The biomedical properties of a chitosan-based periodontal basic fibroblast growth factor delivery system

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Abstract

The aim of this study was to evaluate a CS-HTCC/GP-bFGF thermosensitive hydrogel synthesized using chitosan (CS), quaternized CS (HTCC) and α, β-glycerophosphate (α, β-GP) loaded with basic fibroblast growth factor (bFGF) for possible periodontal tissue engineering applications. The thermosensitive characteristics, substructure, bFGF-release profile, cytocompatibility and alkaline phosphatase (ALP) activity of human periodontal ligament cells (HPDLCs) were determined. An aqueous solution of CS–HTCC/GP–bFGF transformed into a hydrogel within 8–10 min when the temperature increased to 37°C. Scanning electron microscopy showed the substructure to be a three-dimensional, porous structure with interconnected pores. In vitro, bFGF was released over 48h in an artificial saliva buffer with a pH of 6.8, potentially improving the proliferation and ALP activity of HPDLCs. The data demonstrate that the CS-HTCC/GP-bFGF thermosensitive hydrogel was a strong candidate as a local protein delivery system for periodontal tissue regeneration.

Keywords

Periodontal disease; Chitosan; Thermosensitive hydrogel; Basic fibroblast growth factor (bFGF); Periodontal tissue regeneration.

1. Introduction

Periodontal disease is a serious, worldwide public health concern owing to its high prevalence and serious symptoms. It is a chronic infection characterized by the destruction of periodontium with dental plaque as the initial pathogen [1]. These are generally destructive, non-reversible conditions that can result in the formation of pockets between the gingiva and teeth, and possibly tooth loss [2]. Conventional mechanical debridement is considered to be the gold standard for the treatment of periodontal disease [3]. However, such methods cannot completely remove all bacterial biofilms from the root surface within the periodontal pockets. Topical medication is an ancillary treatment to inhibit bacterial deposits from the tooth surface and induce periodontal tissue regeneration [4].

Topical medication is an effective method to treat and prevent oral diseases [5], and drug delivery systems for biologics might improve the drug retention time and efficacy of the treatments [6]. Periodontal pockets provide a natural reservoir for insertion of a delivery device that is readily accessible. The gingival crevicular fluid (GCF) provides a leaching medium for the release of a dosage of drugs and for its distribution throughout the pockets. These features, together with the fact that periodontal diseases are localized to the immediate environment of the pockets, make periodontal pockets a natural site for treatment with local delivery systems.

Basic fibroblast growth factor (bFGF) is a heparin/HS-binding growth factor that not only promotes proliferation, differentiation and numerous other cellular functions in cells derived from the mesoderm and neuroectoderm, but also enhances angiogenesis and therapeutic potentials for tissue
regeneration including bone fracture healing [7]. In dentistry, bFGF has shown therapeutic potential to enhance the proliferation of osteoblasts, periodontal ligament (PDL) cells, and cementoblast angiogenesis for periodontal wound healing [8,9,10]. In vivo studies have shown that bFGF can promote periodontal regeneration at the early regeneration phase when applied to experimental periodontal defects [11,12,13]. By suppressing osteoblastic differentiation of PDL cells, bFGF may have a significant role in wound healing by inducing the growth of immature PDL cells and promoting angiogenesis.

The effect of bFGF is dependent on dose and duration. Therefore, strategies to integrate functional materials into bFGF delivery vehicles are an important candidate for pharmacological stimulation. Controlled delivery systems for bFGF release were evaluated with different methods, such as the encapsulation of heparin–sepharose-bound bFGF in alginate beads [14], bFGF in a succinylated chitosan (CS) nanoparticle [15], gelatin[16], and dextran hydrogels [17], to sustain release.

CS is a well-known natural polysaccharide which is a promising biomaterial in the pharmaceutical and medical fields owing to its many unique features including biocompatibility, biodegradability, bioactivity, antibacterial activity, and the ability to form gels and films [18]. Quaternized CS, N-[(2-hydroxy-3-trimethylammonium) propyl] chloride (HTCC), is derived from the reaction of CS and glycidyltrimethylammonium chloride (GTMAC). This reaction introduces quaternary amino groups into the CS chain to render it soluble in water. Antibacterial and antifungal activity was strengthened, and the bioadhesive properties and permeation effects improved [19,20].

