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Thermoresponsive Hydrogel-based Local Delivery of Simvastatin for the Treatment of Periodontitis

Ningrong Chen[1], **Rongguo Ren**[1], **Xin Wei**[1], **Roshni Mukundan**[1], **Guojuan Li**[1], **Xiaoke Xu**[1], **Gang Zhao**[1], **Zhifeng Zhao**[1], **Subodh M. Lele**[2], **Richard A. Reinhardt**[3], **Dong Wang**[1,*]

[1]Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, Omaha, NE, 68198, USA

[2]Department of Pathology & Microbiology, College of Medicine, University of Nebraska Medical Center, Omaha, NE 68198, USA

[3]Department of Surgical Specialties, College of Dentistry, University of Nebraska Medical Center, Lincoln, NE 68583, USA

Abstract

Except for routine scaling and root planing, there are few effective nonsurgical therapeutic interventions for periodontitis and associated alveolar bone loss. Simvastatin (SIM), one of the 3-hydroxy-3-methylglutaryl-cosenzyme A reductase inhibitors, which is known for its capacity as a lipid-lowering medication, has been proven to be an effective anti-inflammatory and bone anabolic agent that has shown promising benefits in mitigating periodontal bone loss. The local delivery of SIM into the periodontal pocket, however, has been challenging due to SIM's poor water-solubility and its lack of osteotropicity. To overcome these issues, we report a novel SIM formulation of a thermoresponsive, osteotropic, injectable hydrogel (PF127) based on pyrophosphorolated pluronic F127 (F127-PPi). After mixing F127-PPi with F127 at a 1:1 ratio, the resulting PF127 was used to dissolve free SIM in order to generate the SIM-loaded formulation. The thermoresponsive hydrogel's rheologic behavior, erosion and SIM release kinetics, osteotropic property and biocompatibility were evaluated *in vitro*. Therapeutic efficacy of SIM-loaded PF127 hydrogel on periodontal bone preservation and inflammation resolution was validated in a ligature-induced

*Correspondence should be addressed to Dong Wang, Department of Pharmaceutical Sciences, University of Nebraska Medical Center, 986125 Nebraska Medical Center, PDD 3020, Omaha, NE 68198-6125. Phone: 402-559-1995. Fax: 402-559-9543. dwang@unmc.edu.

Author Contributions

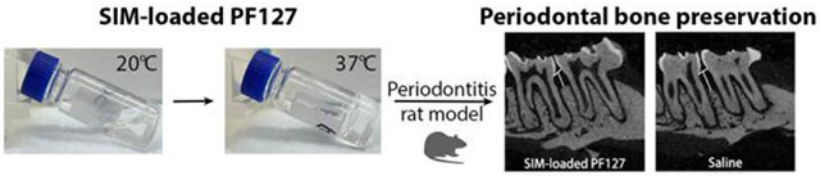
N. Chen prepared and characterized all formulations. She also led all *in vivo* study and drafted the manuscript. R. Ren synthesized F127-PPi. X. Wei, R. Mukundan, G. Li, and Z. Zhao contributed to *in vivo* efficacy study. S.M. Lele performed all histological analysis with X. Xu's assistance. G. Zhao contributed to rheology characterization of the hydrogel samples. R.A. Reinhardt contributed to experimental design and data interpretation. D. Wang led the overall experiment design, data interpretation, manuscript review and finalization.

SUPPORTING INFORMATION

- The Supporting Information is available free of charge.
- The synthesis of F127-PPi
- F127 and SIM-loaded PF127 hydrogels formation (Supporting Figure 1)
- Rheology characterization of SIM-loaded PF127 and control hydrogels (Supporting Figures 2 and 3)
- Biocompatibility study of SIM-loaded PF127 and control hydrogels (Supporting Figure 4)

periodontitis rat model. Given that SIM is already an approved medication for hyperlipidemia, the data presented here support the translational potential of the SIM-loaded PF127 hydrogel for better clinical management of periodontitis and associated pathologies.

Graphical Abstract



Keywords

targeted drug delivery; thermoresponsive hydrogel; poloxamer; pyrophosphate; simvastatin; periodontitis

INTRODUCTION

Periodontitis is a bacteria-induced chronic inflammation that damages the connective tissues and bone that support teeth.(1) It is estimated that 46% of adults 30 years of age or older have periodontitis, representing about 65 million people in the US. Among them, 9% suffer from the advanced stage of periodontitis.(2) Recently, periodontitis has also been implicated to increase the risk of cardiovascular disease, respiratory disease, rheumatoid arthritis, cancer, *etc.*(3–6) Currently, the mainstay of clinical treatments for periodontitis remains to be the scaling and root planing (SRP) for plaque (biofilm) removal(7) followed by local or systemic application of antibiotics to reduce bacterial load at deep pockets and furcations.(8, 9) The long-term utility of antibiotics is known to induce multidrug resistance and cannot reverse damaged periodontal bone.(10, 11) Furthermore, alternative pharmacological therapies including antiseptics, probiotics and nonsteroidal anti-inflammatory drugs only exhibit modest efficacy in the treatment.(12–15) Clearly, there is an unmet need of better therapeutic interventions for periodontitis and associated pathologies.

Many agents with strong anti-inflammatory and bone anabolic effects have been studied preclinically for the feasibility of periodontitis therapy during the past decades. Our laboratory also reported the local delivery of a glycogen synthase kinase 3 beta (GSK3β) inhibitor, 6-bromoindirubin-3’-oxime (BIO), in treating a periodontitis rat model.(16) However, these tested compounds, including BIO have not been approved for clinical use. From a clinical translation perspective, the off-label use of FDA approved medications to treat periodontitis has clear advantages.

Statins (3-hydroxy-3-methylglutaryl-cosenzyme A reductase inhibitors) are a class of medication widely used to treat hyperlipidemia (17) and to prevent cardiovascular diseases in clinics.(18) Their efficacy in treatment of various inflammatory and autoimmune diseases is well documented.(19–22) In addition, statins, such as simvastatin (SIM) are also noted for their potent bone anabolic effect by stimulation of bone anabolic factor expression and

induction of osteoblast differentiation and mineralization.(23–25) Therefore, they have been considered as a class of very promising therapeutic candidates for periodontitis.(26–32) Their clinical translation, however, has been largely impeded by the poor water-solubility, the lack of bone affinity, and the very limited distribution to the skeleton after systemic administration.(33) As a potential solution, a local bone-targeted drug delivery system for statins may be developed to address the issue.

Poloxamer 407 (or Pluronic F127) is a triblock copolymer of ethylene oxide and propylene oxide, which is a Generally Recognized As Safe (GRAS) pharmaceutical excipient and one of the most widely-used thermoresponsive water-soluble polymers.(34) The aqueous solution of F127 at room temperature is able to transform into a hydrogel at body temperature, which is attributed to the formation of partially ordered cubic phases by F127 micelles as the micellar cores dehydrate.(35) This F127-based thermoresponsive hydrogel has become an attractive local drug carrier due to its enhancement of stability,(36) lack of cytotoxicity,(37) excellent biocompatibility,(38) simple drug encapsulation in micellar cores(40) and significant capacity to form a ‘depot’ for in situ controlled drug release.(38, 40, 41) Additionally, various chemical modification of F127 structure could be made to achieve different targeting or stability adjustments.(41–43)

Due to these clear advantages of F127, we seek to use F127 and develop a novel formulation for effective local delivery of SIM for the treatment of periodontitis. To incorporate bone affinity, F127-PPi was synthesized by conjugating osteotropic pyrophosphate (PPi) to the chain termini of F127. The resulting F127-PPi was mixed with F127 at a 1:1 weight ratio to form PF127, which will then be used to incorporate SIM. This simple bone-targeted delivery system has shown great promise in the delivery of GSK3β inhibitor BIO.(16) Its utility for the effective delivery of SIM to the periodontal pocket for the treatment of periodontitis and associated bone loss is the focus of this report.

