Research Article

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Multifunctional hydrogel based on silk fibroin/thermosensitive polymers supporting implant biomaterials in osteomyelitis

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Abstract: The search for novel antibiotic-loaded biomaterials that support the management of osteomyelitis has been extensive in current years. This study developed multifunctional hydrogel to enhance the performance of osteo-conduction and antimicrobial as supporting implant materials for osteomyelitis treatment and prevention. Ciprofloxacin-loaded thermosensitive hydrogels composed of silk fibroin and poloxamers were prepared using the cold method. F1, F2, and F3 formulations resulted in gelation time of within 3 min under 37°C. Physical evaluation showed that the formulations, especially F3, had a proper swelling ratio and 82.34% biodegradability within 14 days. Ciprofloxacin from formulations exhibited an initial burst release within 24 h and continuous delivery, with more than 84% release over 14 days. Released antibiotics showed efficacy against *Staphylococcus aureus* ATCC25923 and eradicating activity against biofilms. Moreover, hydrogels had no cytotoxic effect on MC3T3-E1 cells with 93–106% cell viability. This multifunctional hydrogel showed a promising approach for supporting osteomyelitis treatment and prevention.

Keywords: thermosensitive hydrogel, bone tissue engineering, fibroin, poloxamer, osteomyelitis

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1 Introduction

Osteomyelitis is a serious infection of the bone mostly caused by bacteria, especially Staphylococci. Infections can reach the bone through blood circulation or spread from adjacent tissue. Antibiotic treatment has limited success because the drug cannot easily penetrate the infected bone (1). Surgery is to remove the abscess or necrotic bone tissue, followed by implanting a biomaterial mixed with an antibiotic for local treatment. In clinical practice, antibiotic-loaded polymethyl methacrylate (PMMA) bone cement is inserted into the bone cavity to eliminate the bacteria. (2,3) This local treatment has reduced side effects compared with systemic antibiotic therapy. However, PMMA bone cement has limitations because it is non-degradable and delays bone healing (4). A subsequent, operation is required to remove the biomaterial, causing a second round of pain and new inflammation at the implantation site (5,6). In addition, PMMA has no functional osteo-conductive and osteo-inductive properties to support bone reconstruction (3). Therefore, a biodegradable antibiotic carrier with functional properties would be eminently more suitable (7).

Some thermosensitive polymers including poloxamers exhibit temperature-dependent sol–gel transitions. Poloxamers are water-soluble polymers that can form hydrogels at body temperature. Poloxamers have recently attracted increased attention in biomedical and pharmaceutical applications as hydrogels for drug delivery and tissue engineering (8). Hydrogels are thermosensitive and exist in solution and gel phases depending on the temperature. The delicate balance between portions of the polymer components causes sol–gel phase conversion (9). Hydrogels applied using poloxamers are useful for transdermal, injectable, and ophthalmic medications and therapeutics (10,11). The controllable release of the agent is sensitive to the transformation of the hydrogel, and this has many advantages including biocompatibility and non-toxicity.

Silk is an abundant natural protein polymer produced by insects such as silkworms, spiders, and bees. Mulberry silk, widely used in textile industry, is produced by *Bombyx mori* (12). Fibroin and sericin are the major protein components of silk. Sericin has biological activities (13,14), while fibroin has shown promise for biomedical applications because of its biocompatibility, gradual disintegration, and low immunogenicity. Fibroin promoted stem cell adhesion and differentiation, enhanced tissue healing, and inhibited pathological adherence (15). Silk fibroin has been investigated in bone tissue engineering as an attractive biomaterial for scaffold (16). A composite thermosensitive hydrogel made from silk fibroin and poloxamer showed promise as drug delivery system in tissue engineering and regenerative medicine applications (17,18). Using this system for osteomyelitis treatment would overcome the current limitations.

Therefore, the aim of this study was to develop ciprofloxacin-loaded fibroin thermosensitive hydrogel as a delivery system incorporated with implant biomaterial for treatment and prevention of osteomyelitis. Amounts of silk fibroin and poloxamers (P407 and P188) were optimized to produce thermosensitive hydrogel without chemical crosslinking agents. Hydrogel formulations were characterized by scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, and rheological measurements. Properties of the hydrogel formulations including swelling ratio, degradability, drug release profile, antibacterial activity, and cell proliferation were studied to optimize the potential of hydrogel in supporting osteomyelitis treatment and prevention.

2 Materials and methods

2.1 Materials

Silk cocoons were provided by the Queen Sirikit Department of Sericulture. Poloxamer 407 (Pluronic® F-127) and ciprofloxacin were purchased from Sigma-Aldrich. Poloxamer 188 (Pluronic® F-68) was purchased from HiMedia. Mouse pre-osteoblastic MC3T3-E1 cell lines were provided by the Institute of Biomedical Engineering, Faculty of Medicine, Prince of Songkla University, Thailand. *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213, and clinical isolates of *S. aureus* were obtained from the Faculty of Medical Technology, Prince of Songkla University, Thailand.

2.2 Fibroin preparation

Fibroin was extracted from *B. mori* silk cocoon by boiling in 0.02 M Na_2CO_3 for 30 min. The fibroin was then washed and dried overnight at 70°C before dissolving in 9.3 M LiBr at 70°C for 60 min. The fibroin solution was dialyzed against distilled water for 3 days using a dialysis bag and then centrifuged at 3,500 rpm at 4°C for 20 min. The supernatant was transferred to a new tube and collected at 4°C before use.

2.3 Ciprofloxacin-loaded silk fibroin/ thermosensitive polymer preparation

Ciprofloxacin-loaded fibroin thermosensitive hydrogels were prepared by the cold method. Briefly, 33% w/v of poloxamer 407 (P407) and 5% w/v of poloxamer 188 (P188) were dissolved in cold water at 4°C until a homogeneous solution was obtained. Silk fibroin was then added to the poloxamer solution at various ratios (Table 1). To prepare antibiotic-loaded fibroin thermosensitive hydrogels, ciprofloxacin was added to the mixture at a final concentration of 4 $mg \cdot ml^{-1}$.

