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A Novel Dual Action Monolithic Thermosetting Hydrogel Loaded With Lidocaine And Metronidazole As a Potential Treatment For Alveolar Osteitis

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Abstract

Alveolar osteitis is a complication that can occur after tooth extraction, whereby exposed bone results in severe throbbing pain for the patient and can be prone to infection. The current treatment options are widely regarded as sub-optimal. The aim of this project was to investigate *in vitro* the plausibility of a dual-action monolithic drug-loaded thermosensitive hydrogel that undergoes thermal gelation within the tooth socket and releases both anaesthetic and antimicrobial agents. Hydrogels containing different levels of lidocaine HCl and metronidazole were prepared based upon Carbopol 934P NF and Pluronic F-127 blends. Membrane-less drug release was determined from the set hydrogels into phosphate buffered saline (PBS) at 37 °C as a function of time, following analysis by HPLC. Gelation characteristics and hydrogel dissolution characteristics were also determined. At 23.38% Pluronic F-127, sol-gel transition commenced at 23 °C and gelation was completely at 37 °C (physiological temperature). Setting times varied with Pluronic content and there was an inverse relationship between drug release and Pluronic content. Sustained and dose dependent release of both drugs was observed at therapeutically relevant levels over 24 h, via a combination of diffusion, dissolution and surface erosion processes. Based on the amounts of drugs released, it was determined that hydrogels containing up to 0.5% lidocaine and 0.1% metronidazole exhibited low risk of cytotoxicity to primary human gingival fibroblasts. In an *in vivo* scenario, the sol-phase formulation would make contact with all inner surfaces of a tooth socket prior to transitioning to monolithic gel-phase and provide sustained release of lidocaine and metronidazole at sub-toxic levels, thereby providing simultaneous pain relief, protection from ingress of debris and potentially pathological bacteria.

Key words: Alveolar osteitis; socket infections; Pluronic F-127; thermosetting hydrogel; lidocaine hydrochloride; metronidazole

Introduction

Alveolar osteitis (AO), or 'dry socket', is a post-extraction complication that can occur due to formation failure or premature disintegration of a blood clot within the extraction socket (Figure 1). With an incident rate of over 30 %, AO is seen particularly frequently in mandibular third molar extractions while the overall prevalence for routine tooth extraction is between 0.5 and 5 % (Bowe et al. 2011). Patients with AO commonly experience a non-healing socket often containing debris, with erythema of the surrounding gingiva. The exposed socket bone is exquisitely sensitive to gentle instrumentation and is associated with severe throbbing pain on the second to fourth day after a tooth extraction (Hupp et al. 2013). In addition, 45% of patients who develop AO typically require multiple postoperative visits in order to manage this condition at great cost to the practice and the NHS (Kolokythas et al. 2010). Contributing factors include surgical trauma, bacterial infection, increased local fibrinolysis, nutrient deficiency and use of oral contraceptives (Cardoso et al. 2010, Bowe et al. 2011). Mechanical damage as a result of excessive force or movement during surgery increases the risk of releasing inflammatory mediators that can induce the conversion of plasminogen to plasmin, a fibrinolytic agent, which acts to dissolve the developing blood clot (Birn, 1973). Reducing the insult to the extraction socket, by food debris and microorganisms, is known to accelerate the healing process and relief from pain (Bowe et al. 2011).

Numerous studies have investigated methods of treating or preventing AO using antibacterial, antiseptic, antifibrinolytic or clot supporting agents as well as steroids. Dressings with antibacterial components, topical anaesthetics or combinations of both are often used despite the fact that dressings have been found to delay healing of the extraction socket (Burgoyne et al. 2010). The gold standard among such products is bismuth iodine paraffin paste (BIPP) gauze which is placed as a dressing into the socket. Use of other dressings containing eugenol and zinc oxide is common in order to reduce pain (Navas and Mendoza, 2010).

The provision of pain relief in AO treatments is vital to patient satisfaction as the primary symptom is severe and persistent pain. Lidocaine is an aminoethylamide which has long been used as an intra-oral anaesthetic (Meechan, 2002) and is available in a range of topical formulations. It inhibits the generation and conduction of nerve impulses through interaction with voltage-gated sodium channels in the exposed nerves following surgery (Cummins, 2007). Although effective in terms of pain management, lidocaine treatment does not address bacterial contamination aspects in the pathogenesis of AO; a mouth rinse containing 0.12 % chlorhexidine gluconate has been shown to produce a significant reduction in the incidence of AO after the extraction of mandibular third molars, but did not offer complete prevention (Hermesch et al. 1998). Normal extraction sockets harbour a combination of facultative anaerobes and anaerobic bacteria (MacGregor and Hart, 1970). The presence of facultative anaerobes alone (Rozanis et al. 1976) or a suppurative secretion containing both anaerobic and facultatively anaerobic bacteria (Rodrigues et al. 2011) is known to retard alveolar repair. Metronidazole has been shown to be highly effective against the development of bacteria-mediated AO, reducing incidence by 76.2-80.0 % (Rood and Murgatroyd, 1979). Metronidazole is commonly used, either orally or topically, in endodontic treatment in the UK and across Europe (Segura-Egea et al. 2017), and it is generally well tolerated. As a nitroimidazole prodrug, metronidazole is activated intracellularly only during anaerobic respiration (Freeman et al. 1997), leading to bacterial death by inhibiting DNA synthesis and causing DNA damage (Löfmark et al. 2010).

Although its importance is manifest in terms of sustained drug delivery (Nguyen and Hiorth, 2015), the retention of a drug dose in the treatment of AO is challenging. Formulations such as viscous gels have been evaluated (Burgoyne et al. 2010; Cho et al. 2017), however, these must be applied to the highly sensitive cavity by (unsterile) finger and the drug dose is restricted to the area of application. Thermosensitive hydrogels transition from liquid to gel in response to temperature change. They can be administered using a syringe whilst in the liquid form, benefiting from the ability to adapt to the entire inner cavity surfaces prior to gelation, as well as retention by mucoadhesive attraction (Hoare and Kohane, 2008).

Thermosetting hydrogels are typically co-polymer blends and the current work we used Pluronic F-127 to confer thermal gelation and Carbopol 934P NF to facilitate mucoadhesion and hydrogel retention, and have been extensively investigated in the literature, for example for ophthalmic drug delivery (Wu et al 2019) and nasal delivery (Majithiya et al 2006). Here we utilise the technique in a novel setting. Pluronic F-127, in concentrations of 20-30 % shows an increase of viscosity when heated from 5 °C to physiological temperature (37 °C). Mechanistically, hydrogen bonds between the solvent and the hydrophilic chains of the polymer are broken leading to gelation as temperature increases. This means that Pluronic F-127 is more soluble and less viscous in cold than in warm environments (Escobar-Chavez et al. 2006). Carbopol 934P NF consists of mucoadhesive polyacrylic acid polymers which act to maximise hydrogen bonding to the inner surface of the tooth socket, resulting in greater interfacial contact and better drug bioavailability (Loyd 1994; Singla et al. 2000). Moreover, a drug-loaded thermosetting mucoadhesive hydrogel has the potential to remain in place after gelation, protecting the extraction socket and ensuring maximum therapeutic effect conferred by active drugs formulated into the hydrogel.

In this work we hypothesised that a novel dual-action drug-loaded thermoresponsive hydrogel monolith could be developed which had the potential to alleviate the major symptoms and complications associated with alveolar osteitis, by treating the associated severe pain whilst preventing microbial contamination within the socket. Based upon a combination of metronidazole and lidocaine, we aimed to probe thermal gelation, release, erosion and dissolution properties and also determine safe drug loading levels by assessing cytotoxicity in human gingival fibroblast cultures.