MATERIALS AND METHODS

Materials

Pluronic F127, pyrophosphate and 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Simvastatin (SIM) was purchased from Zhejiang Ruibang Laboratories (Wenzhou, Zhejiang, China). Dense ceramic hydroxyapatite (HA) discs (0.5” diameter × 0.08” thick) were obtained from Clarkson Chromatography Products, Inc. (South Williamsport, PA, USA). Mouse macrophage Raw 264.7 and osteoblast MC3T3-L1 cells were originally purchased from ATCC (Manassas, VA, USA). Dulbecco’s Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM alpha), trypsin-EDTA and penicillin-streptomycin were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Gemini BenchMark (West Sacramento, CA, USA). All other solvents and reagents, if not specified, were acquired from either Acros Organics (Morris Plains, NJ, USA) or Fisher Scientific (Pittsburgh, PA, USA).

Preparation of SIM-loaded PF127 Hydrogel

Pyrophosphorylated pluronic F127 (F127-PPi) was synthesized as previously described.(16) Three micelle formulation methods were tried to incorporate SIM into PF127 hydrogel: film hydration, solvent evaporation and direct dissolution. For the film hydration method, an excess amount of SIM (20 mg) was dissolved in a methanol solution of PF127 (1 mL, 25% w/v mixed F127-PPi and F127 in the ratio of 50:50 w/w). Methanol was evaporated to form a film in the round-bottom flask via rotor evaporator at 60 °C. On the following day, the film was hydrated by ice cooled 0.9% saline for 30 min to prevent formation of gel. For the solvent evaporation method, PF127 (250 mg) and SIM (20 mg) were dissolved in 1 mL of acetone and then added dropwise to ice cooled 0.9% saline under stirring, with acetone being evaporated overnight. For the direct dissolution method, PF127 solution in ice cooled saline (1 mL, 25% w/v) was prepared first and an excess amount of SIM (20 mg) was added and mixed under 4 °C for 48 hr. In all three methods, the final undissolved drug was removed by centrifugation at 12,000 rpm for 30 s followed by passage through a 0.8 μm filter (Scheme 1). SIM-loaded F127 hydrogel was prepared similarly without adding F127-PPi.

Characterization of SIM-loaded PF127 Hydrogel

SIM content in each formulation was measured on an Agilent 1260 Infinity II LC system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with an Agilent C_{18} reverse-phase column (4.6 × 250 mm, 5 μm). A mobile phase of methanol/water (85:15 v/v) at a flow rate of 0.8 mL/min was used for analysis. The SIM-containing samples were applied to the HPLC at a sample volume of 20 μL and the UV detector wavelength was at 238 nm. For quantification of SIM content, a calibration was established with a linear concentration range of 2-20 μg/mL.

The particle size of the SIM-loaded polymeric micelles was analyzed in sterile water by dynamic light scattering (DLS) at 37 °C on a Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, UK).

To investigate the hydrogel formulations' microscopic morphology, F127, PF127, SIM-loaded F127 and SIM-loaded PF127 hydrogels were instantly frozen at −80 °C using liquid nitrogen. After lyophilization, cross-sectional slices of the dried specimens were mounted on stubs, sputter-coated with an approximately 50 nm of gold/palladium alloy using a Hummer V Sputter Coater (Anatech, Battle Creek, MI, USA), and analyzed using a Quanta 200 scanning electron microscopy (SEM) (FEI, Hillsboro, OR, USA).

In Vitro Hydroxyapatite (HA) Binding Assay

To determine the osteotropicity of the formulation, an *in vitro* binding experiment was performed using discs of hydroxyapatite (HA) which is the main content of bone and teeth. SIM-loaded PF127 formulations (0.5 mL, 25% w/v PF127, containing 8 mg/mL of SIM) with different weight ratios of F127-PPi and F127 (0:100, 25:75, 50:50, 75:25, and 100:0 w/w) were prepared respectively and filled in the wells of a 24-well plate. HA discs were then deposited into each well to allow binding to proceed. At different time intervals, 5 μL of each PF127 formulation solution was collected and the concentration of SIM was analyzed

by HPLC. By subtracting the SIM concentration in the well from initial concentration of SIM in PF127 formulation, the percentage of SIM bound to HA discs was calculated.

In Vitro Hydrogel Erosion Study

The erosion of SIM-loaded PF127 hydrogels with different concentrations of PF127 (20%, 25%, 30% w/v) was tested by performing the weight remaining (%) experiment.(44) Samples of polymer solutions (0.5 mL) were injected into the bottom of glass vials and formed gels at 37 °C. Each empty vial's weight before addition of gels was measured as W_v, and the original total weight after addition of gels was measured as W_0. Then 1 mL of phosphate-buffered saline (PBS; pH 7.4) pre-equilibrated at 37 °C was gently laid over the surface of the hydrogels. All samples were incubated in water bath at 37 °C with continuous gentle shaking. The weight of remaining hydrogels together with their vials was measured as W_t at predetermined time intervals after careful removal of the supernatant. To understand the influence of buffer change frequency on the erosion rate, time interval between each time of measurement was set as variable for each concentration of PF127 gel (every 12 hr, every 24 hr, and every 48 hr). After weighing, 1 mL of fresh buffer was refilled in the vials. The gel erosion profiles were obtained by plotting weight remaining (%) against the time course of incubation (n=3).

$$\text{Weight remaining } (\%) = \frac{(W_0 - W_t)}{W_0} \times 100$$

In Vitro Drug Release Study

The drug release of SIM-loaded PF127 hydrogels with different concentrations of PF127 (20%, 25%, 30% w/v) was tested by a traditional membraneless dissolution method.(44) In brief, samples of polymer solutions (200 μL) were injected into the bottom of glass vials and incubated at 37 °C until gels were formed. Subsequently, 1 mL of PBS (pH 7.4) containing 0.5% (w/v) Tween 80 pre-equilibrated at 37 °C was gently added onto the surface of the hydrogels. The same amount of free SIM was added in another vial and used as a control. All samples were incubated in water bath at 37 °C with continuous gentle shaking. To measure the release of SIM, 0.9 mL of releasing medium in each vial was withdrawn at predetermined time intervals and replaced with an equal volume of pre-equilibrated fresh buffer. Here, to understand the effect of buffer change frequency on releasing rate of different concentrations of PF127 gels, the time interval was set to be every 6 hr, every 12 hr, and every 24 hr (n=3). The concentration of SIM in the releasing medium was determined using HPLC.

Thermoresponsive Rheology Characterization of SIM-loaded PF127 Hydrogel

Gelation temperature was determined by measuring the storage modulus (G') and loss modulus (G") of the polymer solution/gel with concentration of 25% (w/v) in PBS (pH 7.4) in the oscillation temperature sweep mode on a rheometer (DHR-2 with a 20 mm parallel plate geometry, TA Instrument, New Castle, DE, USA).(45, 46) The sample was uniformly loaded between the Peltier plate of the rheometer using a syringe and the sample thickness was set as 1000 μm. The linear viscoelastic region of the sample was pre-determined at 50

°C by using the oscillation amplitude function. And the instrument parameters were set as the following: strain of 10%, and angular frequency of 10 rad/s. The G' and G" of the sample were measured from 4 °C to 50 °C with heating step of 1 °C, and soak time of 1 min for each temperature increase. The gelation temperature of the sample was determined as the temperature at the cross over of G' and G" curves.

The shear rate-dependent viscosity of the formulation with a concentration of 25% (w/v) was also performed on the rheometer at different constant temperature, 10, 20, 30, 37 °C. The shear rate ranged from 0.01 to 400 s^{-1}.

