Development of metronidazole loaded in situ thermosensitive hydrogel for periodontitis treatment

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Abstract

Introduction: Periodontal treatment focuses on thorough removal of specific periodontal pathogens, mainly anaerobic gram-negative bacteria, by mechanical scaling and root planning. In case the periodontal abscess is noticed after treatment, a high dose of antimicrobial agents via oral administration is commonly applied. However, this approach increases the risk of antibiotic resistance, reduces efficacy, and possesses systemic side effects. To overcome the mentioned issue, this study focused on thermosensitive hydrogel development to deliver the antibiotic drug metronidazole (MTZ) directly and locally to the oral infection site.

Methods: The thermosensitive hydrogels were prepared by blending 28% w/v Pluronic F127 (PF127) with various concentrations of methyl cellulose (MC) and silk fibroin (SF). Gel properties of sol-gel transition time, viscosity, and gel strength were investigated. Drug dissolution profiles, together with their theoretical models, and gel dissolution characteristics were additionally determined.

Results: All hydrogel formulations exhibited sol-gel transitions at 37 ºC within 1 minute. An increase in MC content proportionally increased the hydrogel viscosity, but decreased its gel strength. In contrast, the SF content showed no significant effect on the gel viscosity, whereas increased the gel strength. The thermosensitive hydrogels also showed prolonged MTZ release characteristics for 10 days in phosphate buffer saline (PBS) at pH 6.6, which followed Higuchi diffusion model. Moreover, MTZ-thermosensitive hydrogel demonstrated delayed dissolution in PBS at 37 ºC of more than 9 days.
Conclusion: MTZ-thermosensitive hydrogels could be considered as a potential local oral drug delivery system to achieve efficient sustained release and might improve the drug pharmacological properties in periodontitis treatment.

Keywords: thermostensitive hydrogel, metronidazole, silk fibroin, sol-gel transition, periodontitis
1. Introduction

Periodontitis is a pathological inflammatory condition of periodontal tissues, including gingiva, periodontal ligament, alveolar bone, and cementum. The major cause of this condition relates with dysbiosis condition that mostly associated with anaerobic gram-negative bacterial loads. Thus, the treatment of periodontitis mainly focuses on the reduction or eradication of periodontal pathogens. The first step of periodontal treatment refers to scaling and root planning, through the elimination of subgingival calculus, by the mechanical removal. However, in some cases, conventional therapy alone is insufficient because bacterial endotoxin penetrated into the root surface. Therefore, the combined treatment with antimicrobial agent, such as local antiseptic agent or systemic antibiotic, is essentially given for increased treatment efficiency. Presently, metronidazole (MTZ) is one of the most widely used antibacterial compounds, which efficiently inhibits anaerobic microorganisms, in periodontal treatment. However, oral administration of MTZ possesses difficulty in delivering antibiotics directly to the infectious site, thus, leads to an insufficient concentration of the drug within the periodontal pocket. Additionally, using high doses of MTZ causes various side effects such as gastrointestinal disorders, resistant bacteria strands development, and supra-infection. To this end, novel approaches for advance periodontal treatments are necessary.

Local drug delivery administration to the oral cavity is a potential approach to overcome these mentioned challenges. This route provides a high concentration of antimicrobial compounds directly to the infected site and minimizes their potential systemic side effects. Nevertheless, for the local route to be effective, the ability to precisely control the drug release at the target site is crucial. For this issue, a drug delivery system such as thermosensitive hydrogel that can carry, protect, and release the drug in a favor manner, proves its benefits.

Over the past decade, thermosensitive hydrogels have become increasingly attractive as carriers for local delivering of the drugs to the sites of action. An ideal thermogelling hydrogel for drug delivery should exhibit a suitable sol-gel transition behavior, namely, the hydrogel is in a solution state below the room temperature while gelling at body temperature. One of the most commonly used material for sol–gel reversible hydrogels is Pluronic F127 (PF127), also known by the non-proprietary name poloxamer 407. It is considered as a biocompatible polymer and has been approved by US Food and Drug Administration (FDA). PF127 is a triblock copolymers of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO–PPO–PEO) that exhibits phase transition temperature at around 25-32 ºC by micellization or micelle aggregation. Unfortunately, due to their low molecular weight and low mechanical strength, PF127 hydrogels are subjected to rapid erosion, thus, possess low stability in physiological conditions. Consequently, burst and uncontrolled drug release takes place, which further reduces the system efficiency. To overcome these, we proposed a novel approach of co-incorporating methylcellulose (MC) and silk fibroin (SF) to the PF127 thermosensitive hydrogels, to improve their properties.