Materials and methods

Materials

Metronidazole (Alfa Aesar) and Pluronic F-127 were purchased from Sigma Aldrich (Gillingham, UK). Lidocaine hydrochloride (MP Biomedicals), phosphate buffer saline

(PBS) tablets, HPLC-grade water, acetonitrile, ammonium acetate, methanol, trifluoroacetic acid, Gibco fetal bovine serum, Gibco DMEM and Gibco DMEM without phenol red were all purchased from Fisher Scientific (Loughborough, UK). Carbopol 934P NF was a gift from Lubrizol, Brussels, Belgium. Primary human gingival fibroblasts were purchased from ATCC (LGC standards, Teddington, UK). The CellTiter-Blue cell viability assay kit was purchased from PromegaUK (Southampton, UK).

Hydrogel preparation

Thermosetting hydrogel formulations were prepared containing Pluronic F-127 and Carbopol 934P NF, deionised water, 0.1 % w/v metronidazole and different levels of lidocaine HCl: 0.25, 0.5, 1, 2, 4 % w/v. The level of Pluronic F-127 and Carbopol 934P NF required to prepare a hydrogel that would set at physiological temperature of 37 °C were 23.38 and 0.13 % respectively (Majithiya et al. 2006) - this was subsequently confirmed in the rheology evaluation herein. To prepare the hydrogels, Carbopol 934P NF was added to deionised water and stirred on a magnetic stirrer (HB502 Bibby, Sterling Limited, UK) at room temperature until completely dissolved. Metronidazole and lidocaine HCl were added and stirring continued until complete dissolution achieved. The solutions were transferred to an overhead stirrer set at 800 rpm (RW20 DZMn, IKA Works Asia) and the Pluronic F-127 added. The mixture was stirred for 45 min and then stored at 2-4 °C for 2 h prior to use. A drug-free (blank) mixture was also prepared.

To investigate setting time, hydrogels were prepared containing Pluronic F-127 at 20, 23.38, 25 and 30 % with 1 or 2 % lidocaine HCl. Drug release was studied using formulations containing 0.25, 0.5, 1 and 2 % lidocaine HCl with 23.38 or 30 % Pluronic F-127 and an additional formulation containing 4 % lidocaine and 23.38 % Pluronic F-127. Carbopol 934P NF and metronidazole concentrations were kept constant at 0.1 %.

Measurement of setting time

Aliquots of 1 mL liquid-phase hydrogels were pipetted in into 2 mL microcentrifuge tubes and placed in a thermoblock heater (FDB03DD Techne, Bibby Scientific, UK) at 37 °C. The hydrogel was checked for solidification by rotating the tubes 180° every 2 sec. The tubes were subsequently returned to 2-4 °C - this step was also used to confirm gelation reversibility (Majithiya et al. 2006). Six replicates were performed for each hydrogel formulation tested. Although unsophisticated, this method proved to be reliable and reproducible.

Determination of thermal sol-gel transition

Thermal sol-gel (gelation) behaviour was determined using a Bohlin C-VOR 200 rotational rheometer (Malvern Instruments Ltd, Malvern, UK) was used with a 40 mm parallel plate and connected to a water bath to maintain the bottom plate at the desired temperature. With the temperature at 15°C, approximately 1.5 mL of sample was removed from the sample stored in the refrigerator and rapidly loaded onto the bottom plate using a syringe, and the gap between the plates set at 1 mm with excess hydrogel removed using a paper tissue. Using a temperature gradient, viscosity was recorded as the temperature increased from 15 - 40 °C at a rate of 1°C/minute. The shear rate was kept constant at 1 s^{-1} throughout the test and 60 data points were collected per run.

Determination of drug release

The unset hydrogels were removed from the refrigerator and 1 mL promptly pipetted into 2 mL microcentrifuge tubes for each time point, each experiment was performed in triplicate, n = 3. Any bubbles present were carefully removed to minimise interference with drug release. The tubes were placed in a thermoblock heater (Techne Dri-block DB-3D, Bibby, Stone, UK) set at 37 °C for approximately 5 min to

allow the hydrogel to completely set. PBS was pre-warmed to 37 °C and 1 mL carefully aliquoted onto the surface of each set hydrogel. The tubes were maintained at 37 °C for the desired contact time (1 min, 5 min, 1 h, 4 h, 18 h or 24 h), after which the PBS was carefully removed and transferred to an autosampler vial for HPLC analysis. Drug release from 23.38 % Pluronic F-127 hydrogels was also investigated for an extended period of up to 48 h.

High performance liquid chromatography (HPLC) analysis

A reverse-phase HPLC method was developed in-house which allowed the simultaneous determination of the two active drugs in a single assay. This was achieved using an Agilent system comprised of a G1379B Degasser, G1311A QuatPump, G1313A ALS autosampler and G1314A VWD UV detector. An isocratic mobile phase of 80 % ammonium acetate (0.2 mol/L), 20 % acetonitrile plus 0.2 % w/v trifluoroacetic acid was used, along with a Kinetex 5 µm C18 150 x 4.6 mm column (Phenomenex, Macclesfield, UK). The detection wavelength was set at 254 nm, with injection volume 20 µL and flow rate 1.5 mL/min. Under these conditions the retention times of metronidazole and lidocaine were found to be approximately 1.5 and 3.0 min respectively. Calibration curves for metronidazole and lidocaine were constructed using concentrations in the range of 31.25 to 1000 µg/mL in methanol and both agents were fully baseline-resolved. An exemplar chromatogram is shown in Figure 2 and chromatographic parameters were determined as follows: $k'_{metronidazole}$ 6.5, $k'_{lidocaine}$ 14, α 2.15, R_s 4.6.

Static hydrogel surface erosion

A straightforward method was developed in-house to simulate hydrogel dissolution/surface erosion within a tooth socket, involving formulations containing 0.25, 0.5, 1 and 2 % lidocaine with 23.38 % Pluronic F-127. After 1 mL aliquots of sample had undergone thermosetting in 2 mL microcentrifuge tubes, 1 mL PBS was carefully pipetted onto the surface of the each and the tubes maintained at 37 °C with

no agitation being applied for up to 42 days, and the hydrogel level determined at weekly intervals. This extended timescale, although not clinically relevant, was used to determine erosion rate.

Cytotoxic/ cell proliferation effects on primary human gingival fibroblasts

The CellTiter Blue (CTB) assay was used to determine the cytotoxicity of the released drugs to primary human gingival fibroblasts at the cellular level. The CTB evaluates cell viability by measuring the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product, resorufin (O'Brien et al. 2000). Based on the amounts of released drugs from the formulations, lidocaine and metronidazole solutions were prepared as shown in Table 2 and tested according to the CTB manufacturer's protocol. Briefly, primary human gingival fibroblasts used between passage 3 and 6 were seeded in a 96-well plate ($3x10^3$) in 100 µL of DMEM supplemented with 2 % (v/v) fetal bovine serum, 2 mM L-glutamine, antibiotic/antimycotic (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_2. After 24 h the medium was removed, and 100 µL of each of the prepared drug dilutions in fresh medium was added to three wells and incubated at 37°C and 5 % CO_2 for 24 h. After the incubation time, the drug-containing medium was removed, and 100 µL of CTB solution (84 µL of fresh DMEM media and 16 µL of CTB reagent) was added to each well. Fluorescence emission was measured using Clariostar plate reader (BMG Labtech) with the excitation/emission wavelengths set to 560/590 nm. The mean background value from cell-free wells incubated dye was subtracted from signals and the data were normalised to control/untreated cells.

Data processing

Drug release was determined in mass per surface area [µg/cm²], mol per surface area [µmol/cm²] and release percentage [µmol %] as a function of total drug in 1 mL sample. Hydrogel surface area was calculated using the measured diameter of the

2 mL microcentrifuge tubes. Statistical tests (ANOVA and student's two-tailed t-test, as appropriate) were preformed using the software Graphpad Instat for Macintosh 2018 and $p < 0.05$ was considered significant.