Cell Culture

Raw 264.7 cells (mouse macrophage) and MC3T3-L1 cells (mouse osteoblast) were cultured in Dulbecco's modified eagle medium (DMEM) and Minimum Essential Medium (MEM alpha), respectively, containing 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin. The culture was maintained at 37 °C in a humidified CO_2 incubator with 5% CO_2 supply.

In Vitro Biocompatibility Assay

The biocompatibility of SIM-loaded PF127 hydrogel (25% w/v) was tested through a colorimetric cell viability assay using 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazoliumbromide (MTT). Briefly, 0.5 mL of each sample solution (PF127, F127, SIM-loaded PF127 and SIM-loaded F127, dissolved in sterile water) was placed into each well of the 24-well plate. Hydrogels were formed in the 37 °C water bath, followed by incubation with 1.5 mL of culture medium in each well for 24 hr in order to obtain the extracts of different sample hydrogels. Cells were cultured in the 96-well plate at a density of 1×10^4 cells per well overnight at 37 °C in a 5% CO_2 incubator. On the following day, the culture medium was replaced with different extracts, free SIM, and fresh medium as control for 1 and 2 days. The SIM concentration in all SIM-containing formulations is 20 μM. MTT solution (10 μL) was then added to each well at the end of the treatment, followed by incubation at 37 °C for 4 hr. Finally, 100 μL of DMSO was added to each well to solubilize the intracellular formazan after removal of the medium. Absorption was recorded using a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA) at 570 nm. The cell viability was determined using the following equation:

$$\text{Cell viability } (\%) = \frac{\text{OD of S}}{\text{OD of C}} \times 100$$

where C is the control group (untreated), and S is the treated cells. Experiments were performed in triplicate for all the samples.

Animal Experiment

Sprague Dawley rats (female, 10-month-old, retired breeders) were purchased from Envigo. Experimental periodontitis was induced by silk ligatures on each rat as previously described.(47) In brief, after anesthesia, 4-0 silk ligatures were tied round the maxillary 2nd molars (M2) on both sides of palate and removed a week later. Once the ligatures were placed, rats

were randomized into six groups (5 rats/group) which include saline, 25% (w/v) PF127 hydrogel, 25% (w/v) F127 hydrogel, SIM-loaded 25% (w/v) PF127 hydrogel (containing 8 mg/mL of SIM), SIM-loaded 25% (w/v) F127 hydrogel (containing 8 mg/mL of SIM), and free SIM acid (dissolved in saline, 8 mg/mL). These formulations (10 μL) were administered locally into the palatal gingiva between the maxillary 1st molar (M1) and M2 on a weekly basis. Three weeks later, all the animals were euthanized, and the entire palates were collected and fixed in 10% formalin prior to micro-computed tomography (μ-CT) and histological analysis. All procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center and conducted following the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

μ-CT Evaluation of Alveolar Bone Quality

Each palate sample was scanned using a high-resolution μ-CT (Bruker SkyScan 1172, Kontich, Belgium) with the following scanning parameters. The X-ray tube voltage was set at 70 kV with the current of 141 μA. A 0.5 mm thick aluminum filter was used to improve imaging quality. Exposure time was set at 720 ms. The X-ray projections were obtained at 0.7° intervals with a scanning angular rotation of 180° and five frames were averaged for each rotation. Reconstructions were performed afterwards to establish 3D-structures using NRecon software.

For the quantitative analysis, the position of each palate was corrected, and both sagittal and coronal section images were saved with DataViewer. To evaluate bone erosion, the distance from cementoenamel junction (CEJ) to alveolar bone crest (ABC) between M1 and M2 was measured in sagittal images. A longer distance between CEJ and ABC indicates more alveolar bone loss. For the analysis of histomorphometric parameters, such as bone volume (BV) and trabecular thickness (Tb. Th), we particularly focused on a rectangular region of interest (ROI) between M1 and M2 in coronal images, which was determined from the distopalatal of M1 to the mesiopalatal of M2 (length), from the palatal side to the buccal side of M1 and M2 (width). This constant rectangular ROI was carefully placed in every slice of total 130 slices below CEJ of M1 and M2. Subsequently, a custom analysis process in which parameters like BV and Tb. Th were calculated in CTan for every sample.

Histological Evaluation of Connective Tissue

After μ-CT analysis, the palates were decalcified for 14 days with change of the 14% EDTA solution every three days. Once completely decalcified, the specimens were paraffin embedded, sectioned (4 μm), stained with hematoxylin and eosin (H&E) for histological evaluation. Infiltration of inflammatory cells such as neutrophils and lymphocytes were evaluated qualitatively in the gingival connective tissues above the alveolar crest between M1 and M2 using a scoring system(48) where 0 is negative, 1 is less than 30% of the affected tissues, 2 is some inflammatory cells (30 - 60%), and 3 is many inflammatory cells (> 60%). Osteoclasts lining the surface of alveolar crest were also assessed using a scoring system(49) where 0 is negative, 1 is a few osteoclasts lining less than 5% of alveolar bone surface, 2 is some osteoclasts (5 - 25%), and 3 is many osteoclasts (25 - 50%).

Statistical Analysis

All data were reported as a mean ± standard deviation (SD). Continuous outcomes among more than two groups were compared using one-way analysis of variance (ANOVA) for one variable data and two-way ANOVA for two variable data by GraphPad Prism 7.0 (GraphPad Software). Tukey's post-hoc test was performed for multiple comparisons. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Preparation and Characterization of SIM-loaded PF127 Hydrogel

Pyrophosphorylated pluronic F127 (F127-PPi) was synthesized as reported previously(16) (see also Supporting Scheme 1). Three different methods including film hydration, solvent evaporation and direct dissolution were used to prepare SIM-loaded PF127 hydrogel (mixed F127-PPi and F127 in the ratio of 50:50 w/w). The highest SIM encapsulation efficiency (EE) in 25% (w/v) PF127 hydrogel was achieved when it was prepared by the film hydration method, while PDI of micelle particle size in the hydrogel was extremely higher than that of hydrogels prepared by other two methods, indicating size distribution is too wide (Table 1). To obtain both high drug EE and narrowly distributed particle size of PF127 micelles in hydrogel, direct dissolution was finally selected as the optimal formulation method in subsequent studies based on these data.

As shown in the SEM images (Figure 1), the microstructure of the lyophilized F127 hydrogel appeared to bear a well-organized sheet-like structure, while the lyophilized PF127 hydrogel, SIM-loaded F127 hydrogel and SIM-loaded PF127 hydrogels exhibited network-like structures with large porosity and were more loosely packed. No apparent microparticular structures were observed.

In vitro osteotropicity property of SIM-loaded PF127 hydrogel was assessed through hydroxyapatite (HA) binding test. SIM-loaded PF127 hydrogels with different ratios of F127-PPi and F127 (0:100, 25:75, 50:50, 75:25, and 100:0 w/w) were studied in the assay (Figure 2). As shown, without PPi element, F127 hydrogel (0% F127-PPi) displayed little binding to HA discs during the entire incubation (< 10%). After 15 min incubation, PF127 hydrogel containing 50% (w/w) F127-PPi had about 49% bound to HA, showing significant differences compared to hydrogel containing 25% (w/w) F127-PPi (39%) and hydrogel containing 75% (w/w) F127-PPi (55%). When measured at 30 min, HA binding of hydrogel containing 50% (w/w) F127-PPi reached 64%, which is significantly higher than that of hydrogel containing 25% (w/w) F127-PPi (56%). But it showed no difference when compared with hydrogel containing 75% (w/w) F127-PPi (65%). This is similar when incubation was extended to 2hr. The percentage of binding reached 77% for hydrogel containing 50% (w/w) F127-PPi, showing no significant decrease compared with hydrogel containing 75% (w/w) F127-PPi (81%). And it was also found that between hydrogels containing 75% and 100% (w/w) F127-PPi, there was no difference in binding capacity throughout the study. Based on these results, the formulation of 50:50 (w/w) ratio of F127-PPi and F127 was used in further experiments as it showed strong osteotropicity with relatively low PPi content.