2.4 Gelation time

Gelation time was determined by the tube inversion method. Briefly, 1 ml of hydrogel in the solution phase was added to a tube and then placed in a water bath at 37°C. Gelation time was observed every 5 s by inverting the tube horizontally. The gelation time was recorded when the liquid transformed into a gel and did not flow.

Table 1: Compositions of ciprofloxacin-loaded fibroin thermosensitive hydrogels with ciprofloxacin at a final concentration of 4 $mg \cdot ml^{-1}$

Formulation	Fibroin (5% w/v)	Poloxamer P407 (33% w/w) + P188 (5% w/w)
F1	32	68
F2	34	66
F3	36	64

2.5 Morphological characteristics

The morphological characteristics of ciprofloxacin-loaded fibroin thermosensitive hydrogels were investigated by SEM analysis (JEOL, Japan). Freeze-dried hydrogels were cross-sectioned, mounted onto stubs, and coated with gold. The prepared samples were observed using a SEM.

2.6 FTIR

FTIR analyses of the freeze-dried ciprofloxacin-loaded fibroin thermosensitive hydrogels were performed using a spectrometer (Bruker, Germany). All infrared spectra were recorded with 100 scans at 4.0 cm^{-1} resolution for wavelengths ranging between 4,000 and 400 cm^{-1}.

2.7 Rheological properties

The rheological properties of ciprofloxacin-loaded fibroin thermosensitive hydrogel were analyzed using a rheometer with a 60 mm diameter plate geometry at a gap of 1 mm. Hydrogel solutions were added to the plate of the rheometer (TA Instruments, USA) at 25°C. Dynamic temperature scanning was performed at 25–45°C at a heating rate of 0.5°C·min^{-1}. The storage modulus (G') and loss modulus (G'') were recorded under constant strain of 0.1% and frequency 1 Hz.

2.8 Swelling ratio

Swelling ratios of the freeze-dried hydrogels were determined by immersing in phosphate-buffered saline (PBS) at 37°C for 24 h. After removal from PBS, the hydrogels were immediately weighed. The swelling ratio was calculated using the following equation:

$$\text{Swelling ratio} = \frac{(W_s - W_d)}{W_d}$$

where W_s and W_d represent the weights of the swollen hydrogel and the dry hydrogel, respectively.

2.9 Enzymatic degradation

The biodegradation of ciprofloxacin-loaded fibroin thermosensitive hydrogels was studied in lysozyme solution and weight loss was measured. In brief, the formed hydrogels were freeze-dried and measured for initial dry weight (W_i). The dried hydrogels were then immersed in 2 mg·ml^{-1} of lysozyme in PBS and further incubated at 37°C. At the specific time, the hydrogels were removed and freeze-dried to obtain the final weight (W_t). Enzymatic degradation evaluated by percentage weight loss was calculated using the following equation:

$$\text{Weight loss}(\%) = ((W_i - W_t)/W_i) \times 100$$

2.10 Drug release

Ciprofloxacin-loaded fibroin thermosensitive hydrogels were prepared as described above and incubated at 37°C until the hydrogels were formed. Ten milliliters of deionized water were added on top of the hydrogels at 37°C. At the specific time point, the release medium was collected and replaced by the same volume of fresh deionized water. Release of ciprofloxacin from the hydrogels was determined using a microplate reader (Multiskan Sky Thermo Scientific, USA) at a wavelength of 309 nm.

2.11 Cytotoxicity evaluation

Ciprofloxacin-loaded fibroin thermosensitive hydrogels were prepared and incubated at 37°C until the hydrogel was formed. Then, α-minimum essential medium (α-MEM) was added on the top of the hydrogels and incubated at 37°C for 24 h before collection. MC3T3-E1 osteoblastic cell line was cultured in α- MEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic at 37°C, 5% CO_2, and 95% humidity. MC3T3-E1 cells were seeded on the 96-well plates with a density of 1×10^4 cells/well and cultured in completed α-MEM. After 24 h, the culture medium was removed and replaced with hydrogel extracts. The cells were then incubated at 37°C, 5% CO_2, and 95% humidity for 24 h. Untreated cells were used as controls. Cell viability was measured using the MTT assay. Briefly, MTT solution was added to each well and incubated for 4 h. The MTT solution was removed, and dimethyl sulfoxide solution was added to dissolve the formazan crystals. The optical density at 570 nm (OD570nm) was recorded using a microplate reader (Multiskan Sky Thermo Scientific, USA). Percentage of cell viability was calculated using the following formula:

$$\text{Percentage of cell viability} = \frac{\text{OD570 nm treated cells}}{\text{OD570 nm untreated cells}} \times 100$$

2.12 Antibacterial efficacy of released antibiotics

All bacterial strains were grown on Luria-Bertani (LB) agar at 37°C. Bacterial colonies were suspended in LB broth and grown into the log phase. The bacteria were then adjusted to 0.5 McFarland standard. Antibacterial activities of the hydrogels were tested by the Kirby-Bauer disk diffusion method on Mueller-Hinton agar plates using ciprofloxacin (5 µg) and the paper disk absorbed 20 µl of ciprofloxacin from the drug release experiment (1, 2, 4, and 8 h). All isolates were interpreted following the Guidelines of the Clinical Laboratory and Standards Institute (19).

2.13 Quantitative biofilm production

Twenty milliliters of bacteria at 0.5 McFarland standard were filled in 96-well flat-bottom microplates with 180 µl of tryptic soy broth (TSB) containing 1% glucose and incubated at 37°C for 24 h. The medium was used as a negative control, while *S. aureus* ATCC 29213 known as a biofilm-producing reference strain, and clinical isolates of *S. aureus* were used in this study. After incubation, the wells were washed three times with PBS (pH 7.2) to remove the planktonic bacteria. The biofilm was fixed with absolute ethanol for 20 min and air-dried overnight. Biofilm mass was stained with 2% Hucker's crystal violet for 15 min and washed with running tap water. The stain was dissolved using 33% v/v glacial acetic acid and the absorbance at 570 nm was measured using a microplate reader (Multiskan Sky Thermo Scientific, USA). All assays were performed in triplicate. The biofilm mass was calculated and graded following a previous study (20).