Results

Thermosetting hydrogels loaded with metronidazole and lidocaine were successfully prepared that were fluid, i.e. flowed freely, at room temperature (<23 °C) but underwent sol-gel transition to a state of full gelation at physiological temperature (37 °C) (Figure 3).

Rheological evaluation

A sol-gel transition is essentially a change from a liquid state to a semi-solid gel state. Figure 4 shows profiles for viscosity as a function of temperature for the 23.38 % Pluronic hydrogel containing either no drug (blank) or metronidazole with 0.25, 0.5, 1 and 2 % lidocaine. Below 23°C the viscosity of the hydrogel was at baseline; there was then a sharp increase in viscosity that started to plateau at approximately 1500 Pa s, and reached a peak at approximately 35°C, indicating that at this temperature the hydrogel had reached its maximum viscosity for this shear rate. Interestingly, the same behaviour was observed irrespective of the amount of lidocaine and metronidazole present, including the blank.

Effect of Pluronic F-127 content on gelation time

Figure 5 shows the influence of Pluronic F-127 content and lidocaine HCl on the hydrogel sol-gel transition time, demonstrating that the higher the Pluronic F-127 content, the faster the hydrogel sets. There was a significant difference in setting time between hydrogels with different Pluronic F-127 concentrations ($p < 0.001$). In fact, at the higher Pluronic F-127 concentrations of 25 and 30 %, the hydrogel started setting

at room temperature – outside of this range were not considered. For each replicate set of hydrogels covering the range of Pluronic F-127 concentrations, the setting rate in seconds as a function of percent Pluronic F-127, revealed no significant difference between the mean setting rate with 1 and 2 % lidocaine HCl (p 0.9109).

Drug release

The release of metronidazole and lidocaine are summarised in Table 1. Release is shown after 1, 4 and 24 hours from thermoset gels based on either 23.38 or 30% Pluronic F-127 and loaded with 0.1 % w/v metronidazole and different levels of lidocaine HCl: 0.25, 0.5, 1, 2, 4% w/v (n = 3 ± SD). Release data in terms of mass per area (1 cm diameter, 0.785 cm^2) are useful in relating drug dosages to therapeutic effect. The release of lidocaine per surface area was found to be related to its initial concentration, with higher lidocaine concentration hydrogels having released a greater amount at each time point with both 23.38 and 30% Pluronic F-127 (Figure 6 (i) and (iii)). Other than between the 0.25 and 0.5% lidocaine hydrogels, a significant difference was recorded (p <0.05) between all lidocaine concentrations with 23.38% Pluronic F-127 at each time point. For 30% Pluronic F-127 hydrogels there was a significant difference in lidocaine release between all loading concentrations and time points except 0.25 and 0.5%, and 1 and 2% after 1 h of incubation. The release of metronidazole as a function of time was comparable regardless of lidocaine or Pluronic F-127 content, except with a combination of 0.25% lidocaine and 30% Pluronic F-127 (Figure 6 (ii) and (iv)). Release profiles for both drugs from all hydrogels tested exhibited a rapid burst in the first 2-4 h followed by slower release over the remainder of the testing period. There was no significant difference between drug release in mass per surface area after 24 h from formulations containing 23.38 and 30% Pluronic F-127 (p >0.05).

Drug release in terms of mol per area is useful to determine molar relationships between the release of lidocaine and metronidazole from the binary hydrogel, which would indicate potential drug-drug interaction in the release process. Table 1 shows there was no apparent correlation or molar ratio pattern of drug release, and release

of each drug was independent of the presence of the other drug. Lidocaine release in mols decreased nearly proportionately after 24 h incubation with decreasing lidocaine concentration in the formulation. On the other hand, metronidazole remained at a consistent level (approximately 2 μmol/cm^2). Consideration of % release is useful in determining the overall performance of the hydrogel, where low percentage would indicate excess retention and high/burst release potential over-loading. In hydrogels with 23.38 % Pluronic F-127 (Table 1) lidocaine was more readily released than metronidazole. After 4 h incubation, 25-35% lidocaine was found to have been released compared to approximately 10% metronidazole, and 60 compared to 20% at 24 h. There was no difference between the percentage release profiles of either drug from 23.38% Pluronic F-127 hydrogels with different initial lidocaine concentrations (p >0.05). Percentage release for both drugs from 30% Pluronic F-127 hydrogels were also independent of initial lidocaine concentration, as shown in Table 1. For metronidazole release there was a significant difference after 24 h for each lidocaine concentration, whereas there was no significant difference in percentage lidocaine release (p >0.05). Lidocaine percentage release was lower from 30% than from 23.38% Pluronic F-127 hydrogels at all time points, reaching a maximum of 26.4% of the loaded dose at 24 h.

As the percentage release of lidocaine increased rapidly with time from 23.38% Pluronic F-127 hydrogels, an additional 48 h time point and lidocaine concentration of 4% was investigated. At 48 h, there was no significant difference (p >0.05) between the percentage lidocaine released from the hydrogels with lower loading concentrations of 0.25 and 0.5%, both of which were >90% (Figure 7). However, with 4, 2 and 1% lidocaine, release after 48 h incubation was only 68.7, 78.7 and 85.4% of the loaded dose, respectively, indicating that with increased lidocaine concentration the maximal release percentage may be decreased.

Hydrogel surface erosion/dissolution rate

Gel dissolution profiles, % versus time up to 35 days, are presented in Figure 8. Generally, the profiles were sigmoidal in form, with lower dissolution up to 7 days

before the rate increased up ~28 days, prior to depleting thereafter (Figure 8). *Post hoc* tests revealed a significant difference between the erosion/dissolution rate of hydrogels containing 1 or 2% lidocaine and those containing 0.25 and 0.5 % (p <0.05).

Cell Viability Assay

Primary human gingival fibroblasts were used in order to estimate the cytotoxic effect of lidocaine and metronidazole on the wound within a tooth extraction cavity. Both drugs were tested separately and in combination according to the concentrations released from 23.38% Pluronic F-127 formulations containing 0.1% metronidazole and either 0.25, 0.5, 1, 2 and 4 % lidocaine. The results presented in Figure 9 show that the prolonged treatment (24 h) with 0.1% of metronidazole and 0.25% of lidocaine were well-tolerated by the fibroblasts, with cell viability comparable to the control. However, lidocaine showed a concentration-dependent effect on cell viability with significantly reduced cell viability for concentrations higher than 0.5% (sample D). A similar pattern was obtained using lidocaine in association with metronidazole, indicating a toxic effect at higher lidocaine concentrations.

Discussion

Thermosetting hydrogels based upon Pluronic F-127 has been well-researched as a drug delivery excipient in a wide variety of settings (Escobar-Chavez et al. 2006) and it has also been proposed as a vehicle for the delivery of biological entities such as marrow- and dental-derived mesenchymal stem cells (Vashi et al. 2008; Diniz et al. 2015) for tissue engineering purposes. In the current work we used Pluronic F-127 and Carbopol 934P NF for the specific purpose of producing a monolithic body of hydrogel within the tooth extraction socket, having adopted the shape of the socket whilst in the sol phase, in order to simultaneously deliver anaesthetic and antimicrobial drugs *in situ*. As well as modulating setting properties the presence of

Carbopol 934P confers additional mucoadhesive properties which further aids retention within the tooth socket.