To understand if SIM-loaded PF127 formulation would maintain the same thermoresponsive property as regular F127 formulation, additional rheological experiments were conducted. As shown in Supporting Figure 1, both the blank F127 and the SIM-loaded PF127 formulations were liquid at 10 °C. When elevated to 20 °C, both transitioned into hydrogel. According to the rheology characterization (Supporting Figure 2), the values of storage modulus (G') and loss modulus (G") were equal at 14 °C, which was designated as the gelation temperature (T_{gel}). When G' and G" had the second crossover at 27 °C, that value is designated as the syneresis temperature (T_{syn}). Viscosity of the formulations was also measured under four different temperatures, 10, 20, 30 and 37 °C as a function of shear rate in the range of 0.01 to 400 s^{-1}. As shown in Supporting Figure 3, the viscosity of SIM-loaded PF127 solution/hydrogel increased significantly as temperature was raised.

Sustained Hydrogel Erosion and Drug Release

All SIM-loaded PF127 hydrogels (F127-PPi:F127 = 50:50 w/w) stayed at the bottom of glass vials without detachment. As shown in Figure 3, 20% (w/v) PF127 hydrogel disintegrated completely in the shortest duration regardless of frequency of buffer changing, while 25% and 30% (w/v) PF127 hydrogels had a more sustained erosion process. Thus, in general, the rate of gel erosion decreased as the concentration of PF127 increased. In addition, it was found the frequency of buffer exchange affected gel erosion kinetics to some extent. This effect was particularly obvious in the first 48 hr with significant difference in percentage of remaining gel at the same time point for the same concentration of PF127 hydrogels.

As shown in Figure 4, the release of SIM from the PF127 formulation was generally sustained. Higher polymer concentration led to a slower SIM release kinetics, which corresponded to the erosion profiles (Figure 2). Moreover, the release rate was also influenced by the frequency of fresh buffer replacement. More frequent buffer change (every 6 hr) resulted in faster SIM releasing when compared to less frequent buffer change (every 12 hr or every 24 hr). Dissolution of free SIM was used as a control in the study. As shown in Figure 4, the dissolution of unformulated SIM was very slow, confirming the effectiveness of the PF127 formulation in solubilization of SIM and its sustained release. Based on the gel erosion and drug releasing kinetics, the SIM-loaded PF127 formulation with 25% (w/v) concentration was selected for *in vivo* assessment.

Biocompatibility of SIM-loaded PF127 Hydrogel

The biocompatibility of SIM-loaded PF127 hydrogel was assessed on MC3T3-L1 and Raw 264.7 cells using MTT assay. These cells were exposed to the extracts of different formulations, including PF127, F127, SIM-loaded PF127, and SIM-loaded F127 hydrogels and free SIM solution. As shown in Supporting Figure 4, for both MC3T3-L1 cells and Raw 264.7 cells, there was a slight reduction in viability among different treatments after 24 hr and 48 hr, but all above 80%.

Micro-Computed Tomography (μ-CT) Evaluation of Alveolar Bone Quality

Alveolar bone loss is an essential feature of periodontitis, and preservation of bone quality is important in the treatment of the disease. After 3 weeks of treatment, the SIM-loaded PF127

hydrogel treated group exhibited smaller distance from cementoenamel junction (CEJ) to alveolar bone crest (ABC) between the maxillary 1st molar (M1) and 2nd molar (M2) (0.57 mm) (Figure 5A and 5B). In contrast, the saline treated groups demonstrated significantly longer distance in the same site (0.79 mm, $P < 0.05$). Blank PF127 and F127 hydrogels had the distance of 0.76 and 0.71 mm respectively, and both of SIM-loaded F127 hydrogel and free SIM treated groups had the distance of 0.67 mm, all showing no statistically significant differences compared to saline control group. For further validation of the bone quality in different groups, quantitative analysis on parameters such as bone volume (BV) and trabecular thickness (Tb. Th) was performed (Figure 5C and 5D). Comparison of animals in different groups illustrated significant difference in BV (0.33 mm^3 vs. 0.21 mm^3) and Tb. Th (0.059 mm vs. 0.033 mm) for the SIM-loaded PF127 hydrogel treated group compared to the saline control group ($P < 0.01$). SIM-loaded PF127 hydrogel treated animals also had significantly higher value in Tb. Th. than PF127 hydrogel and F127 hydrogel (both at 0.034 mm) ($P < 0.01$). When comparing the other groups (PF127 hydrogel, F127 hydrogel, SIM-loaded PF127 hydrogel and free SIM treated) to saline treated group, none of the parameters demonstrated statistically differences except Tb. Th of free SIM group (0.052 mm vs. 0.033 mm, $P < 0.05$).

Histological Evaluation of Connective Tissue

The histological analysis further confirmed the anti-inflammatory effect and remarkable bone preservation of SIM-loaded PF127 in the region of connective tissue between M1 and M2 and the surface of alveolar bone crest (ABC). Standard H&E (Figure 6A) revealed significantly less neutrophil and lymphocyte infiltration and less osteoclast lining was observed in SIM-loaded PF127 hydrogel treated group while serious inflammation infiltration was found in saline, PF127 and F127 control groups. Histology scores of neutrophils and lymphocytes were shown in Figure 6B and 6C, based on the grading criteria described above. The neutrophil score of SIM-loaded PF127 hydrogel group had a statistically significant difference compared to saline and PF127 group, while SIM-loaded F127 hydrogel group and free SIM-loaded PF127 group did not exhibit a difference. SIM-loaded PF127 hydrogel group achieved the lowest score of lymphocytes ($P < 0.01$) when compared to saline group, while SIM-loaded F127 hydrogel group and free SIM-loaded PF127 group had the second lowest scores ($P < 0.05$). Osteoclast lining on the bone crest was also evaluated according to the grading criteria. It was found that osteoclasts in SIM-loaded PF127 hydrogel treated group was significantly less than those in the saline control group (Figure 6D).

DISCUSSION

In addition to being a highly effective treatment for hyperlipidemic,(18) simvastatin (SIM) is also known to be anti-inflammatory (20) and bone anabolic (25) which can be extremely beneficial for the clinical management of periodontitis. On molecular level, previous studies have suggested that the statins' bone anabolic and anti-inflammatory activities may be associated with downregulation of metalloproteinase 9 (MMP-9) and TNF-α, upregulation of IL-10, IL-1 receptor-like 1 (IL1rl1), IGF-1 and bone morphogenetic protein-2 (BMP-2).

Other potential signaling pathways that may have been involved include FGF7, and the Wnt/β-catenin pathway.(50)

Although regular reduction of bacterial biofilm (scaling and root planing) and administration of antibiotics in the conventional therapy for periodontitis is effective against disease progression,(7, 8) the managements to suppress host inflammatory response and stimulate new bone formation are required to be given, especially in cases with moderate to advanced disease stages, because complete removal of biofilm is unrealistic within deep periodontal pockets. Usually, a surgical procedure is needed for debridement of bony defect and establishment of a microenvironment for bone regeneration. However, recent clinical trials suggested that nonsurgical treatment with the bone anabolic agent SIM could induce new bone formation in periodontitis therapy, reducing patients' morbidity and cost.(51, 52) These findings support SIM as a promising therapeutic candidate for periodontal bone preservation and regeneration. SIM's poor water solubility (0.0122 mg/mL)(53) and lack of osteotropicity, however, have impeded its clinical utility. As shown in Figure 4, free SIM indeed showed a very slow dissolution/release kinetics. According to Noyes–Whitney equation(54), this slow kinetics may be explained by the micron-size of the SIM particles, the poor water-solubility of SIM, the limited presence of surfactant in the releasing medium (0.5 w/v% Tween 80) and the rather gentle agitation used in the drug release study.