2.14 Biofilm eradication activity of released antibiotics

Determination of the biofilm eradication activities of the hydrogel extracts against bacteria was carried out by the biofilm production assay, as described above. After 24 h, the wells were washed three times with PBS, added with 200 µl of the extract and incubated at 37°C for 18 h. The biofilm mass was washed and evaluated by 2% Hucker's crystal violet staining assay. Bacteria in TSB containing 1% glucose served as an untreated control. All experiments were performed in triplicate. Percentages of biofilm eradication were calculated as follows:

$$\%\text{Biofilm eradication} = \frac{\text{Untreated OD570 nm} - \text{Treated OD570 nm}}{\text{Untreated OD570 nm}} \times 100$$

The eradication effects of hydrogel extracts and ciprofloxacin on mature bacterial biofilms were also observed by microscopy. Briefly, the treated and untreated bacteria were stained with 0.1% crystal violet and observed under an inverted light microscope (Thermo Fisher Scientific, USA).

2.15 Statistical analysis

Statistical analyses were performed using analysis of variance, with the level of significant difference defined as $p < 0.05$.

3 Results and discussion

3.1 Preparation and characterization of ciprofloxacin-loaded silk fibroin thermosensitive hydrogels

Ideally, thermosensitive hydrogel should be in liquid form at room temperature and converted to hydrogel form under body temperature (21). Antibiotics can be loaded and delivered to the infection area directly to eliminate bacteria that have colonized the bone tissue. Moreover, hydrogel could act as a scaffold to support cell attachment and cell proliferation. Biocompatibility, low cytotoxicity, and favorable rheological properties have made poloxamers appealing for use in bone tissue engineering (8). However, poloxamers have weak gel strength and rapid dissolution of the gels formed limits their performance in drug delivery systems (22,23). Silk fibroin has been widely used in bone tissue engineering applications because of its good mechanical strength, controllable degradation and drug release, and biological activities (24,25). In this study, fibroin was incorporated with P407 and P188 to form fibroin thermosensitive hydrogels and improve functional properties. Ciprofloxacin, a broad-spectrum bactericidal antibiotic against osteomyelitis pathogens such as *Staphylococcus aureus* being the most common bacteria, *Pseudomonas aeruginosa* for long bone infection (26), and *Aggregatibacter actinomycetemcomitans* for jawbone infection (27) *etc*., was selected to load into the hydrogel.

In this study, 33% w/v of P407 was combined with 5% w/v P188 and mixed with different ratios of fibroin.

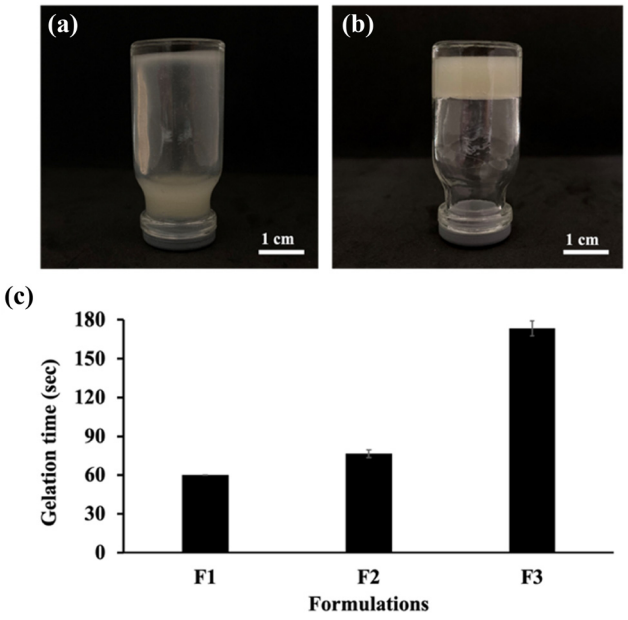

Figure 1: Representative photographs of ciprofloxacin-loaded fibroin thermosensitive hydrogels (F3): (a) 25°C, (b) 37°C, and (c) gelation times at 37°C.

Preparation of the thermosensitive hydrogel was successful using an uncomplicated procedure without chemical crosslinking agents. As shown in Figure 1a and b, the hydrogel remained in the solution at room temperature and became hydrogel at 37°C, which was suitable to use in human body. Gelation times of the three hydrogel formulations were determined by the tube inversion method. The resulting gelation time was found to be within 3 min (Figure 1c). Increasing the poloxamer content of thermosensitive hydrogel enhanced gelation behavior, resulting in a shorter gelation time (28). The gelation time indicated that all hydrogel formulations had a desirable gelation behavior for osteomyelitis treatment, compared to previous reports (29,30).

A SEM examination of the hydrogels was conducted to evaluate the degree of porosity that supported cell diffusion, secretable therapeutic molecules, and nutrients for cell survival (31). Results showed that the ciprofloxacin-loaded fibroin thermosensitive hydrogels had a dense, porous and expanded network structure (Figure 2a–c), indicating the formation of a stable hydrogel (32). Pore size and pore size distribution of the three hydrogel formulations were measured and analyzed at magnification of 100× (Figure 2d). F1 displayed an irregular pore size distribution with pore size ranging from 8.11 to 110.44 μm. An increase in fibroin ratio led to a homogeneity of pore size (33). Uniformly scattered porosity was found in F2 and F3 with 24.02 ± 5.18 μm and 40.90 ± 6.57 μm pore sizes, respectively. The crosslinking density in the hydrogels was also associated with the content of poloxamer. High concentration of poloxamer probably conferred small pore size, due to its high-water binding capacity (34–36). Cross-sectional morphology of the hydrogels showed a porous structure layer, resembling the structure of periosteum and suitable for bone formation (37). Therefore, ciprofloxacin-loaded fibroin thermosensitive hydrogels showed promise as a supportive network for bone cells during the infection curing period.