Setting time and temperature

Beneficial properties of a hydrogel for use in a clinical setting include efficiently fill and adhere to the exposed inner surface of the socket, followed by rapid gelation at physiological temperature. Rheological analysis showed that the hydrogel formulations undergo sol-gel transition between 23-34 °C, with rapid gelation onset at 23 °C, prior to attaining maximum viscosity at 37 °C (Figure 4) – this is of crucial importance for *in situ* gelation as it suggests stability and retention at physiological temperature of the socket (Majithiya et al. 2006). Surprisingly, rheological determination of thermal gelation revealed consistent behaviour irrespective of drug loading suggesting that, at the levels used, drug molecules present within the matrix did not modulate the physicochemical changes that occur when gelation occurs, although such effects have been reported previously (Gilbert el al 1987).

At lower concentrations Pluronic F-127 solutions show Newtonian behaviour, whereas a shift to pseudoplastic and plastic behaviour is observed for more concentrated solutions (Lenaerts et al. 1987). In the current work, gelation was found to occur within 60 s and, as expected, hydrogels with higher concentrations of Pluronic F-127 were found to undergo faster thermosetting (Figure 5). Hydrogels with higher concentrations of Pluronic F-127, i.e. >30%, could provide more rapid setting time, although this might not allow enough time for the hydrogel in the sol phase to fully adopt the shape of the socket. Levels of Pluronic F-127 below 20% were not considered relevant in terms of providing a useful drug delivery system.

Pluronic F-127 undergoes marked swelling upon heating, leading to a conformational change of the polymers due to the temperature-dependent desolvation. As the polymer micelles expand it is thought they form pseudo-crosslinks because the hydroxy groups - made accessible by desolvation - are able to interact and form

hydrogen or polar bond (Escobar-Chávez et al. 2006). The progressive increase of viscosity with temperature causes desolvation and swelling of the micelles with temperature. With increasing concentration and also the degree of micelle swelling, the intermicellar distance that is necessary for polymers to interact is reduced. The micellar phase is thought to be stable at low temperature and as the temperature increases it transforms into a cubic structure and then hexagonal-packed cylinders (Escobar-Chávez et al. 2006).

Drug release

Determining drug release from formulations are useful in indicating potential drug bioavailability and therapeutic effect. In the current system, *in situ* drug release arises via a combination of two physicochemical processes: surface erosion and dissolution of the monolith in the presence of saliva and diffusional release of drug molecules through and exiting the monolith. A direct drug-release experimental protocol was used in this work, based on that reported previously by Morishita et al. (2001) - other approaches include diffusion cells (Shin and Kim 2000) and dialysis (Nie et al. 2011). Here we used pH 7.4 PBS as release medium in place of human saliva and it is feasible that electrolyte content and concentration could modulate release data, although as both are comprised largely of water, this effect would be expected to be minor. Lidocaine and metronidazole were both released from all dual-action hydrogels in a time-dependent manner at clinically relevant levels indicating there would be a continuous drug release from a hydrogel under in use conditions, providing sustained local pain relief and antimicrobial prophylaxis for the patient (Figures 6 and Table 1). Although drug release is largely governed by surface erosion or dissolution of the gel matrix, the physicochemical properties of lidocaine and metronidazole would be important and it was observed that the release of lidocaine, bearing a positive charge, was greater lower than that of co-formulated metronidazole which is neutral. Erosion by saliva within the buccal cavity could be limited if an occlusive strip were to be placed across the tooth socket opening. A further distinction needs to be made between the drugs released from the monolith surface into the gum area (erosion and diffusional release) and that which releases internally and becomes bioavailable across the gel/tissue/bone interface (diffusional

release only). After 8 hours, Morishita et al. (2001) found that between 30-60% of loaded insulin was released after 8 h from F-127 formulations and Nie et al (2011) reported up to 90% release of paclitaxel – here we found ~13% metronidazole and 37% lidocaine released after 8 h. The presence of co-formulated lidocaine may act to enhance the binding of metronidazole to the polymers in the binary hydrogel, or metronidazole has greater affinity for the polymers, thereby inhibiting metronidazole release – which is a desirable characteristic in terms of maintaining inhibitory levels within the matrix.

In the current work, higher Pluronic F-127 content was found to reduce the percentage of lidocaine released, with more than double the percentage of loaded drug being released from hydrogels containing 23.38% compared to those with 30% (Figure 8). This may have been due to greater steric hindrance in release due to the increased presence of polymer chains in the hydrogel matrix, although increasing viscosity is also known to affect drug release (Morishita et al. 2001), or preferential interaction between metronidazole and the hydrogel matrix. Further enhancements in drug release have been reported by the inclusion of enhancers in the formulations such as bile salts (Shin and Kim 2000), or long chain fatty acids (Morishita et al. 2001).

Static hydrogel erosion/dissolution

Static incubation with PBS was intended to simulate the scenario between the set hydrogel within the socket and moist environment of the buccal cavity due to the presence of saliva. As anticipated, the volume of the hydrogels decreased during incubation with PBS, indicating that the polymer was soluble in the PBS – up to 12% after 7 days for all hydrogels. Dissolution profiles were unexpectedly sigmoidal in form. After 7 days, dissolution was the same for each hydrogel ($p > 0.05$), however, beyond 7 days hydrogels containing 1 or 2% lidocaine dissolved more rapidly than those containing 0.25 and 0.5% ($p < 0.05$). The release of drug, in particular the more water-soluble lidocaine would leave behind vacancies allowing the ingress of PBS, thereby increasing the rate of dissolution. Dissolution-controlled drug release is

known to be important in the use of Pluronic F-127 hydrogels and direct contact between the monolithic hydrogel surface and the release medium was found to dissolve the hydrogel in the membrane-less release model used by Nie et al. (2011). After four weeks all hydrogels had decreased to less than a half of the starting volume. It is feasible that release of water from the hydrogel matrix could have contributed to monolith volume reduction, although no stiffening due to such dehydration was noted.

The extended incubation times, although not clinically relevant, allowed the determination of longer-term erosion/dissolution effects. Dissolution tailed after 28 days but did not reach 100% dissolution. This was unexpected but may have been due to compaction in the lower reaches of the gel monolith, leading to reduced dissolution by PBS. When used in the clinical situation, it is not expected that the hydrogel be in place long term but rather offer temporary pain relief and protection from food ingress and microbial contamination when AO occurs, until the normal healing process resumes e.g. up to 7 days.

Cell viability assay

Cell viability was determined in order to indicate potential adverse effects of the released drugs within the socket, and the appropriate model – a primary human gingival fibroblast cell line was used as such cells are exposed within a newly created socket after tooth extraction. In this study, the CellTiter-Blue cell viability assay is a commercial assay kit containing solutions of the cell-permeable fluorogenic resazurin, that is less cytotoxic, more sensitive and faster to perform than the more frequently used tetrazolium-based (MTT) assay (Riss et al. 2016). Results showed that the drug released from 0.1% metronidazole and 0.25% lidocaine formulation did not decrease the viability of the cells significantly, meanwhile higher concentrations of lidocaine showed a toxic effect, particularly above 0.5%. These results are in line with the literature. A study conducted by Villarruel et al. (2011) demonstrated a decrease in cell viability according to the concentration and exposure time after lidocaine treatment. They revealed the presence of apoptotic corps in human gingival fibroblasts after 24 h of 0.01 mM lidocaine treatment. A previous study using MTT and WST-1 assays indicated that lidocaine has a cytotoxic effect on human oral

mucosa fibroblast viability (Oliveira et al. 2014). Their cell viability results showed that concentrations between 1-5% reduced cell viability - concentrations that were the similar to those formulated within the hydrogels in the current work, but significantly higher than the released bioavailable amounts observed here. Several studies support the safety and efficacy of metronidazole in human oral cell culture. Ferreira et al. (2010) compared different concentrations of metronidazole, ciprofloxacin hydrochloride and clindamycin hydrochloride, demonstrating that concentrations of 5 and 50 mg/L of metronidazole showed the highest cell viability even after 72 h and 96 h compared to the other antibiotics. Similar results were obtained by Chuensombat et al. (2013) when comparing metronidazole cytotoxicity with two other antibiotics, minocycline and ciprofloxacin, confirming metronidazole safety in human dental pulp cells and apical papilla cells. Poloxamers are generally regarded as safe, In terms of polymer safety and Majithiya et al. (2006) found no adverse effects when using Pluronic F-127 and Carbopol 934P for the intranasal delivery of sumatriptan. Carbopol 934P NF consists of polyacrylic acid polymers which are also associated with low toxicity (Loyd, 1994). Based up the data obtained using primary human gingival fibroblast cells, the indications are that the formulation and the drugs released from it would not pose a risk of adverse effects within the tooth socket.