To address this challenge, we incorporated SIM into a bone-targeting thermoresponsive hydrogel (PF127 hydrogel) delivery system. Pyrophosphate (PPi) was conjugated to pluronic F127 to synthesize F127-PPi through a straightforward two-step reaction which has improved the bone-affinity of the delivery system.(55) It was confirmed in the previous study that with the modification on chain termini, the copolymer didn't alter the gelation and viscous properties of F127, and this novel hydrogel system was effective in delivery of a glycogen synthase kinase 3 beta inhibitor (BIO) without off-target side effects.(16) Bisphosphonates and oligopeptides have been used extensively to incorporate osteotropicity to drug delivery systems.(56) While oligopeptides have the advantages of biodegradability, their ability of bone binding is weak, and the synthesis can be expensive. On the contrary, bisphosphonates are stronger bone-targeting agents with much lower cost. However, long-term use of bisphosphonates such as alendronate has been considered to contribute to higher risk of osteonecrosis of the jaw (ONJ) and atypical fracture development.(58) Therefore, biodegradable PPi with similarly strong bone affinity which has been widely applied in food and oral care industry was selected in the PF127 formulation as a much safer bone-targeting moiety.

In the core-shell structure of F127 micelles, the hydrophilic corona (formed by the ethylene oxide block of the copolymer) provides dispersion stability to the micelle, while hydrophobic propylene oxide core functions as a microenvironment for the encapsulation of hydrophobic compounds.(59) Of the three formulation methods used to prepare SIM-loaded PF127 hydrogel, direct dissolution attained both high drug encapsulation efficiency (EE) and narrow micelle particle size distribution, which was chosen as the best protocol for hydrogel preparation. In contrast, the other two methods were abandoned as film hydration showed the widest particle size distribution, while solvent evaporation showed the lowest EE. After preparation, it was verified that SIM-loaded PF127 hydrogel preserved the thermoresponsive

feature as F127. Upon elevation of the temperature, the micellar cores of free-flowing solution of PF127 become dehydrated leading to an entropic change of state into a partially ordered cubic liquid crystalline gel state.(60) In other words, the formed in situ thermoresponsive hydrogel maintains its fluidity at low temperature (< 14 °C) that is suitable for local injection through thin needles. Once administered *in vivo*, PF127 solution solidifies into a hydrogel at body temperature, which would help to maintain a long retention time of drug payload in the periodontal pocket. Although the SEM images of the lyophilization hydrogel specimens may not fully reflect the hydrated hydrogel morphology, some interesting differences were observed. The well-organized stacked-sheet-like structure observed in the lyophilized F127 hydrogel (61) was disrupted when F127-PPi and/or SIM was introduced into the formulation, displaying network-like structures with large porosity. Importantly, no irregular micro-particular structure was observed in the SIM-containing hydrogels, suggesting complete micellization of SIM in the SIM-loaded F127 and SIM-loaded PF127 hydrogel formulations. The long-term retention of the gel is the premise of sustained release of SIM over time. In essences, the SIM-loaded PF127 hydrogel is physically cross-linked SIM/PF127 micelles. Different from the free SIM control, the SIM released from PF127 hydrogel is in the micelle form. The release is through a dilution/solubilization/erosion process at the hydrogel/releasing medium interface. From *in vitro* hydrogel erosion and SIM release profiles, it could also be inferred that with higher PF127 concentration, prolonged gel erosion and drug release kinetics may be achieved *in vivo* to provide the necessary bone anabolic and anti-inflammatory activity of SIM. It is imperative to recognize that the hydrogel erosion process we observed in this study is attributed the gradual dissolution of the physically crosslinked hydrogels into micelle solutions rather than polymer degradation, because F127 is hardly degradable under physiological conditions. The bone-targeting property of PF127 hydrogel was validated by an *in vitro* hydroxyapatite (HA) binding test. Importantly, the affinity to bone ensures the effective delivery of payload to bone associated cells (*e.g.* osteoblasts, osteoclasts, osteocytes and chondrocytes) which could help reduce the frequency of dosing and overcome the drug's lack of osteotropicity.

When treated on a ligature-induced experimental periodontitis rat model, SIM-loaded PF127 hydrogel exhibited an excellent therapeutic impact on alveolar bone preservation and anti-inflammation according to μ-CT and histological analyses. The μ-CT results demonstrated that it remarkably reversed alveolar crest erosion and retained bone volume (BV) when compared with saline control group, while SIM-loaded F127 hydrogel and free SIM exhibited limited therapeutic impact. The histological evaluation also suggested that SIM-loaded PF127 hydrogel showed superior anti-inflammatory effects and inhibition of osteoclastic bone resorption when compared to saline. And as expected, both PF127 and F127 hydrogels did not have any significant impact on periodontitis.

As for SIM-loaded PF127 hydrogel's safety profile, the previous study(16) has demonstrated this drug delivery system's biocompatibility *in vitro*. As a FDA-approved medication, SIM has a fully established safety profile, which is superior to the BIO used in the previous study.(16) Moreover, in our current *in vivo* efficacy study, the effective drug dose used was equivalent to 80 μg of SIM per week, which is much lower than the dosing level reported previously.(47, 62) From the safety prospective, no abnormal body weight change,

histological abnormality or any abnormal behavior was observed during the study, supporting the safety of the formulation.

CONCLUSION

We have successfully developed a bone-targeting thermoresponsive hydrogel drug delivery system (PF127) composed of pyrophosphorolated pluronic F127 (F127-PPi) and regular pluronic F127 (1:1). This thermoresponsive hydrogel system can effectively incorporate simvastatin (SIM) and the hydrogel formulation demonstrated strong osteotropicity and excellent biocompatibility. When evaluated in a rat periodontitis model, the formulation was found to effectively preserve the periodontal bone and mitigate inflammation. The evidence presented in this study supports the SIM-loaded PF127 injectable formulation as a promising SIM formulation candidate for better clinical management of periodontitis and associated pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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D. Wang and R. Ren are co-inventors of a PCT patent application covering the PF127 technology. D. Wang claims an equity position in Bohe Biotechnology, a start-up company that has licensed the technology for further preclinical and translational development. The rest of the coauthors declare no competing interest.