MC is one of the water-soluble cellulose derivatives, which has a unique property to induce reversible sol-gel transitions by the hydrophobic interactions in aqueous solution with increasing temperature. MC is recognized by the US FDA as a highly biocompatible material. It has been used for biomedical applications including dermal wound repair, regenerative medicine, cell sheet engineering and bone regeneration. Additionally, SF from Bombyx mori silkworms has been widely explored as biomaterials for decades due to its versatile strengths, biocompatibility, controllable degradability and excellent mechanical properties. SF can generate sol-gel transition by β-sheet assembly under physiological conditions such as ionic surfactant, pH, concentration, and temperature. Therefore, we hypothesized that MTZ-thermosensitive hydrogel based on PF127 with SF and MC could
improve the properties of hydrogels, to match the periodontitis treatment, including increased gel strength, slow erosion, and sustained drug release. This study aimed to develop MTZ-thermosensitive hydrogels composed of the combination of PF127, MC, and SF, at various ratios and concentrations, for intra-periodontal pocket local drug administration. The hydrogels were prepared using physical mixing method. The hydrogel gelation time was investigated by sol-gel transition at different temperatures. The hydrogel viscosity was determined at storage temperature for ensuring that the hydrogel can be administered after long-time storage. Furthermore, the gel strength was investigated at 37 °C to determine its suitability in the oral cavity. Finally, the drug dissolution was studied in phosphate buffer solution at pH 6.6 to investigate the effect of biocompatible polymers content on the drug release rates from hydrogels.

2. Materials and methods

2.1. Materials
Degummed silk yarns of *Bombyx mori* were collected from Bodin Thai Silk Khorat Co., Ltd. (Khorat, Thailand). PF127 (MW 12,500 g/mol) was purchased from BASE Corporation (Bangkok, Thailand). MC (M0512; viscosity 4,000 cP, MW 88,000 g/mol and DS 1.5-1.9) was obtained from Sigma-Aldrich (USA). MTZ injection (Medizole injection 5mg/mL) was purchased from Utopan Co., Ltd. (Samutprakan, Thailand). Sterile water for injection (SWI) was obtained from A.N.B. Laboratories Co., Ltd. (Bangkok, Thailand). Methanol (HPLC grade) were purchased from Sigma-Aldrich (USA). Triethylamine (TEA) (DNA and peptide synthesis grade) was purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). Potassium phosphate monobasic (KH2PO4) ReagentPlus® was purchased from Elago Enterprises Pty. Ltd. (Australia).

2.2. Silk fibroin extraction
Aqueous silk fibroin (SF) was prepared and characterized by a standardized method reported in previous literature. Briefly, 5 g of degummed silk yarns was cut into short fibers and added in a mixed solution of CaCl2: H2O: Ca(NO3)2: EtOH at 30:45:5:20 weight ratio. Then, the wetted fibers were heated at 900 W in microwave for 2 min to form a clear solution. The resulting SF solution was dialyzed in a snakeskin pleated dialysis tube (10,000 MWCO) against distilled water at room temperature for 3-5 days to remove residual salts. The aqueous SF solution was centrifuged at 10,000 rpm, 4 °C for 30 min to remove the impurities and silk aggregates formed during dialysis. Following centrifugation, SF solution was lyophilized using freeze dryer (Heto PowerDry LL3000, Thermo Fisher, USA) at 1×10⁻⁴ Torr and - 55 °C, and was kept in sealed plastic bags at - 20 °C until used.

2.3. Preparation of MTZ-thermosensitive hydrogel
The polymer solutions were separately prepared before hydrogel preparation. SF solution was prepared by dissolving the freeze-dried SF in sterile water. MC and PF127 solutions were prepared by separately dispersing the powders in water with gentle mixing, followed by storing in a refrigerator until the solutions were clear.