Clinical considerations

The thermoset hydrogels successfully released both metronidazole and lidocaine, as shown in Figures 6-9. The metronidazole minimal inhibitory concentration (MIC) for anaerobes is 4 mg/L (EUCAST breakpoint tables, accessed Apr-2019). Even with release of only 4% of the incorporated 0.1 % metronidazole after 30 min from the 23.38% Pluronic F-127 hydrogel (Table 1) local concentrations of 40 mg/L would be achieved in this time, approximating to 10-times the MIC. Despite inconclusive evidence regarding whether bacteria are involved in AO pathogenesis, metronidazole prophylaxis is known to reduce incidences (Rood and Murgatroyd 1979), potentially indicating there is a particular contributory role for anaerobic bacteria. Direct topical application of metronidazole as a hydrogel component could, therefore, be expected to prevent, or halt progression of, AO by the same means as prophylactic oral metronidazole. Given the increasing responsibility for antibiotic stewardship in terms

of not over-prescribing drugs such as metronidazole, it may be pertinent that a product including this drug should be reserved for persistent cases. That said, general use may be defensible prophylactically because, unlike larger systemic antibiotic doses, this binary product would deliver lower doses of both drugs directly to the exposed bone within the AO socket rather than the systemic circulation. Thus, prophylactic use at the time of extraction could provide distinct benefits at the time of extraction, although its use as a treatment in cases of non-healing AO would be seen as the primary application.

Lidocaine gel is used topically for the treatment of localised neuropathic pain arising from damaged nerves (Casale et al. 2017). A 2% lidocaine viscous jelly administered to patients with AO immediately following tooth extraction showed a decrease in pain perception and increase in pain relief (Betts et al. 1995). In another study, a thermosetting gel containing 2.5% prilocaine and 2.5 % lidocaine was found to produce a significantly greater reduction in pain compared to a eugenol strip (Burgoyne et al. 2010). Lidocaine has proven efficacy in the treatment of AO, especially in the first minutes after surgery. In this work, a dual-action hydrogel with 2% lidocaine HCl and 0.1% metronidazole showed burst drug release in the first hour followed by slower sustained release which would provide patients with ongoing pain relief. Concentrations of 0.5% lidocaine, and particularly above this level, showed toxicity in human gingival fibroblasts cultures, however, these data must be considered alongside the fact that *in situ*, the hydrogel would be interacting with a tissue surface, and only the surface cells would be prone to deleterious effects. A trade-off may be possible between risk of cytotoxicity and the dual benefits of providing sustained anaesthesia and protection from microbial contamination. Generally, the aqueous nature of hydrogels is known to be a feature that is conducive to re-epithelialisation in wound healing, although this would need to be confirmed for the current product in subsequent experimentation.

From a practical perspective it is anticipated that products based on this system would be stored at 2-4 °C within the dental surgery, in order to ensure the formulation is in the sol phase prior to use. It is intended that this be product administered into the sockets created by tooth extraction as a liquid, which can be performed using a

syringe, which preludes the need for aseptic manual application, e.g. as in a regular gel application scenario. As the socket temperature will be between 30-37 °C, as soon as the clinician administers the solution it will start to undergo sol-gel transition, filling the socket and increasing in viscosity. As mentioned earlier, it may be beneficial to apply an occlusive covering strip to limit erosion and to exclude food and drink. There is also potential applicability in cases of smaller tooth sockets and multi-rooted teeth with narrow sockets, although this product is less likely to be appropriate for very narrow, capillary-type cavities.

The timescale over which the thermosetting hydrogel should remain in place remains to be determined, however, for comparison the currently used BIPP impregnated ribbon gauze is typically intended to remain in place for 3 days, at which time it has to be removed. The current hydrogel formulations could readily be removed at any time by cold (room temperature) aqueous/saline irrigation following reversal of the sol-gel transition, without risk of dislodging the blood clot. This will enable the dentist to safely and readily inspect wound-healing progress, with subsequent re-administration of the hydrogel as necessary.

Conclusions

Novel thermosensitive dual-action hydrogels demonstrated sustained release of lidocaine and metronidazole over at least 24 h, at sub-toxic levels up to 0.5% lidocaine. The use of a thermosetting hydrogel represents a significant improvement to current treatment options for AO, including: pain relief, ease of application, delivery efficacy with protection for all surfaces within the entire extraction socket, protection of the forming blood clot and reducing the risk of developing microbial infection.

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Legends to figures

Figure 1 Left: blood clot is formed after removal of tooth. Image source: http://www.drleachman.com/a-prf-socket-preservation/ Right: AO present, no blood clot formed after removal of tooth. Image Source: www.identalhub.com

Figure 2 HPLC chromatogram showing the resolution of metronidazole and lidocaine using the optimised method, thereby allowing the simultaneous analysis of both analytes. Chromatographic parameters were determined as follows: $k'_{metronidazole}$ 6.5, $k'_{lidocaine}$ 14, α 2.15, R_s 4.6. This exemplar shows the drugs released from hydrogel composed of 0.13 % Carbopol 934P NF, 0.1 % metronidazole, 0.5 % lidocaine and 30 % Pluronic F-127.

Figure 3 Depiction of hydrogel thermosetting. A: liquid gel at 2-4 °C. B: thermoset formulation at 37 °C.

Figure 4 Change in viscosity across a temperature gradient for the 23.38% Pluronic F-127 gel containing metronidazole and lidocaine HCl, showing transition temperature range and attainment of equilibrium (plot representative of 3 determinations).

Figure 5 Comparison of the effect of varying Pluronic F-127 content on the setting time of hydrogels loaded with 0.1% metronidazole and lidocaine concentration (1% (triangles) or 2% (squares)) (n=6 ± SD). Note: levels of Pluronic F-127 below 20% would set too slowly to provide a useful drug delivery system, and above 30%, setting would be unmanageably rapid.

Figure 6: Profiles for the mass of drug released per surface area for lidocaine (dashed lines) and metronidazole (dash-dotted lines) over 24 h from tested hydrogel

formulations containing 2% (squares), 1% (triangles), 0.5% (open circles) or 0.25% (crosses) lidocaine and either 23.38% (i) & (ii) or 30% (iii) & (iv) Pluronic F-127 (n=3 ± SD).

Figure 7 Percentage release of lidocaine over 48 h from hydrogels containing 23.38% Pluronic F-127 and 4% (filled circles), 2% (squares), 1% (triangles), 0.5% (open circles) or 0.25% (crosses) lidocaine (n=3 ± SD).

Figure 8 Dissolution profiles (% vs time) of set hydrogel containing 23.38% Pluronic F-127 in the presence of PBS. Gels were loaded with: 0.1% metronidazole and 2% (squares), 1% (triangles), 0.5% (open circles) or 0.25% (crosses) lidocaine HCl (n=3 ± SD).