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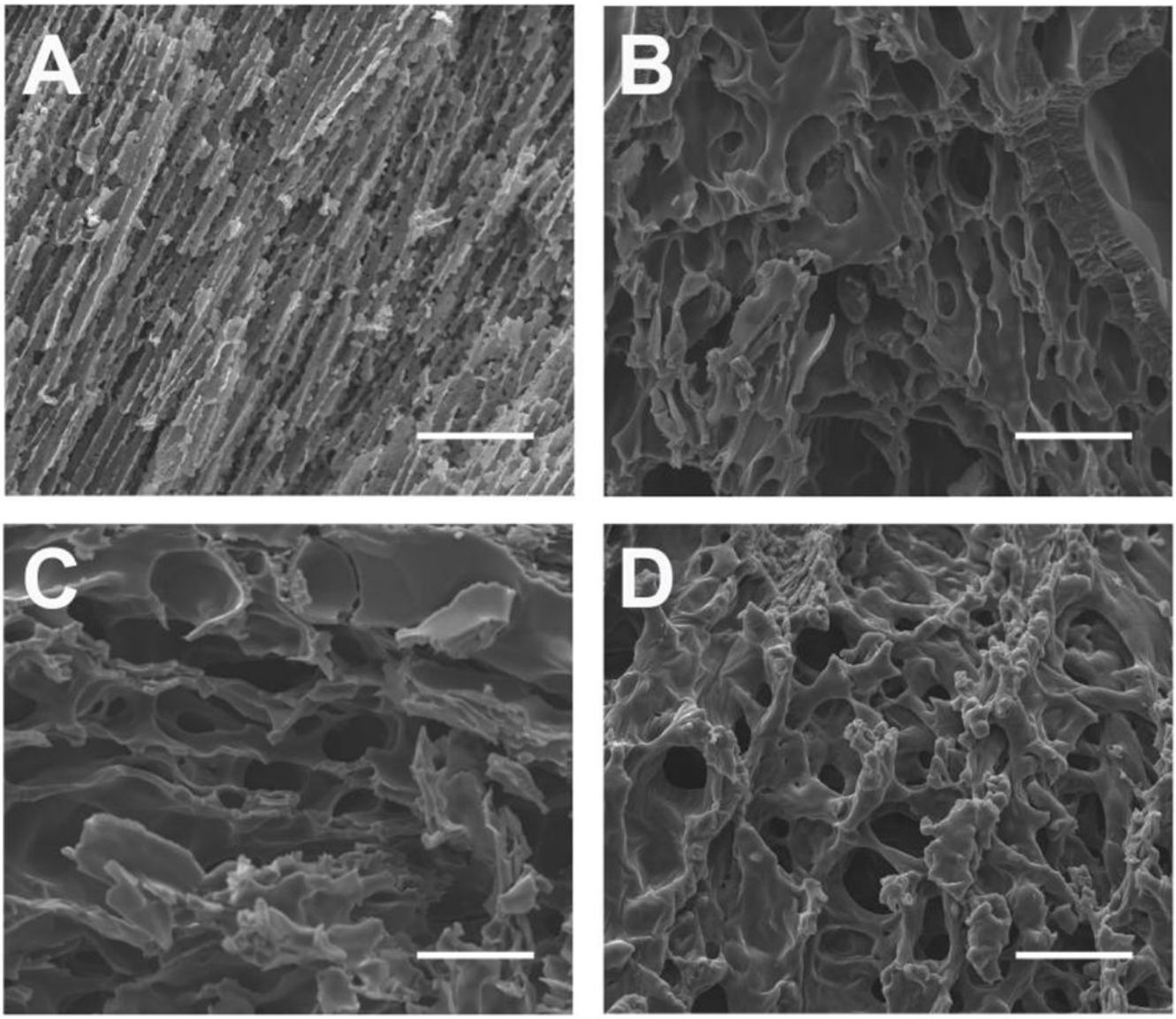


Figure 1.

Scanning electron microscopy (SEM) images of lyophilized F127 (**A**), PF127 (**B**), SIM-loaded F127 (**C**) and SIM-loaded PF127 hydrogels (**D**). The hydrogels were prepared at 37 °C and instantly frozen at −80 °C with liquid nitrogen. After lyophilization, cross-sectional slice of the dried specimens were sputter-coated with gold/palladium alloy, and imaged with a Quanta 200 SEM. Scale bar = 150 μm.

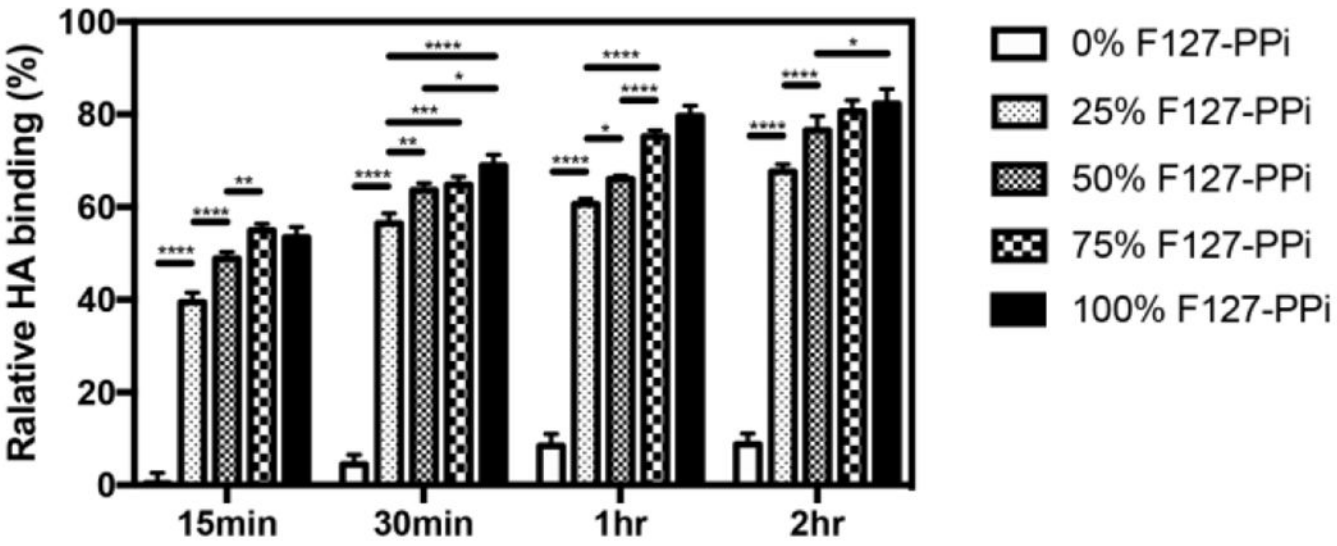


Figure 2.

The binding of SIM-loaded PF127 formulations to hydroxyapatite (HA). The w/v PF127 concentration was maintained at 25% for all tested samples. The weight ratios of F127-PPi and F127 were tested at 0:100, 25:75, 50:50, 75:25, and 100:0 w/w. The binding (incubation) time were set at 15 min, 30 min, 1 hr and 2 hr. Two-way ANOVA and Tukey's post-hoc test were used for statistical analyses. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

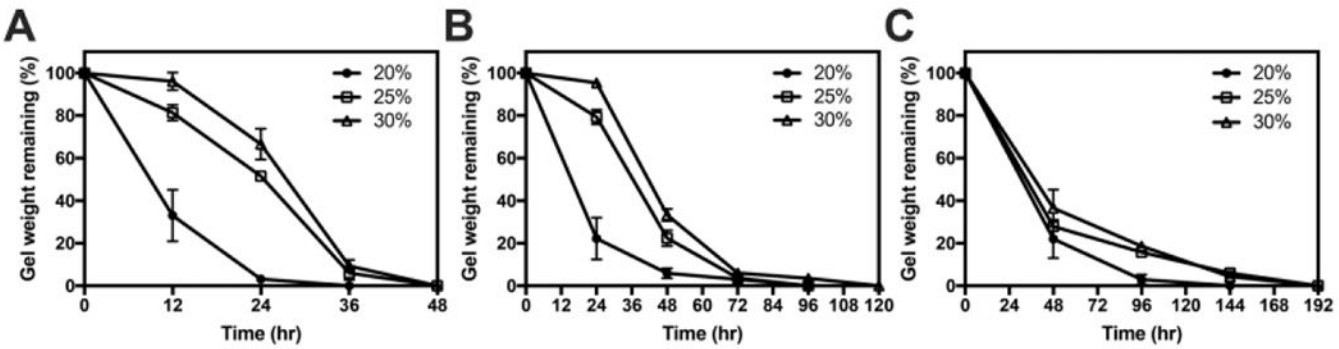


Figure 3.

In vitro dissociation of SIM-loaded PF127 hydrogel. The erosion profile of SIM-loaded PF127 hydrogels with different concentration of PF127 (20%, 25% and 30% w/v) in PBS at 37 °C versus time when the fresh buffer was replaced and weight was measured every 12 hr (**A**), every 24 hr (**B**), or every 48 hr (**C**). Results are shown as the percentage of gel remaining weight. Data are expressed as mean ± SD (n=3).