MTZ-thermosensitive hydrogel was prepared by physical mixing method. MTZ was mixed with SF solution under gentle stirring at room temperature for 10 min. Then, MC solution and PF127 solution were added into the mixture with gentle stirring for 5 min. The final volume was adjusted by sterile water and further stirred for 30 min. Hydrogels were prepared by varying concentrations of SF and MC, while the concentration of PF127 and MTZ were kept constant at 28% and 0.05% w/v (Table 1).

2.4. Determination of sol-gel transition of MTZ-thermosensitive hydrogel
A vial-inverting method was employed to determine the occurrence of the sol-gel transition. The sol formation was determined as flowing liquid and the gel formation as a non-flowing gel when the vial was inverted. 1 mL of thermosensitive hydrogel solution was transferred into a sealed test tube which were subsequently immersed into a water bath at 4 ± 0.5 ºC, 25 ± 0.5 ºC, and 37 ± 0.5 ºC. The gelation time of thermosensitive hydrogel were determined as the initial time point at which the solution did not flow when tilted.

2.5. Viscosity test of MTZ-thermosensitive hydrogel
The viscosity of MTZ-thermosensitive hydrogel solutions were measured at 4 ± 0.5 ºC by a Brookfield rotational rheometer model DV-III (Brookfiled engineering labs, USA) fitted with a parallel plate configuration (40 mm in diameter) with a rotating rate of 6 rpm. The 0.5-mL hydrogel solutions were placed at the parallel plate with the temperature controller and subjected to the viscosity analysis.

2.6. Gel strength test of MTZ-thermosensitive hydrogel
Gel strength of the samples were analyzed by a texture analyzer (TA.XT. plus, Charpa Techcenter Co., Ltd., Thailand). 1 mL of the hydrogel solution was placed in a 5-mL vial, thereby avoiding the air into the samples and assuring generation of a smooth upper surface. Then, the hydrogel solution was incubated at 37 ± 0.5 ºC for 15 min to form hydrogel. A hemispherical probe P/0.5HS (50 mm in diameter) was compressed into the hydrogel sample to a defined depth of 4 mm. Three replicate analyses were performed at 37 ± 0.5 ºC for each formulation, providing the same conditions for each measurement.

2.7. Metronidazole dissolution test and hydrogel erosion study
The drug dissolution profiles were evaluated by in vitro dissolution method at 37 ± 0.5 ºC. 1 mL of the MTZ-thermosensitive hydrogel was transferred into 10-mL vial and kept at 37 ºC for 15 min to form hydrogel. Then, 1 ml PBS pH 6.6 was added into the vial as a dissolution medium (sink condition, as MTZ aqueous solubility is 10 mg/mL at pH 2.5-8) and the temperature was maintained at 37 ºC in an incubator with shaking at 50 rpm. At each time point, 0.5 mL of the sample was withdrawn, and the same volume of fresh dissolution medium pre-warmed to 37 ± 0.5ºC was replaced. The withdrawn samples were collected to analyze the amount of MTZ by HPLC, modified from Trivedi et al.25 Briefly, the samples were diluted with a mobile phase (20 mM KH₂PO₄: methanol at a ratio of 70:30 (v/v) with TEA 0.1 % v/v) and subjected to HPLC (Shimadzu, Japan), equipped with SPD-20A UV-Visible detector. The separation was done with a Vertisep C18 column (5 µm, 4.6 x 250 mm, USA), at a flow rate of 1.0 mL/min and a detection wavelength of 364 nm. The standard curve was constructed in the range of 5-100 µg/mL with a regression equation of y = 10706x – 2950.1 and a correlation coefficient (R²) of 0.9997. The MTZ amount was calculated based on the calibration curve and the results were reported as an average cumulative drug release percentages of three determinations.

To understand the mechanism of drug release, correlation coefficients (r²) for various models (zero order, first order and Higuchi model) were determined for all samples by Microsoft Excel 2018. The release model having the highest r² value was considered as the fitted model to assess the drug release kinetics.

In terms of hydrogel erosion study, same experimental settings with the dissolution study were conducted. At each time point, 0.5 mL of the sample was withdrawn and freshly replaced. The remaining hydrogels after erosion was collected and photographed.