Figure 9 Percent viability human gingival fibroblast cells after 24 h treatment. (A) control, (B) 0.1% metronidazole, (C) 0.25% lidocaine, (D) 0.5% lidocaine, (E) 1 % lidocaine, (F) 2% lidocaine, (G) 4% lidocaine, (H) 0.1% metronidazole + 0.25% lidocaine, (I) 0.1% metronidazole + 0.5% lidocaine, (L) 0.1% metronidazole + 1% lidocaine, (M) 0.1% metronidazole + 2% lidocaine, (N) 0.1% metronidazole + 4% lidocaine. The cell viability was determined as percentage of untreated cells. Bars represent mean ± SEM of at least three independent measurements. Data were analysed using 1-way ANOVA, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

Figure 1

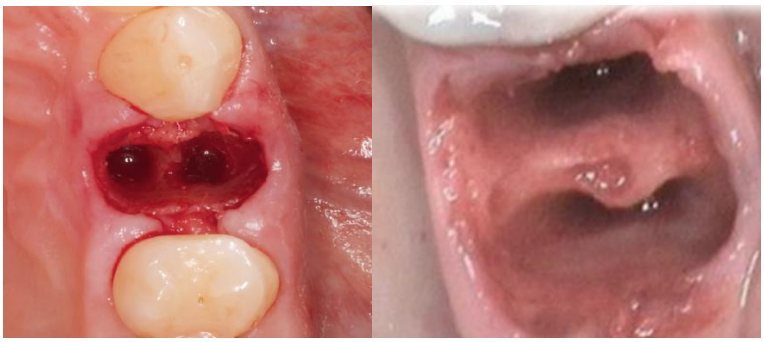
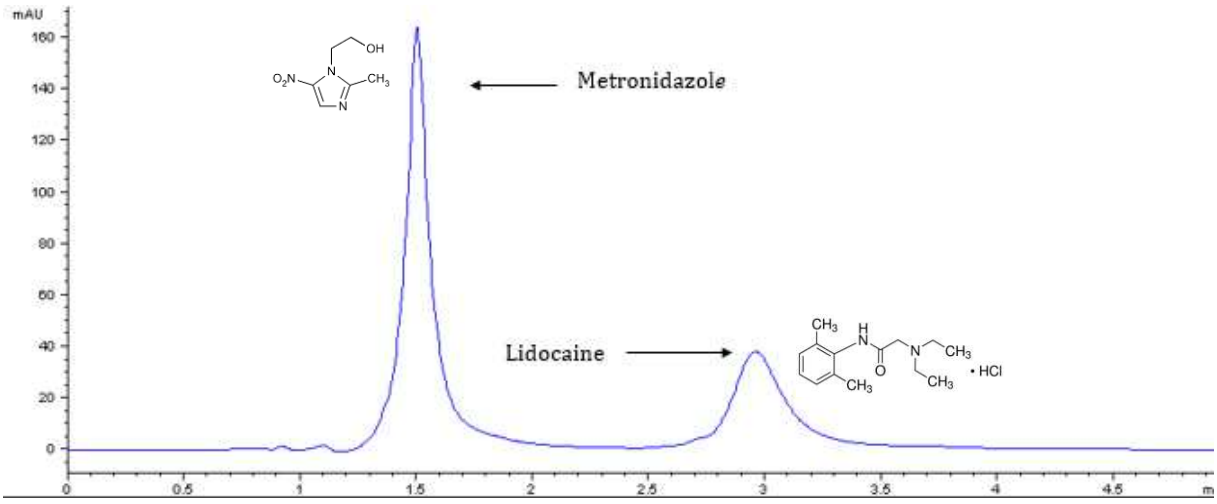


Figure 2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140
120
100
80
60
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20
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
m
Metronidazole
Lidocaine
OH
N
O_2N
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H
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· HCl

Figure 3

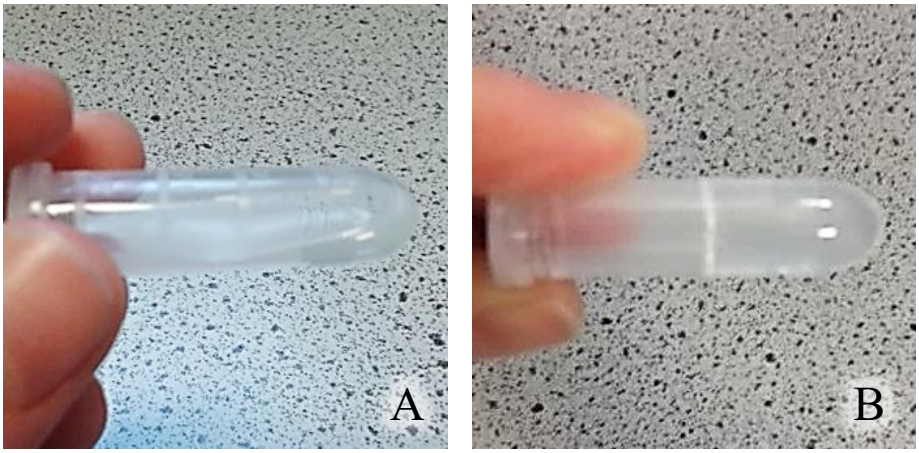
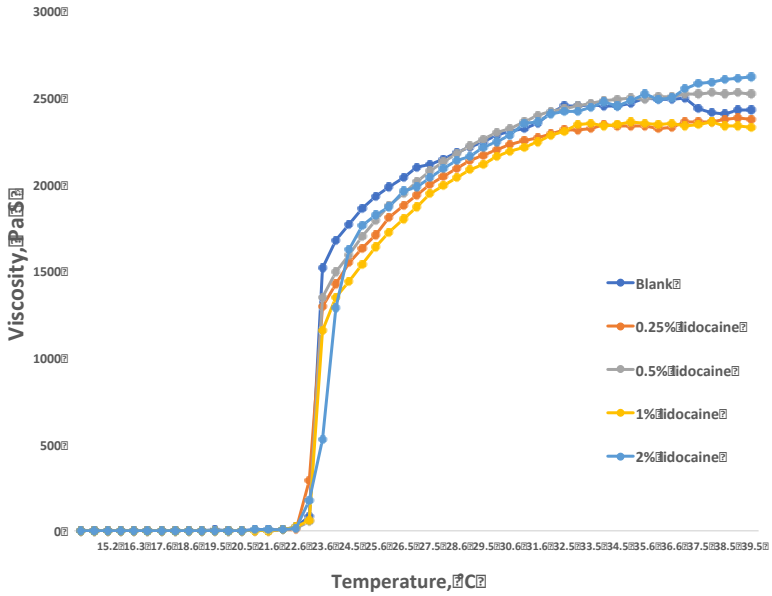


Figure 4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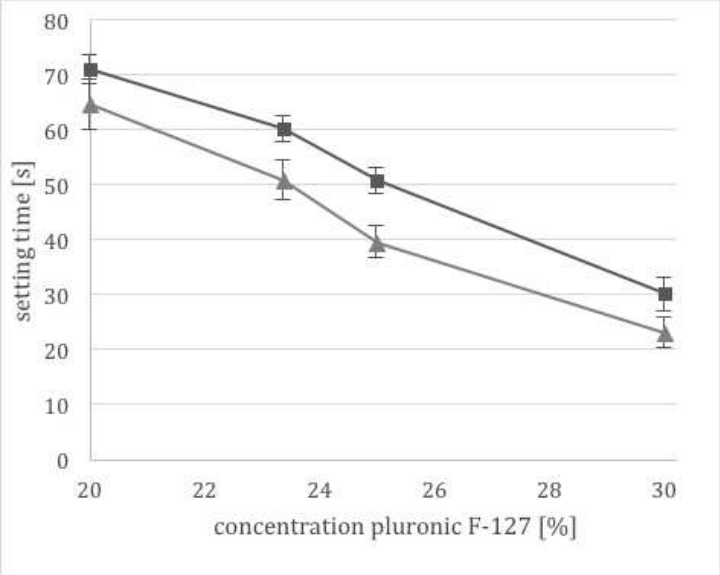
Viscosity,
Blank
0.25%
0.5%
1%
2%
Temperature,