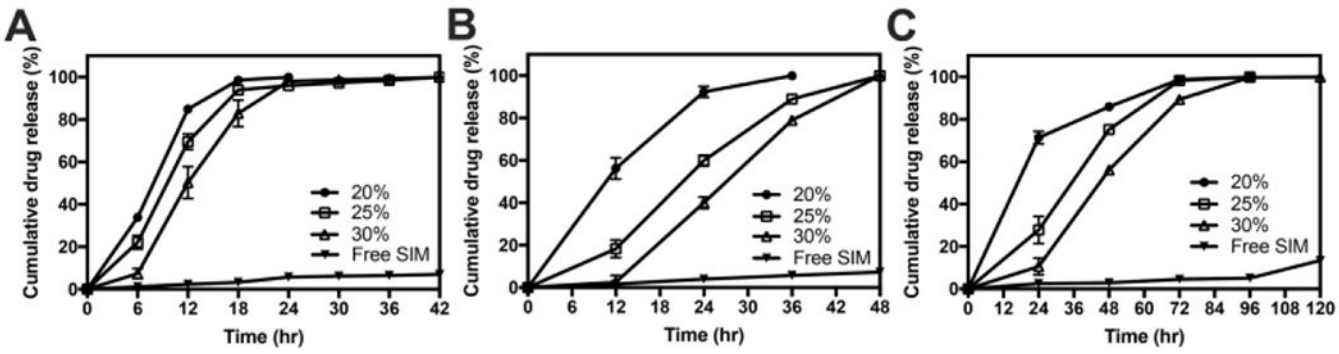


Figure 4.

In vitro cumulative release profiles of SIM from SIM-loaded PF127 hydrogels with different concentrations of PF127 (20%, 25% and 30% w/v) in PBS at 37 °C. Dissolution of free SIM was set as control. The releasing buffer was replaced and measured for SIM concentration every 6 hr (**A**), 12 hr (**B**), or 24 hr (**C**). Data are expressed as mean ± SD (n=3).

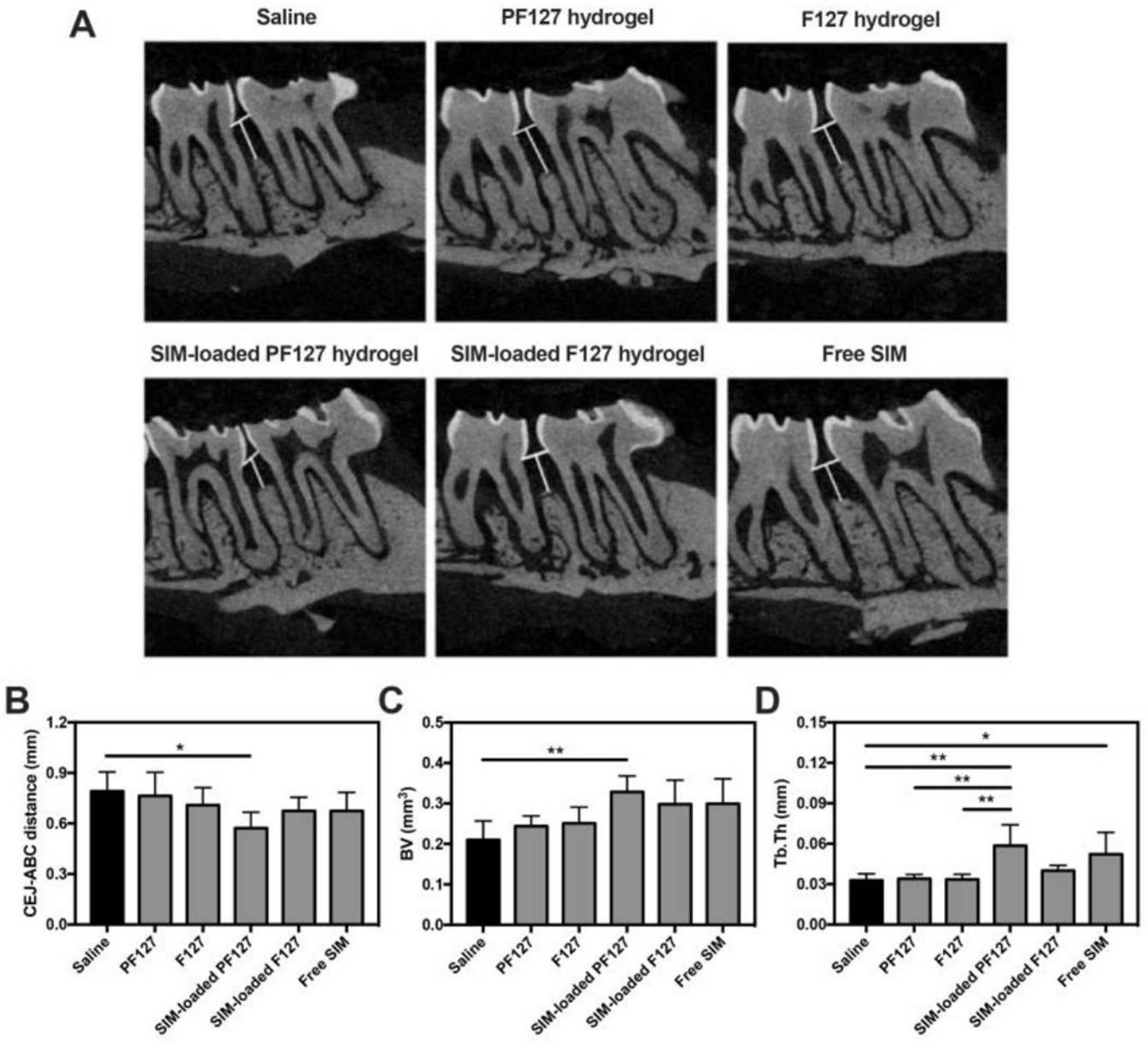


Figure 5.

In vivo evaluation of alveolar bone quality after different treatments. (**A**) Representative μ-CT sagittal images of the maxilla of cementoenamel junction (CEJ) to alveolar bone crest (ABC) after 3 weeks of treatment. White vertical lines indicate CEJ-ABC distance. The images for each group were selected from the samples showing the average length from CEJ to ABC during quantitative analysis. (**B**) Measurement of the linear distance from CEJ to ABC. SIM-loaded PF127 hydrogel treated group exhibited significantly shorter distance when compared to saline control group. μ-CT morphometric analysis showing the quantitative parameters bone volume (BV) (**C**) and trabecular thickness (Tb. Th) (**D**) of alveolar bone after 3 weeks of treatment. Data are expressed as mean ± SD. $*P < 0.05$, $**P < 0.01$ (one-way ANOVA with Tukey's post-hoc test for multiple comparisons).