2.8. Statistical analysis
The results are expressed as mean ± standard deviation (SD). Differences between the groups were compared by one-way ANOVA followed by Tukey’s post hoc test. The results were considered to be statistically significant at p < 0.05.

3. Results and discussions
3.1. Sol–gel transition time of MTZ-thermosensitive hydrogels

To determine the suitability for in situ application, as well as storage conditions, of the formulations, the sol–gel transition tests were conducted in 3 different temperatures, including the storage temperature at 4 ºC, the room temperature at 25 ºC, and the application periodontal pocket site temperature at 37 ºC.

In our preliminary study, by varying the PF127 concentrations from 10% to 30% w/v, we found that the 28% PF127 + 0.05% MTZ solution can transform into hydrogel within 1 min at 37 ºC. At lower PF127 concentration of 18%, only the blank PF127 solution formed hydrogel, whereas the PF127 + 0.05% MTZ remained in the solution form. This suggests that PF127 and the drug MTZ might interact via non-covalent bonds, consequently disrupt hydrophobic interaction during PF127 gelation process, resulting in increased sol–gel transition temperature of PF127. Therefore, 28% PF127 was chosen for further studies. However, the PF127 hydrogel had low mechanical properties, which might result in hydrogel leaking from the periodontal pocket after administration, as well as rapid drug release. Therefore, to increase the mechanical properties of PF127 hydrogels, MC and SF were used as gel strength enhancers.

To this end, three groups of formulations, including PF/MC, PF/SF, and PF/MC/SF, at three different concentrations (0.25, 0.5, and 0.75% w/v) of the enhancer, were prepared (Table 1). Then, the gelation time at 4 ºC (storage temperature), 25 ºC (room temperature), and 37 ºC (bodily temperature), was determined (Table 2). At 4 ºC, all samples remained in low-viscosity liquid form for at least 6 months, suggesting their stability for long-term storage. At 25 ºC, the gel was formed after at least 15 min, allowing adequate time for periodontal administration. After administered to the site of action, at 37 ºC, the solutions formed gel almost immediately within less than 1 min (Figure 1), which further stick on the dental cavity for longer time. Thus, the drug could perform its action without being washed down to the gastrointestinal tract, consequently enhance its efficacy and reduce the systemic side effects. Ultimately, these thermosensitive-hydrogel should be stored at 4 ºC (solution form), and quick apply (not more than 15 min) to the periodontal pocket site after getting out from the fridge. The mechanism of sol–gel transition of thermosensitive-hydrogel is shown in Figure 2. First, at a concentration higher than PF127 critical micelle concentration (CMC, 1-7 g/L), the polymer formed polymeric micelles consisted of a PPO hydrophobic core enclosed by the hydrophilic PEO blocks. MTZ was mostly resided in the PPO hydrophobic core. When the temperature increased to a higher value (37 ºC in our case) than the PF127 lower critical solution temperature (LCST), the polymer solubility reduces by partial dehydration, leading to the decrease in PF127 CMC (0.09 g/L at 37 ºC). Consequently, more micelles were formed, leading to formation of packed micellar structure due to enhanced particles contact, ultimately forming gel. The MC and SF molecules could entangle with hydrophilic PEO shell of the PF127 micelles by intercellular packing, thus, leading to gel property enhancement.

3.2. Viscosity of MTZ-thermosensitive hydrogel

All hydrogels were determined their viscosity at 4 ºC in the solution form, to ensure the hydrogel can be subjected into the periodontal infection site (Figure 3). All hydrogel formulations had viscosity of less than 500 cP, demonstrating a low viscosity, which is beneficial to be administered to the lesion site easily. Additionally, the MC content could influence the viscosity properties of the hydrogel. When the MC content increased from 0.25 to 0.75 %w/v, the viscosity of PF/MC dramatically increased from 158.00 to 476.17 cP. Whereas, an increase in SF content, in both PF/SF and PF/MC/SF hydrogel, showed no observable changes in viscosity. This can be explained by the inherent hydrophilicity of MC and SF. MC can form hydrogen bondings with water and/or PF127, consequently increases the gel connecting networks, resulted in enhanced viscosity. In contrast, less hydrophilic SF...
might has limited interactions with the polymer compared to MC, thus, did not affect the gel viscosity.