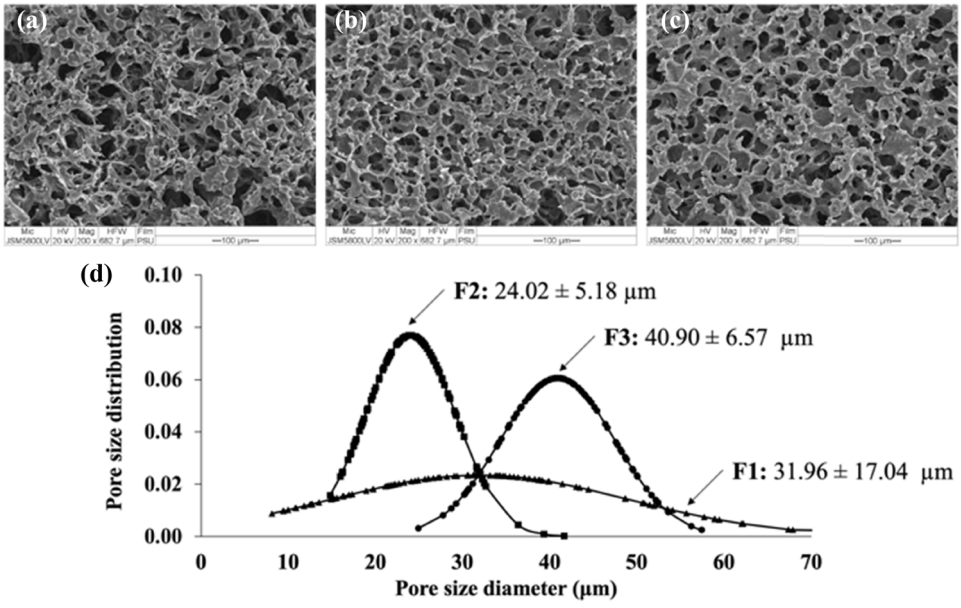

Figure 2: SEM images (200×) of ciprofloxacin-loaded fibroin thermosensitive hydrogels. (a) F1, (b) F2, (c) F3, and (d) pore size distributions.

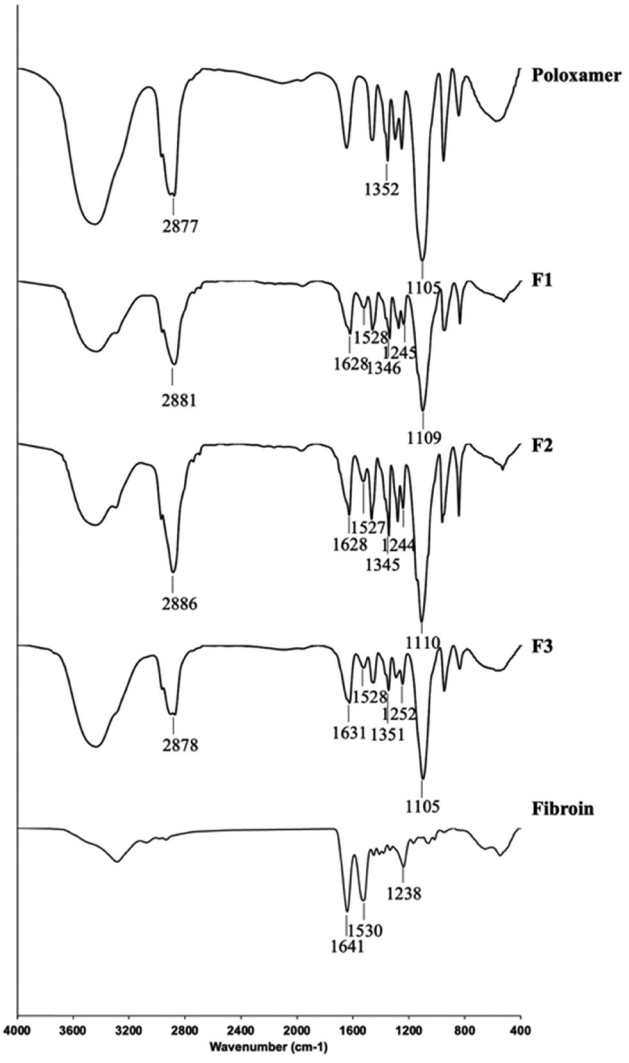

Figure 3: FTIR spectra of poloxamer, ciprofloxacin-loaded fibroin thermosensitive hydrogels, and fibroin.

The chemical compositions of fibroin and poloxamer, and interaction of fibroin/poloxamer thermosensitive hydrogel formulations were analyzed by FTIR spectroscopy. Major peaks of silk fibroin were found at 1,700–1,600, 1,600–1,500, and 1,350–1,200 cm^{-1} corresponding to amide I (C═O stretching), amide II (N–H bending), and amide III (C–N stretching) groups, respectively (38). As shown in Figure 3, the band for amide I was observed at 1,621 cm^{-1}, amide II at 1,530 cm^{-1}, and amide III at 1,238 cm^{-1}. FTIR spectrum of poloxamer was observed at 2,877 cm^{-1} (aliphatic C–H stretching), 1,352 cm^{-1} (in-plane O–H bending), and 1,105 cm^{-1} (C–O–C stretching). The amide I and amide II peaks of hydrogel shifted to lower wavenumbers for all formulations. The shifting of the wavenumbers might be the result of the intermolecular interaction between fibroin and poloxamer (18). Moreover, aliphatic C–H stretching and C–O–C stretching of poloxamer of all hydrogel formulations shifted to higher wavenumbers, indicating molecular motion into the hydrogel (39,40).