Figure 5


80
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setting time [s]
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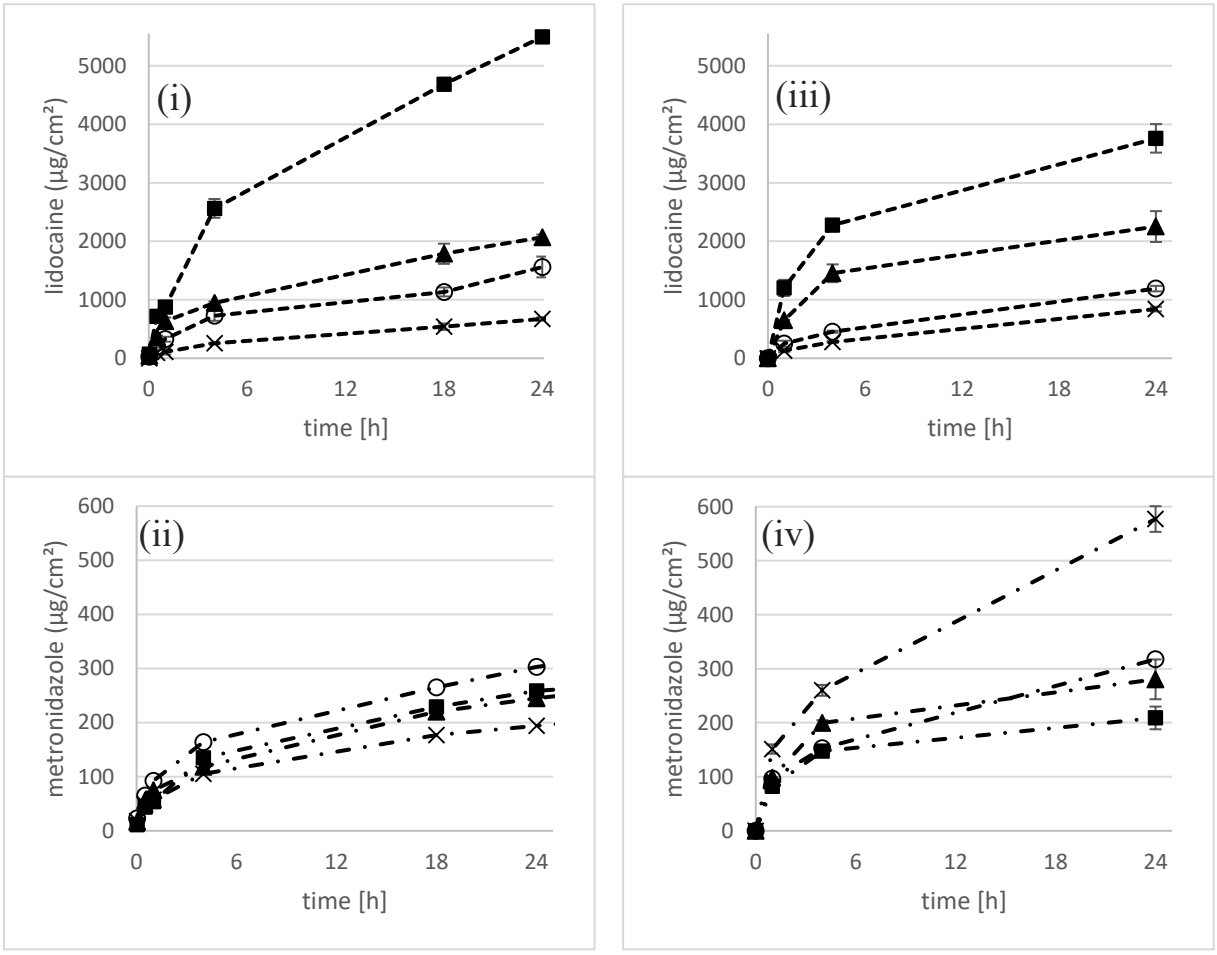
24
26
28
30
concentration pluronic F-127 [%]

Figure 6


(i)
lidocaine (µg/cm²)
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time [h]
(iii)
lidocaine (µg/cm²)
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4000
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time [h]
(ii)
metronidazole (µg/cm²)
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(iv)
metronidazole (µg/cm²)
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time [h]