In our previous study, a novel thermosensitive hydrogel was successfully designed and prepared. This hydrogel was based on CS and its derivative, quaternized CS (HTCC) with α, β-glycerophosphate (α, β-GP) without any additional chemical cross-linkers [21,22].

2. Experimental

2.1 Materials

CS was prepared in our laboratory [23] as 1080 kDa (molecular weight) with 86% deacetylation. GTMAC was obtained from Dongying Guofeng Fine Chemical Company, Limited (Shandong, China). α, β-GP was provided by Kaiyuan Pharmaceutical & Chemical Company, Limited (Shanxi, China). bFGF was purchased from the Zhuhai Yisheng Pharmaceutical company, Limited (Zhuhai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bFGF kits and ALP kits were purchased from the Sigma Chemical Company (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were provided by Gibco (Carlsbad, CA, USA). All other chemicals were of analytical grade.

2.2 Synthesis of the CS-HTCC/GP-bFGF thermosensitive hydrogel

HTCC was synthesized by the reaction of CS and GTMAC [24]. The bFGF–loaded hydrogels were fabricated with the following procedure. First, CS and HTCC (2% w/v) were mixed and progressively added to a 0.1 M aqueous lactic acid (LA) solution at room temperature by magnetic stirring until complete dissolution. Then, the bFGF solution was added to the CS-HTCC solution under stirring (CS-HTCC: bFGF). The α, β-GP aqueous solution (50% w/v) was prepared in deionized water. Both the aqueous α, β-GP solution and the CS-HTCC:bFGF solution were chilled in an ice bath for 15 min. Finally, the aqueous α,β-GP solution was added drop-wise to the CS-HTCC:bFGF solution. The resulting solution was stirred for 20 min. The preparation of CS-HTCC/PG-bFGF followed the optimum formulation of our previous study with (CS+HTCC) (2% w/v), CS/HTCC (5/1 w/w), α, β-GP (8.33%w/v) and a final bFGF concentration (20 or 40 µg/ml).

2.3 Characterization of the thermosensitivity

The test tube inverting method [25] was used to evaluate the thermosensitivity of the CS-HTCC/GP-bFGF thermosensitive hydrogel at 37°C.
2.4 Morphological studies
Samples of the CS-HTCC/GP-bFGF solution and thermosensitive hydrogels were observed after being frozen in liquid nitrogen and lyophilized for 48 h. Samples were then coated with platinum by ion sputtered gold under vacuum and investigated using a scanning electron microscope (KYKY2800B, KYKY Technology Development Limited, Beijing, China).

2.5 In vitro release behavior
To measure the release behavior of bFGF in vitro, the hydrogel sample was placed in a 2 mL microcentrifuge tube. Artificial saliva buffer [26] was used as the release medium to simulate normal gingival crevicular fluid (GCF). The sample was incubated at 37 °C. At predetermined time points, the release medium was collected. The bFGF concentration in the release medium was measured using bFGF ELISA kits.

2.6 In vitro cytocompatibility study
2.6.1 HPDLC culture
HPDLCs were isolated and cultured according to our previous study [27]. Primary HPDLCs were obtained from premolars that had been extracted from healthy patients (11–16 years old) for orthodontic reasons. The human PDL tissues were obtained from the center of the tooth root surface with a surgical scalpel. The tissues were cultured in DMEM supplemented with 10% (v/v) FBS and a 1% (v/v) penicillin–streptomycin solution (5000 units/ml penicillin and 50 µg/ml streptomycin; Sigma Chemical Co.) at 37°C, 5% CO2 in a humidified atmosphere. The medium was changed every 2–3 days. When the HPDLCs cultured from the tissue fragments reached confluence, the cells were trypsinized and split at a ratio of 1:2 with a 0.25% trypsin solution. Cells at passages 3–5 were used for this study.

2.6.2 Preparation of the CS-HTCC/GP-bFGF thermosensitive hydrogel extract
According to ISO standards, the ratio between the surface of the sample and volume of the medium was 0.5cm²/ml [28]. A CS-HTCC/GP-bFGF thermosensitive hydrogel was soaked in culture medium at 37°C for 24 h. The culture medium was then filtered through a 0.2-µm filter. The extracts containing almost all soluble compounds of the hydrogel in the culture medium were obtained and diluted with DMEM containing 10, 12.5, 25 and 50 % FBS (v/v).