All hydrogels were tested for their rheological properties including complex viscosity, storage modulus (G') and loss modulus (G'') of the aqueous solutions as a function of temperature. At temperatures below 30°C, values of complex viscosity, G', and G'' were low in all formulations. However, all values rapidly increased by several orders of magnitude, with G' higher than G'' when temperature increased above 30°C (Figure 4). The thermosensitive hydrogel had higher mechanical properties when a specific temperature was reached. Interestingly, G' larger than G'' corresponded to fibroin content, implying partial hydrogel formation due to molecular organization of thermosensitive polymers as a result of the interference of silk fibroin molecule, fibroin aggregation, or fibroin-poloxamer molecular interaction. Fibroin enhanced the mechanical properties of poloxamer hydrogels, making them suitable as osteo-conductive scaffold (41).

3.2 Physical properties of ciprofloxacin-loaded silk fibroin thermosensitive hydrogels

Swelling behavior is an important property of tissue engineering scaffolds which involves the diffusion of nutrients and biological signals and plays an important role in tissue regeneration (42). Figure 5a shows the swelling results of ciprofloxacin-loaded fibroin thermosensitive hydrogels in PBS at 37°C for 24 h. The highest swelling ratio was found in F3, followed by F2 and F1, respectively. Swelling ratios of F2 and F3 were significantly higher than F1. The swelling ratio of the hydrogels followed a content-dependent manner of poloxamer and fibroin. Low poloxamer and high fibroin contents of F2 and F3 caused higher hydrogel swelling ratio. Poloxamer is a tri-block copolymer composed of a hydrophobic core of polypropylene oxide (PPO) surrounded by two hydrophilic chains of polyethylene oxide. With higher poloxamer ratio, PPO blocks are enlarged, forming transient 3D polymer gel formation and increased chain entanglement. This reduces the quantity of water entering the polymer framework and decreases the swelling ratio (43). Results demonstrated that addition of fibroin, a natural polymer, enhanced the capacity to absorb water and nutrients without destroying the physical properties of thermosensitive hydrogel.

Biodegradability is one of the most important properties of biomaterials in biomedical applications. The biodegradable hydrogel should be matched with new bone

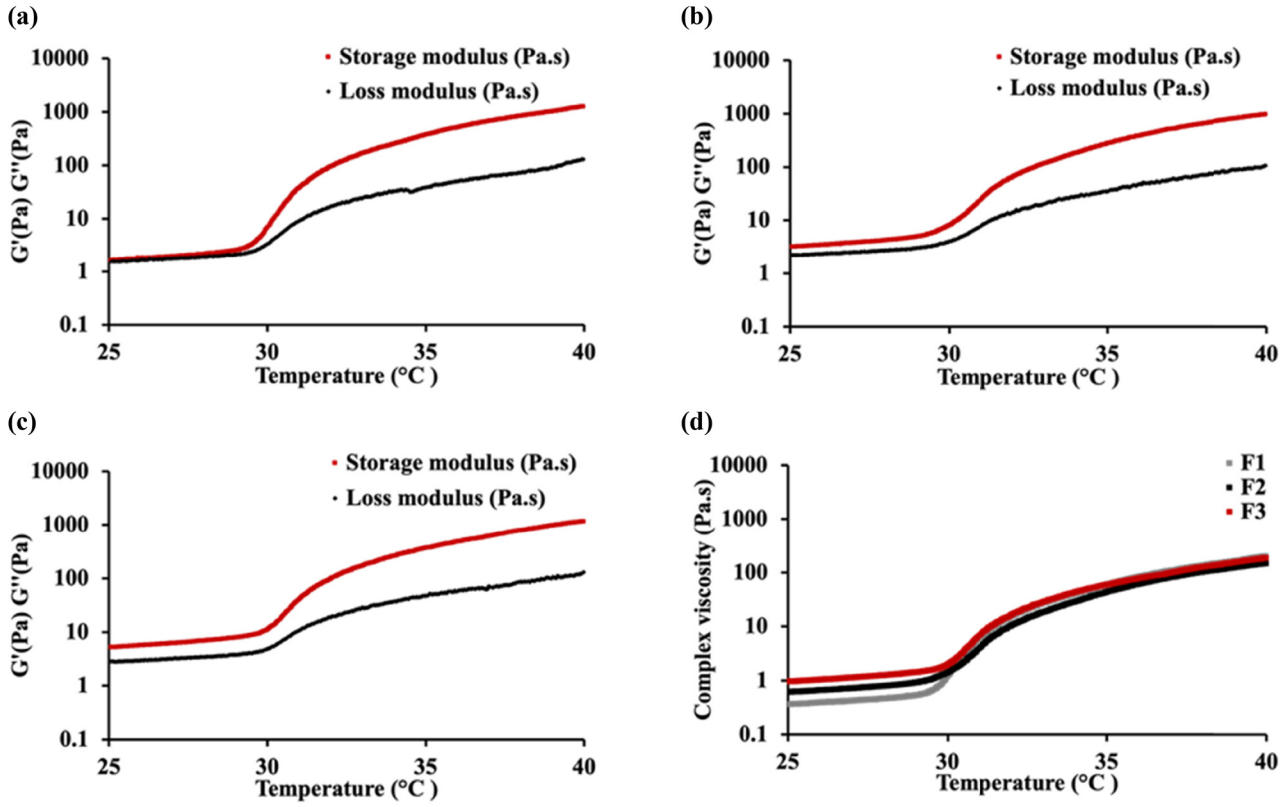

Figure 4: Rheological analysis of ciprofloxacin-loaded fibroin thermosensitive hydrogels. (a) F1, (b) F2, (c) F3, and (d) complex viscosity of ciprofloxacin-loaded fibroin thermosensitive hydrogels.

regeneration and gradually replaced by new bone tissue without additional surgery to remove the materials (44). All three hydrogel formulations showed fast degradation behavior during the first 3 days (Figure 5b), caused by dissolution of the hydrogel surface layers. After 14 days, weight losses of F1, F2, and F3 hydrogels were 79.21%, 79.30%, and 82.34%, respectively. No significant differences in weight loss were observed between formulations. A bone tissue engineering study found that injectable chitosan/silk fibroin thermogelling hydrogels degraded by 80% after 10 days (44), while thermosensitive chitosan/silk sericin hydrogels loaded with longan seed extract demonstrated a degradation rate of 66% after 10 days (45). The results suggested that our hydrogels exhibited excellent biodegradability for bone tissue engineering applications.