3.3. Gel strength of MTZ-thermosensitive hydrogel

All hydrogels were determined their gel strength at 37 ºC and the results are shown in Figure 4. When increasing the MC concentration from 0.25% to 0.75%, the PF/MC hydrogel gel strength proportionally decreased from 2234.97 to 1143.70 g. On the other hand, the PF/SF hydrogel gel strength slightly increased from 1809.20 to 2196.17 g when the SF concentrations increased from 0.25% to 0.75%. Thus, it is possible that the hydrophobic SF enhanced the mechanical strength of the hydrogels by forming the rigid β-sheet, which induced by heat at 37 ºC. Whereas the hydrophilic MC increased hydrogel flexibility by forming hydrogen bonding with water and hydrophilic PEO shell of polymeric micelles. Interestingly, when combining both MC and SF in the formulations, the gel strength of PF/MC/SF decreased slightly when increasing the SF concentrations. This phenomenon might be explained by the interactions between MC and SF, as MC could swell and coated on the SF surfaces, altering its properties. Nevertheless, more experiments are needed to explain this issue.

3.4. Drug dissolution study and erosion behavior of MTZ-thermosensitive hydrogels

To mimic the conditions of periodontal infection, a sink condition with PBS at pH 6.6 and 37 ºC, was chosen to be the medium for studying the dissolution behavior of MTZ-thermosensitive hydrogels. Notably, all PF/SF formulations were dissolved rapidly in the dissolution medium, and all PF/MC formulations were not strong/rigid enough for experiment conduction. Thus, both MC and SF are necessary to improve the gel properties, as MC enhances gel viscosity and SF increases its strength. Figure 5A demonstrated the cumulative release percentages of MTZ over time from PF/MC/SF formulations at different SF concentrations of 0.25%, 0.5%, and 0.75% w/v. With no significant differences, all formulations showed a sustain dissolution profile of MTZ up to 80% within 10 days. Furthermore, these dissolution data fitted well with the Higuchi model (Table 3), with r² values of > 0.98, indicating that the drug released via diffusion-controlled mechanism.

As expected, the hydrogel erosion in dissolution medium was observed. As shown in Figure 5B, the hydrogels were dissolved sustainably, with a remaining weight of approximately 40% after 9 days of the dissolution study (data not showed). This was attributed to the hydrophilicity of both PF127 and MC, which are subjected to dissolution in aqueous medium. Since the drug MTZ located in the PPO hydrophobic core of PF127 micelles, its diffusion-controlled release profile is governed by the PF127 and/or MC dissolution. This fact, therefore, benefits the periodontitis treatment, as the systems release high initial dose in the first day (approximately 30%) that is adequate for fast action; followed by a decreasing release rate over the next 9 days that is suitable for maintaining the drug efficacy without the need of re-administration.

MTZ is commonly used to inhibit infection from anaerobic microorganisms such as Porphyromonas gingivalis. The MIC of MTZ against this bacteria isolated is in the range of 0.063-0.514 µg/mL. From our data, the amount of MTZ release in the first time point (the first day) was approximately 120 µg/mL for all formulations, which was more than 200 times higher than the MIC of MTZ on P. gingivalis. In addition, the amount of MTZ release in the 9th day was approximately 13 µg/mL, which was still more than 20 times higher than the required MIC. Thus, the release amount of drug reached the therapeutic concentration for at least 9 days.

4. Conclusions

This study successfully developed and characterized the novel metronidazole loaded PF/MC/SF thermosensitive hydrogels as in situ drug delivery systems for the treatment of
periodontitis. The hydrogels remained in low-viscosity solution form for at least 6 months at 4 °C (storage temperature) and rapidly formed hydrogel at 37 °C within 1 minute after injection into the dental pocket. Silk fibroin significantly enhanced the gel strength, whereas methyl cellulose increased its viscosity. Furthermore, the hydrogels exhibited sustained metronidazole released for 10 days, which benefit for reducing administration frequency. The drug dissolution profile of PF/MC/SF was governed by 2 different mechanisms including hydrogel erosion by fluid at the administration site (periodontal pocket) and the diffusion of the drug through the hydrogels. In conclusion, these novel thermosensitive hydrogels possess great potential in controlling drug release in periodontal pocket, which might improve its pharmacological properties. Thus, this could be further investigated in vitro and in vivo to become a clinical treatment in the future.

