. Clinical potential

The sustained presence of biofilm in the gum pocket vicinity is the prime causative agent of firstly gingivitis, then periodontitis. In the current work the chlorhexidine released from the mucoadhesive films was consistently active against all seven test microbes including both Gram-positive anaerobes and clinical isolates of Gram-negative anaerobes in all microbiological testing. In addition to planktonic bacteria, the activity of the mucoadhesive film was also established against mixed species biofilm in the current research. On the other hand, the low amount of chlorhexidine that penetrated the gingiva is beneficial as it forms a small superficial reservoir, without significant penetration, promoting substantivity (Garcia-Caballero et al. 2013). At the levels released from the thin films, chlorhexidine does not pose a risk of cytotoxic effects. Unlike chlorhexidine-only film, the amount of co-formulated diclofenac released and penetrated into the gingiva showed a demonstrable knock-down effect on local inflammation – lidocaine and particularly betamethasone also show activity. The presence of diclofenac in the film does not reduce the potency of the antimicrobial effect of the chlorhexidine. From the results obtained it is clear that the activity of chlorhexidine was not compromised by the presence of the anti-inflammatory or anaesthetic, and *vice versa*.

For the polymer combination used here, the thin films dissolve within 2 hours, although drug released thereafter would continue to exert their dual action. As a marketable product, the thin film lends itself to self-administration and repeated application until chronic inflammation resolves and the biofilm clears or reduces to a level that no longer stimulates degradation processes within the tooth socket. Additionally, they have the potential to be self-administered without health professional intervention.

## 5. Conclusions

In this work polymeric thin films incorporating microbicide and anti-inflammatory agents have been successfully prepared, that show dual action in terms of activity against a panel of relevant bacteria implicated in the aetiology of periodontal disease whilst simultaneously eliciting downregulation of PGE<sub>2</sub> – a key mediator in periodontal disease progression. At the concentrations used, the films also show no adverse cytotoxic effects in a keratinocyte cell line. Together these properties indicate such thin films may confer major beneficial effects when used by sufferers of periodontal diseases in arresting the progression of debilitating symptoms.

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## Legends to figures

Figure 1 Translucent, polymer based drug-loaded mucoadhesive thin films were successfully prepared. Average thickness approximately 100  $\mu\text{m}$ .

Figure 2 The effect of plasticiser PEG400 on the dissolution time of thin films ( $n=3 \pm \text{SD}$ ), showing the inverse relationship between PEG400 and dissolution time.

Figure 3 Drug release profiles for chlorhexidine and diclofenac over 24 h from a  $0.785 \text{ cm}^2$  (1cm diameter) disc,  $n=3 \pm \text{SD}$ . 3a: mass of chlorhexidine and diclofenac from individual films containing either 25 mg chlorhexidine or 10mg diclofenac; 3b: mass of chlorhexidine and diclofenac released simultaneously from combination film containing 25 mg chlorhexidine and 10mg diclofenac; 3c: percent chlorhexidine and diclofenac released simultaneously from combination film containing 25 mg chlorhexidine and 10 mg diclofenac.

Figure 4 Drug release profiles over 24 h from a  $0.785 \text{ cm}^2$  (1cm diameter) disc,  $n=3 \pm \text{SD}$ . 4a: mass of chlorhexidine and betamethasone released simultaneously from combination film containing 25 mg chlorhexidine and 10mg betamethasone; 4b: mass of chlorhexidine and betamethasone released simultaneously from combination film containing 25mg chlorhexidine and 50 mg betamethasone; 4c: mass of chlorhexidine and lidocaine released simultaneously from combination film containing 25 mg chlorhexidine and 10 mg lidocaine.

Figure 5 Chlorhexidine and diclofenac extracted from porcine gingiva after application of 1 cm diameter disc of combination film for 2 or 6 h,  $n = 3 \pm \text{SD}$ .

Figure 6 Prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ) levels extracted from *ex vivo* porcine gingival tissue following various treatments for 3 h,  $n= 2-3 \pm \text{SD}$ . Data plotted in order of decreasing  $\text{PGE}_2$  content, illustrating the increasing anti-inflammatory effect, left to right (\* $p < 0.5$ , \*\* $p < 0.1$ ).

Figure 7 Cytotoxicity determinations using the MTT assay at 1, 6 and 24 h,  $n = 3 \pm \text{SD}$ . Primary human gingival fibroblasts cells were incubated with levels of drug released from films at 24 h.

Figure 8 Mean zone of inhibition (ZOI) for 1cm diameter films containing chlorhexidine (red), and in combination with diclofenac (blue), lidocaine (green) and betamethasone (blue) against a

panel of bacteria relevant to periodontal disease,  $n = 3 \pm \text{SEM}$ . Note: blank film and films loaded with diclofenac, lidocaine or betamethasone demonstrated no zone of inhibition (not shown).

Figure 9 Mean log cell recovery in the number of colony forming units (CFU) of Gram-positive facultative anaerobes in biofilms suspension incubated in an aerobic condition after exposure to three different formulations and three sets of control for 1 h, 2 h, 6 h, 18 h (o/n),  $n=3 \pm \text{SEM}$ .

Figure 10 Mean log cell recovery in the number of colony forming units (CFU) of Gram-negative facultative anaerobes in biofilm suspension incubated in an aerobic condition after exposure to three different formulations and three sets of control for 1 h, 2 h, 6 h, 18 h (o/n),  $n=3 \pm \text{SEM}$ .

<b>Test formulation</b>	<b>Working formula (in 50 mL)</b>	<b>Amount</b>
Diclofenac and chlorhexidine	HPMC PEG400 Carbopol 917 Diclofenac sodium Chlorhexidine digluconate (20%) Deionised water	0.175g 0.25g 0.075g 10mg or 50mg 0.125mL to 50mL
Betamethasone and chlorhexidine	HPMC PEG400 Carbopol 917 Betamethasone valerate Chlorhexidine digluconate (20%) Deionised water	0.175g 0.25g 0.075g 10 or 50mg 0.125mL to 50mL
Lidocaine and chlorhexidine	HPMC PEG400 Carbopol 917 Lidocaine hydrochloride Chlorhexidine digluconate (20%) Deionised water	0.175g 0.25g 0.075g 10mg 0.125mL to 50mL

Table 1 Formulations for drug combination thin films containing 50% w/w PEG400 (in the case of single drug films, the amount of drug was as listed, whereas the second drug was omitted).

<b>Drug</b>	<b>Mobile phase</b>	<b>Detection wavelength</b>	<b>Retention time</b>
Chlorhexidine	50:50 acetonitrile:water with 0.5% v/v TFA	254nm	1.27min
Diclofenac	50:50 acetonitrile:water with 0.5% v/v TFA	254nm	3.29min
Lidocaine	50:50 acetonitrile:water with 0.5% v/v TFA	254nm	1.29 min
Betamethasone	85:15 methanol:water with 0.1% TFA	254 nm	2.2min

Table 2 HPLC conditions and retention times for the 4 drugs used in thin film preparation.