The thermosensitive hydrogel-based drug delivery systems have been extensively studied for sustained release of therapeutic agents (46,47). In this study, ciprofloxacin-loaded fibroin thermosensitive hydrogels were measured for continuous release of ciprofloxacin up to 14 days. As shown in Figure 5c, an initial burst release of ciprofloxacin from all formulations was exhibited within the first day, leading to cumulative release of up to 73%. After the first day, ciprofloxacin was continuously released, reaching 84% after 14. Cumulative release of the F3 formulation was significantly higher than the other two formulations during the first 4 h, with higher poloxamer ratio leading to lower cumulative release. Increase in poloxamer content caused high cross-link density of the hydrogel, with drug diffusion more difficult through the hydrogel matrix (48). Hydrogel formulations with higher cross-link density provide a more restrictive environment for drug diffusion, leading to lower release rates (49). The F3 formulation was an ideal antibiotic carrier with high initial release followed by sustained release as an acceptable release profile for osteomyelitis treatment (50).

3.3 Biological properties of ciprofloxacin-loaded silk fibroin thermosensitive hydrogels

The MTT assay was performed to investigate the cytocompatibility of the hydrogel extracts on MC3T3-E1 cells. Cell viabilities after treatment with F1, F2, and F3 hydrogel

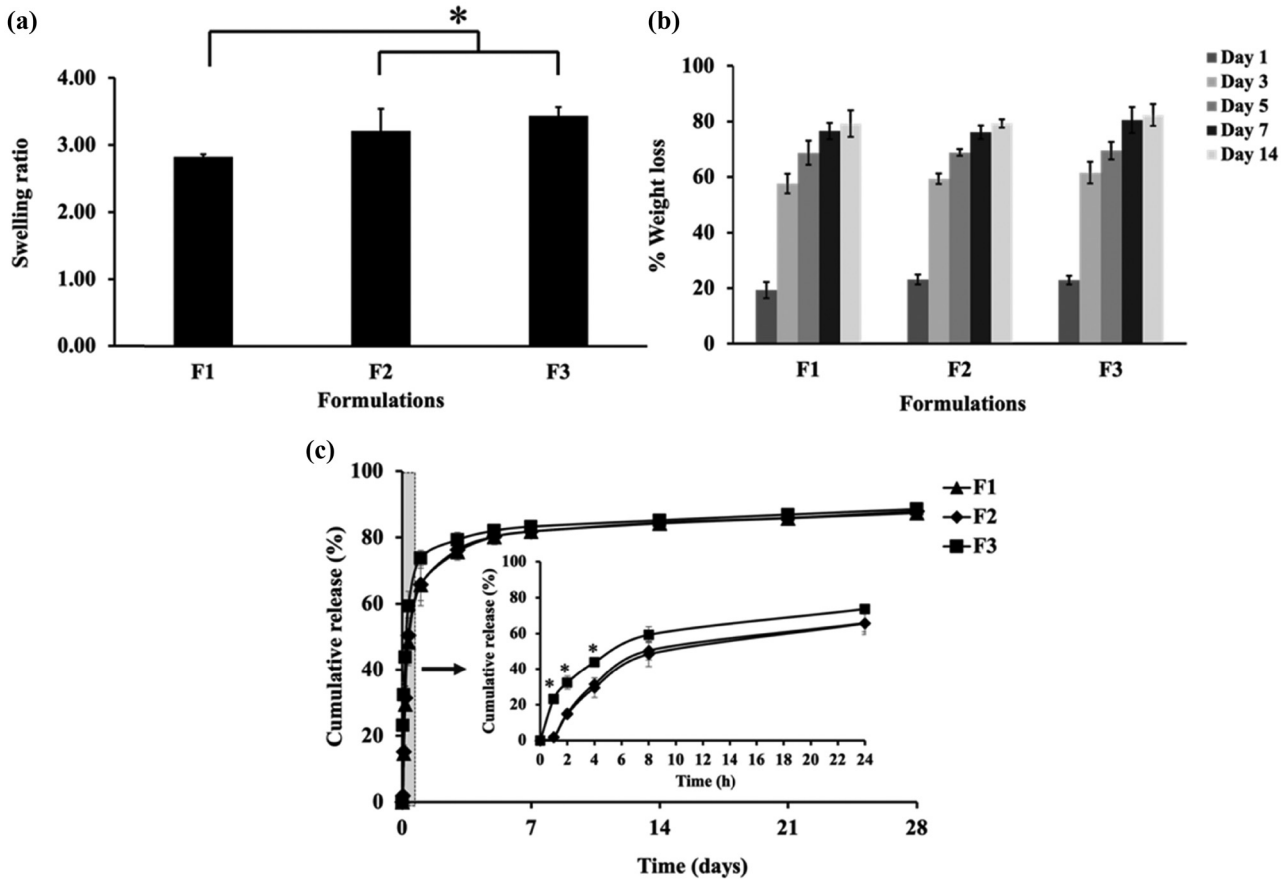

Figure 5: Physical properties of ciprofloxacin-loaded silk fibroin thermosensitive hydrogels. (a) Swelling ratio *represents a statistically significant difference between F2 and F3 formulations and the F1 formulation ($p < 0.05$); (b) weight loss after 1, 3, 5, 7, and 14 days; (c) cumulative release of ciprofloxacin as a function of time *represents a statistically significant difference between the F3 formulation and the F1 and F2 formulations ($p < 0.05$).

extracts were 106%, 100%, and 93%, respectively (Figure 6a). No significant differences in cell viability were found between the treated groups. Results revealed that the hydrogel had no cytotoxic effects on cell survival and showed good compatibility with osteoblast cells as a promising biomaterial vehicle to treat osteomyelitis.