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Conflict of interest
The authors declare none.
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| Formulation     | MC (% w/v) | SF (% w/v) |
|-----------------|------------|------------|
| PF/MC 0.25      | 0.25       | -          |
| PF/MC 0.5       | 0.5        | -          |
| PF/MC 0.75      | 0.75       | -          |
| PF/SF 0.25      | -          | 0.25       |
| PF/SF 0.5       | -          | 0.5        |
| PF/SF 0.75      | -          | 0.75       |
| PF/MC/SF 0.25   | 0.25       | 0.25       |
| PF/MC/SF 0.5    | 0.25       | 0.5        |
| PF/MC/SF 0.75   | 0.25       | 0.75       |
Table 2. Sol-gel transition time of thermosensitive hydrogels at 4, 25, and 37 ºC. The results were presented as mean ± SD (n = 3). The time unit at 25 ºC and 37 ºC is minute and second, respectively.

| Formulation\textsuperscript{a,c} | Gelation time |                  |                  |
|----------------------------------|---------------|------------------|------------------|
|                                  | 4 ºC\textsuperscript{b} | 25 ºC (min)      | 37 ºC (sec)      |
| PF/MC 0.25                       | N/A           | 17 ± 1           | 33 ± 2           |
| PF/MC 0.5                        | N/A           | 39 ± 2           | 48 ± 3           |
| PF/MC 0.75                       | N/A           | > 120            | 61 ± 3           |
| PF/SF 0.25                       | N/A           | 26 ± 1           | 35 ± 2           |
| PF/SF 0.5                        | N/A           | 20 ± 2           | 28 ± 2           |
| PF/SF 0.75                       | N/A           | 16 ± 1           | 25 ± 2           |
| PF/MC/SF 0.25                    | N/A           | 19 ± 1           | 35 ± 2           |
| PF/MC/SF 0.5                     | N/A           | 28 ± 2           | 30 ± 2           |
| PF/MC/SF 0.75                    | N/A           | 40 ± 2           | 29 ± 1           |

PF: Pluronic F127, MC: methyl cellulose, SF: silk fibroin
\textsuperscript{a}Thermosensitive hydrogel composed of 28% PF127 and 0.05% w/v MTZ.
\textsuperscript{b}N/A = could not form hydrogel for at least 6 months
\textsuperscript{c}For PF/MC/SF formulations, the MC concentration was fixed at 0.25% w/v
| Formulation\(^a\) | r\(^2\) value | Zero order | First order | Higuchi model |
|------------------|----------------|------------|-------------|---------------|
| PF/MC/SF 0.25    | 0.7139         | -0.5380    | 0.9872      |
| PF/MC/SF 0.5     | 0.7331         | -0.5090    | 0.9865      |
| PF/MC/SF 0.75    | 0.7012         | -0.5420    | 0.9811      |

PF: Pluronic F127, MC: methyl cellulose, SF: silk fibroin

\(^a\)Thermosensitive hydrogel consisted of 28% PF127, 0.05% w/v MTZ, and 0.25% w/v MC
**Figure 1.** Sol-gel transition images of MTZ-thermosensitive hydrogel, composed of silk fibroin, methyl cellulose, and Pluronic F127, by vial-inverting method at 25 °C and 37 °C.
**Figure 2.** The mechanism of micellar packing formation of MTZ-thermosensitive hydrogel.
Figure 3. Viscosity of MTZ-thermosensitive hydrogel at 4 °C. Data are presented as mean ± SD (n = 3). Different letters (a, b, c, d) present statistical differences, $p < 0.05$. 
Figure 4. Gel strength of MTZ-thermosensitive hydrogels at 37 °C. Data are presented as mean ± SD (n = 3). All data was significantly different from each other (p < 0.05).
Figure 5. (A) Cumulative release of MTZ from thermosensitive hydrogel in PBS pH 6.6 within 10 days and (B) the erosion of MTZ-thermosensitive hydrogel (PF/MC/SF 0.75) in PBS pH 6.6 within 9 days. Data are presented as mean ± SD (n = 3).