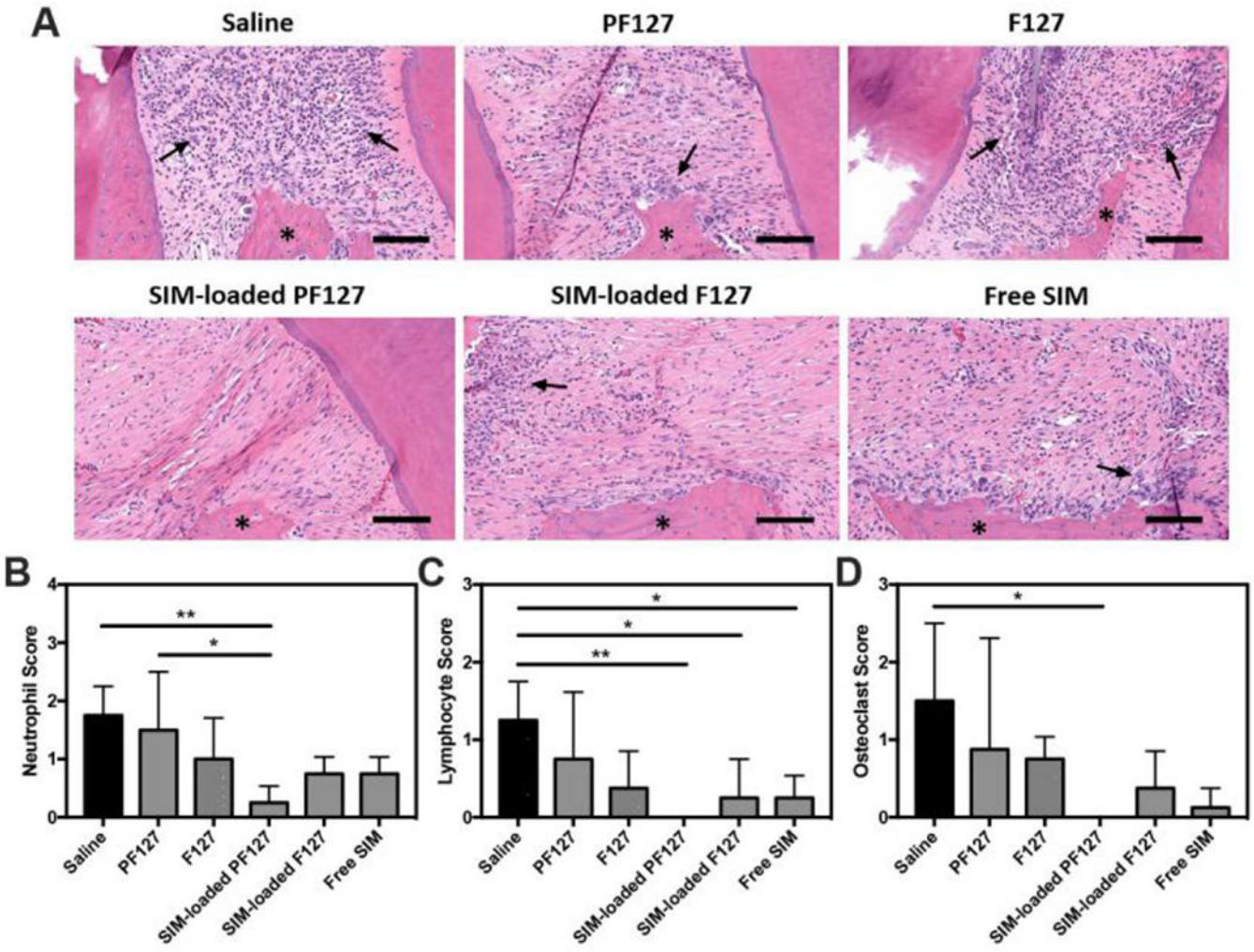
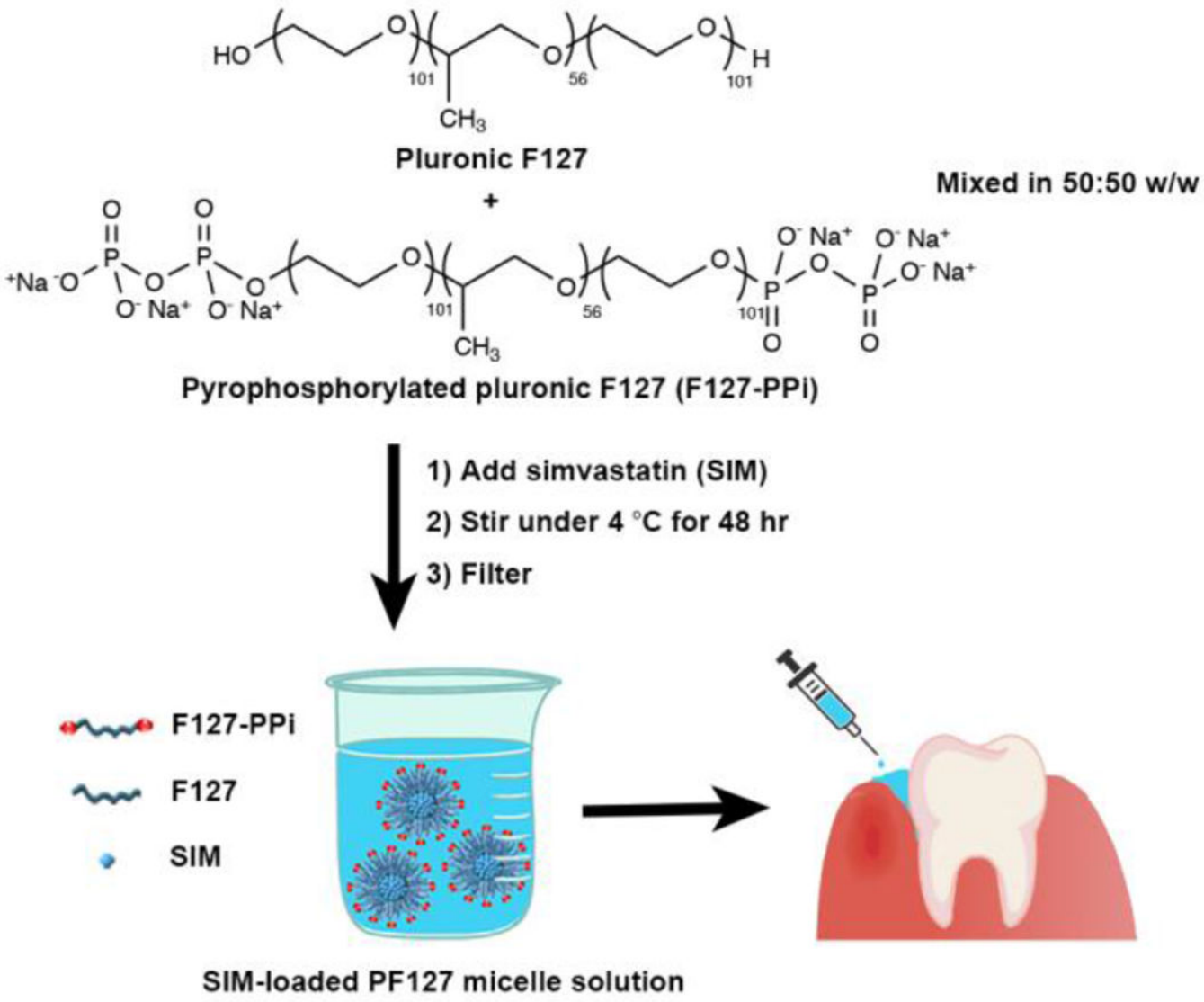


Figure 6.

Histological analysis on connective tissue and alveolar bone between the first molar (M1) and the second molar (M2) after different treatments. (**A**) Representative histological images of connective tissue between M1 and M2 after 3 weeks of treatment captured under 200× magnification. Asterisks indicate alveolar bone crest (ABC). Black arrows indicate inflammation infiltrate. Scale bar = 100 μm. (**B**-**D**) Qualitative analyses of neutrophil, lymphocyte and osteoclast scores of all treatment groups. $*P < 0.05$, $**P < 0.01$ (one-way ANOVA with Tukey's post-hoc test for multiple comparisons).



Scheme 1.
Preparation of SIM-loaded PF127 hydrogel formulation through direct dissolution method.

Table 1.

Comparison of Formulation Method on SIM Encapsulation Efficiency (EE) and Particle Size of PF127 Micelle in SIM-loaded PF127 Hydrogel (n=3)

Formulation Method	SIM EE (%)	Micelle Size (nm)	PDI
Film Hydration	66.5 ± 4.8	31.7 ± 21.9	0.335
Solvent Evaporation	36.2 ± 5.5	56.9 ± 14.1	0.136
Direct Dissolution	40.3 ± 5.5	19.3 ± 3.2	0.090

EE (%) = $(W_t/W_i) \times 100\%$ where W_t is the amount of drug loaded in micelle of hydrogel and W_i is the total amount of drug added initially during preparation.