Growth inhibition zones of the test bacteria were measured to evaluate the antibacterial effects of the hydrogels using the agar disk diffusion assay. Ciprofloxacin released at 1, 2, 4, and 8 h was assessed for antibacterial activity against *S. aureus*, the main pathogen of osteomyelitis. Growth inhibition zone mean diameter against *S. aureus* ranged from 13 to 27 mm (Table 2), while hydrogel extracts at 8 h impacted bacteria similar to ciprofloxacin disk as the positive control. The inhibition zones gradually increased in a time-dependent manner with antibiotic release from the hydrogel (Figure 6b) corresponding to drug release profiling.

Biofilms, multicellular communities of bacteria, could adhere to any kind of surface. The bacteria within the biofilm became adaptively resistant, requiring higher concentrations of antibiotic treatment (51). In this study, biofilm eradicating effect of ciprofloxacin-loaded fibroin thermosensitive hydrogel extracts was observed under the microscope, which showed the reduction in biofilm compared to untreated biofilm (Figure 6c). The hydrogels gave up to 42.32 ± 8.20% reduction in biofilm mass in clinical isolates of *S. aureus*. However, no biofilm eradication activity was seen on strong biofilm producing bacteria including standard strains (Figure 7). This finding concurred with a previous study that found biofilm eradication concentrations of ciprofloxacin in strong biofilm producing *S. aureus* at up to 4-folds higher than in moderate biofilm producing *S. aureus* (52). Strong biofilm masses required high concentrations of ciprofloxacin for removal.

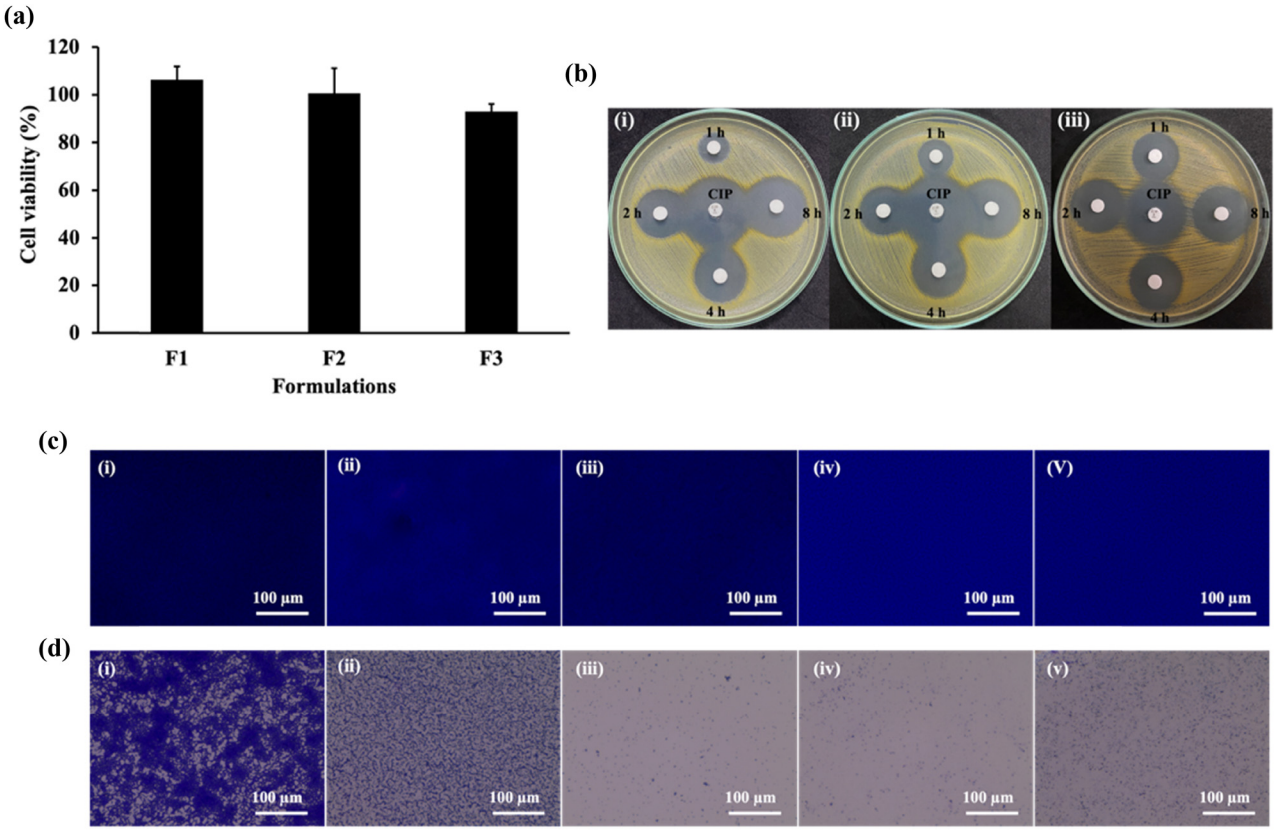

Figure 6: Biological properties of ciprofloxacin-loaded silk fibroin thermosensitive hydrogels. (a) Effect of hydrogel extracts at 24 h on the MC3T3-E1 cell line; (b) Antibacterial activity of drug released from hydrogels, (i) F1, (ii) F2, (iii) F3; (c) micrographs showing the effects of hydrogel extracts on mature bacterial biofilms of *S. aureus* ATCC 29213; and (d) representative clinical strain of *S. aureus*, (i) untreated bacterial biofilm, (ii) ciprofloxacin, (iii) F1, (iv) F2, and (v) F3.