Figure 7

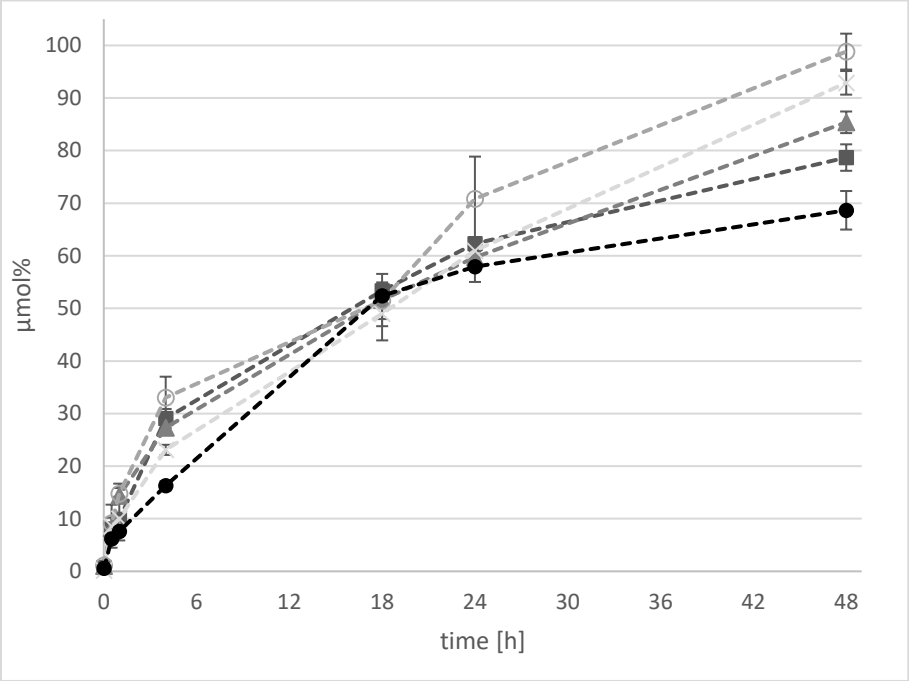


Figure 8

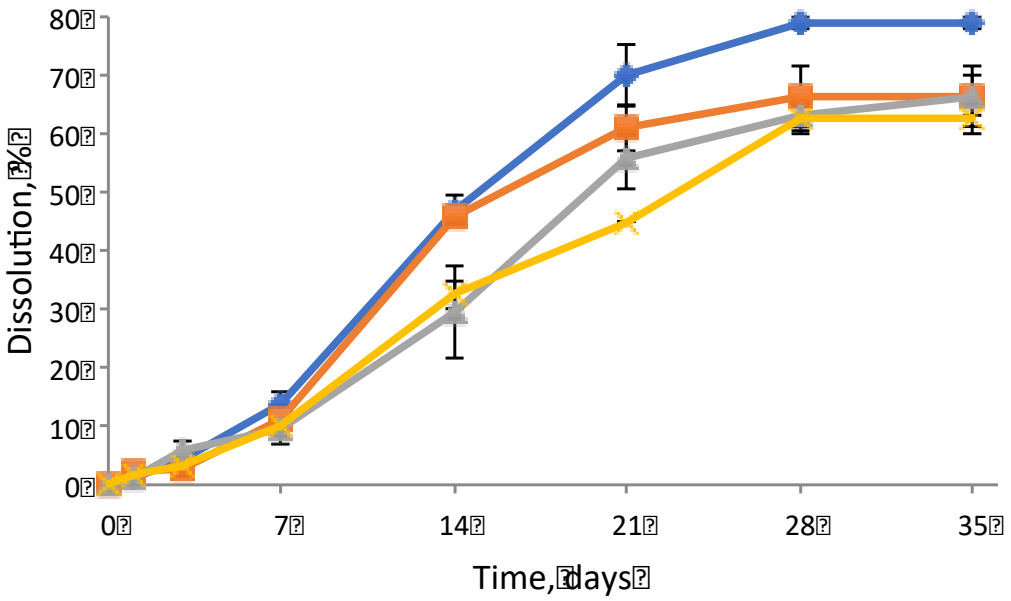
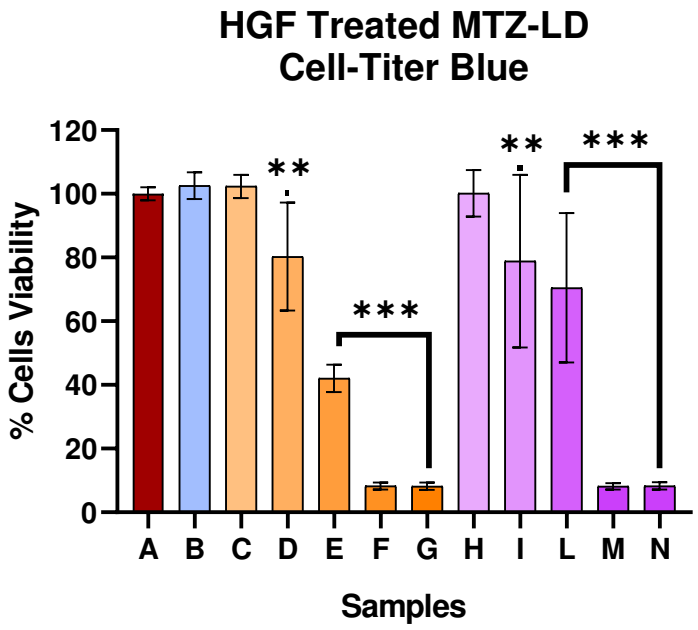


Figure 9


HGF Treated MTZ-LD
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% Cells Viability
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80
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40
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***
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***
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L
M
N
Samples

Formulation	1h		4h		24h		1 h		4h		24h		1h		4h		24h	
	µg/cm²						µmol/cm²						%					
	metro	lido	metro	lido	metro	lido	metro	lido	metro	lido	metro	lido	metro	lido	metro	lido	metro	lido
0.25% lidocaine, 30% Pluronic F-127	151.3±8.6	128.4±22.2	260±10.1	281±12.7	577±23.8	841±39.8	0.88±0.05	0.47±0.08	1.52±0.06	1.04±0.05	3.34±0.14	3.11±0.15	11.9±0.68	4.03±0.70	20.42±0.80±	8.83±0.40	45.32±1.87	26.42±1.25
0.5% lidocaine, 30% Pluronic F-127	96.4±15.0	248±451.3	153±3.38	454±17.3	317±5.8	1191±47	0.56±0.09	0.92±0.19	0.89±0.02	1.68±0.06	1.85±0.03	4.40±0.18	7.57±1.18	3.91±0.81	3.91±0.80	12.0±0.27	24.9±0.45	18.7±0.75
1% lidocaine, 30% Pluronic F-127	98.1±1.37	648±3.4	199.5±5.3	1453±152	280.4±37	2251±263	0.57±0.01	2.39±0.01	1.17±0.03	5.37±0.56	1.64±0.22	8.31±0.97	7.71±0.11	8.05±0.04	15.67±0.41	18.1±1.89	22.0±2.89	27.98±3.3
2% lidocaine, 30% Pluronic F-127	82.4±5.8	1201±138	147.7±0.46	2275±33	208.9±21	3759±243	0.58±0.03	4.44±0.51	0.86±0.01	8.4±0.12	1.22±0.12	13.9±0.9	4.08±0.29	4.72±0.54	7.33±0.02	8.9±0.13	10.4±1	14.76±0.96
0.25% lidocaine, 23.38% Pluronic F-127	57.4±7.6	110.7±46	105±3.4	254.6±10.6	194.1±2.8	672±28	0.34±0.04	0.38±0.16	0.61±0.02	0.88±0.04	1.13±0.02	2.33±0.1	4.51±0.6	10±4.2	8.26±0.27	23.1±0.96	15.24±0.22	61±2.5
0.5% lidocaine, 23.38% Pluronic F-127	92.2±4.7	326±41.7	164±10.2	728±88	303±5.4	1560±177	0.54±0.03	1.13±0.14	0.96±0.06	2.52±0.3	1.77±0.03	5.4±0.62	7.24±0.37	14.8±1.9	12.9±0.8	33±4	23.8±0.4	70.8±8.1
1% lidocaine, 23,38% Pluronic F-127	75.3±4.9	636±62	118.3±2.6	946±23	244±3.4	2068±47	0.44±0.03	2.2±0.22	0.69±0.02	4.17±0.1	1.43±0.02	9.1±0.2	5.92±0.39	14.4±1.41	9.29±0.2	27.3±0.67	19.2±0.27	59.7±1.36
2% lidocaine, 23.38% Pluronic F-127	53.7±4.9	876±85	134.6±6.5	2562±160	258±5.7	5494±105	0.31±0.03	3.03±0.3	0.77±0.04	8.87±0.56	1.51±0.03	19.1±0.36	4.21±0.38	9.93±0.97	10.6±0.51	29.1±1.8	20.3±0.45	62.3±1.2

Table 1 Summary of mass, mols and % of metronidazole (metro) and lidocaine (lido) released after 1, 4 and 24 hours from thermoset gels based on either 23.38 or 30% Pluronic F-127 and loaded with 0.1 % w/v metronidazole and different levels of lidocaine HCl: 0.25, 0.5, 1, 2, 4 % w/v (n = 3 ± SD).

% drug loaded in the formulation	Concentration of drug loaded in the formulation	Drug/s released, μg/cm²	Drug/s released, μg	Drug/s levels tested in CTB assay, mM
Control, drug-free	0	0	0	0
0.1% metronidazole	1 mg/mL	302.92	237.8	1.39
0.25% lidocaine	2.5 mg/mL lidocaine	672.06	527.6	1.95
0.5% lidocaine	5 mg/mL lidocaine	1560.90	1225.3	4.52
1% lidocaine	10 mg/mL lidocaine	2068.02	1623.4	5.99
2% lidocaine	20 mg/mL lidocaine	5494.26	4312.8	15.92
4% lidocaine	40 mg/mL lidocaine	10214.59	8018.0	29.60
0.25% lidocaine + 0.1% metronidazole	2.5 mg/mL lidocaine + 1 mg/mL metronidazole	672.06 + 302.92	527.6 + 237.8	1.95 + 1.39
0.5% lidocaine + 0.1% metronidazole	5 mg/mL lidocaine + 1 mg/mL metronidazole	1560.90 + 302.92	1225.3 + 237.8	4.52 + 1.39
1% lidocaine + 0.1% metronidazole	10 mg/mL lidocaine + 1 mg/mL metronidazole	2068.02 + 302.92	1623.4 + 237.8	5.99 + 1.39
2% lidocaine + 0.1% metronidazole	20 mg/mL lidocaine + 1 mg/mL metronidazole	5494.26 + 302.92	4312.8 + 237.8	15.92 + 1.39
4% lidocaine + 0.1% metronidazole	40 mg/mL lidocaine + 1 mg/mL metronidazole	10214.59 + 302.92	8018.0 + 237.8	29.60 + 1.39

Table 2 Summary of drug levels used in the cytotoxicity experiments. These figures were determined based upon the drug or drugs released at 24h over an area of 1 cm^2.