3.4 Proposed multifunctional hydrogel with implantation for osteomyelitis treatment

For osteomyelitis, in some cases, the patients have to obtain the surgery with implant biomaterials (4). The main implant materials for surgery are metals. For implantation into the defect area, cements were used for bone tissue bonding (53). In some cases of infection, the patients need implant biomaterials supporting with antimicrobial bone cement (54). With this approach, our multifunctional hydrogel was proposed as soft antimicrobial bone cement with scaffolding like function for tissue growth. After removal of some debris, our multifunctional hydrogel was inserted into the defect area and fixed with implant biomaterials of plate and screws (Figure 8).

There are two main components: (1) polymer binder and (2) inorganic compound. Critically, the bone cement often shows high heat releasing which is harmful to the local tissue around implant materials (55). To create the low or non-heat releasing of bone cement is the alternative approach to prevent local tissue damaging. For our hydrogel

Table 2: Inhibition zone diameters of ciprofloxacin-loaded silk fibroin thermosensitive hydrogels against *Staphylococcus aureus* ATCC 25923

Test		Inhibition zone (mm)			
		1 h	2 h	4 h	8 h
Ciprofloxacin-loaded fibroin thermosensitive hydrogel	F1	13.00 ± 0.71	21.50 ± 0.71	22.50 ± 0.71	25.00 ± 1.41
	F2	15.50 ± 0.71	22.00 ± 1.41	24.00 ± 0.00	27.00 ± 0.00
	F3	20.00 ± 0.00	22.00 ± 1.41	24.00 ± 0.00	25.50 ± 0.71
Ciprofloxacin disk		28.50 ± 1.64			

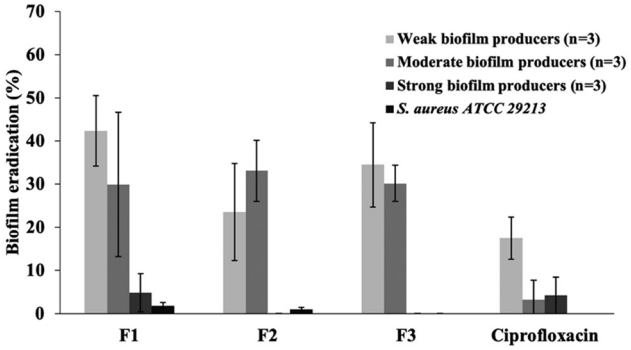

Figure 7: Biofilm eradication of ciprofloxacin-loaded silk fibroin thermosensitive hydrogels against *Staphylococcus aureus* ATCC 29213 and clinical strains of *S. aureus.*

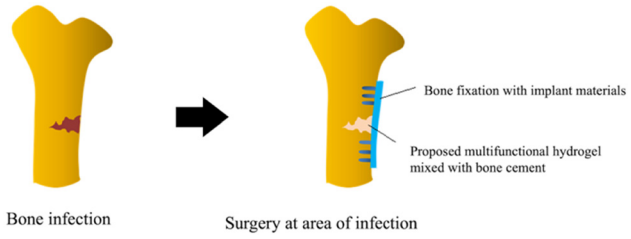

Figure 8: Implant materials incorporated with bone cement in orthopedic surgery; (a) Bone cement incorporated with fixation of implant materials and (b) bone cement incorporated with medical device of total knee replacement.

based on thermosensitive polymer, it was selected as based binder because it shows low heat releasing. Second, it has suitable performance of hydrophilicity which supports the interaction to inorganic compound (56). Third, hydrogel-based material exhibits function to support angiogenesis leading to promotion of bone formation (57). Fourth, to enhance the biological performance which is suitable for osteomyelitis treatment, our hydrogel was added with antimicrobial drug. Furthermore, silk fibroin was mixed with hydrogel because of its osteo-conductive performance supporting bone formation (58). Our hydrogel exhibited multifunction which is proposed as supporting implant biomaterials for osteomyelitis treatment.

Nevertheless, thermosensitive hydrogel normally forms physical crosslink formation which exhibits low stability (59,60). This leads to non-sufficient mechanical strength to support bone cement during biomaterials implantation. Hence, our hydrogel needs the development on mechanical performance supporting bone cement fitting for biomaterials implantation. To modify the molecules of thermosensitive hydrogel with reactive groups is an alternative approach to form chemical crosslink to enhance mechanical strength of bone cement (61). Another method is to formulate a certain amount of inorganic compound with good distribution in hydrogel. This supports to enhance the mechanical strength of bone cement (62).

In the view of biological performance, our hydrogel needs the bioactive molecules which enhance osteo-induction and angiogenesis. For instance, molecules are bone morphogenetic proteins and vascular endothelial growth factors. For antimicrobial function, certain encapsulated drug is an alternative method to prolong the sustainable releasing during biomaterial implantation. This is a model to inhibit the recurrent of infection at the local area of implantation.

4 Conclusion

Ciprofloxacin-loaded thermosensitive hydrogels composed of silk fibroin and poloxamers were successfully developed using the cold method. Hydrogel formulations F1, F2, and F3 had slightly different physical properties. F3 tends to be the optimal ratio with uniformly scattered porosity, high swelling ratio, and up to 82.34% biodegradability within 14 days. In addition, ciprofloxacin from F3 exhibited a significant initial burst release at the first 4 h and continuous delivery, with more than 84% release over 14 days. However, no significant differences among three formulations were found in biological properties. The hydrogel exhibited antibiotic properties against the pathogen of osteomyelitis, with good results against weak and moderate biofilm producing bacteria. Furthermore, the thermosensitive hydrogel showed good cytocompatibility with 93–106% osteoblast cell viability. Therefore, this multifunctional hydrogel was suitable for supporting implant materials without the need for additional surgery and showed promise as a new approach for supporting implant biomaterials in osteomyelitis.

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draft, and writing – review and editing; Dennapa Saeloh Sotthibandhu: conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, resources, supervision, validation, visualization, writing – original draft, and writing – review and editing.

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