2.6.3 MTT assay
Cytotoxicity evaluation of the CS-HTCC/GP-bFGF thermosensitive hydrogel was performed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HPDLCs were seeded into 96-well flat-bottom microplates (Costar, USA) at a density of approximately 3×10⁴ cells/well in medium containing 10% (v/v) FBS. The medium was replaced after 24 h and the cells were divided into four different groups that were treated with DMEM containing different concentrations of CS-HTCC/GP-bFGF hydrogel extracts: group 1, negative controls; group 2, 12.5% (v/v); group 3, 25% (v/v); group 4, 50% (v/v). After culturing for 1, 3 and 5 days, 200 µl of the MTT solution (2 mg/ml diluted in phosphate buffered saline (PBS)) was added to each well and incubated for 4 h. Subsequently, 200 µl of dimethyl sulfoxide (DMSO) was added to each well. The plates were then shaken until the crystals dissolved and the absorbance was determined at 490 nm with a microplate reader (Bio-Rad Model 550; Bio-Rad, Hercules, CA, USA). All reported values were the means of triplicate samples and this test was repeated twice.

2.6.4 Measurement of the ALP activity
The ALP activity in the cells was measured by p-nitrophenyl phosphate (p-NPP) substrate reactions using Sigma ALP assay reagents. After culturing for the indicated amount of time, cells were washed twice with PBS and incubated with 50 µl of 0.2% Triton X-100 for 20 min with gentle shaking. The cells were then incubated with 100 µl of substrate (10 mM p-NPP, 1 mM MgCl₂) for 30 min at 37 °C. The reaction was stopped by adding 100 µl of 1 M NaOH. The p-nitrophenol formed was measured
at 405 nm using a microplate reader (Bio-Rad Model 550). Cell numbers were evaluated using a hemacytometer. ALP activity data were converted to units of ALP per $10^4$ cells (scale down).

2.7 Statistical analysis
All quantitative data were expressed as means±standard deviation (SD). The P-values were calculated with one-way ANOVA using SPSS13.0 statistical software (SPSS, USA) with P < 0.05 considered statistically significant.

2.8 Results
2.8.1 Characterizations of thermosensitivity
CS-HTCC/GP-bFGF remained in a liquid state for a desired period of time when the temperature was less than 37 °C (Fig. 1a) and turned into a solid gel when the temperature was 37 °C (Fig. 1b). The sol–gel transition time was 8–10 min. The temperature-dependent phenomenon indicated that the CS-HTCC/GP-bFGF hydrogel exhibited good thermosensitivity (Fig. 1b) 2.8.2 Morphological studies
The morphologies of the freeze-dried CS-HTCC/GP-bFGF (Fig. 1c)solution and thermosensitive hydrogel (Fig.1d) were examined by scanning electron microscopy. Compared with the solution of CS-HTCC/GP-bFGF, the CS-HTCC/GP-bFGF thermosensitive hydrogel showed a three-dimensional, porous structure with interconnected pores. Additionally, porous microstructures with different chamber diameters and distributions were observed.

Fig. 1. (a) CS-HTCC/GP-bFGF solution and (b) the formed CS-HTCC/GP-bFGF hydrogel at 37 °C. Scanning electron microscopy images of (c) the CS-HTCC/GP-bFGF solution and (d) thermosensitive hydrogel.

2.8.3 bFGF release behavior in vitro
The in vitro release tests of bFGF from CS-HTCC/GP-bFGF were conducted and the release profiles are presented in Fig. 2. An artificial saliva buffer solution with a pH of 6.8 simulated a normal condition, while pH 4.0 represents the acid environment of a pathologic status of oral cavity surroundings. Fig. 2 (a) illustrates the release behavior of the CS-HTCC/GP-bFGF hydrogel with different amounts of bFGF in the pH 6.8 artificial saliva buffer. Almost 35% and 55% of the total bFGF were released in the first 16 h from the 20 and 40 µg/ml groups, respectively (Fig. 2a). This
release was followed by a relatively slow and controlled release period. Furthermore, compared with that of the 40 µg/ml bFGF-hydrogel, the cumulative release rate of the 20 µg/ml hydrogen decreased over a 48-h period. The release profiles of bFGF from artificial saliva buffers with different pH values are shown in Fig. 2. The bFGF release started with an initial burst effect and continued with a controlled and steady release period. At pH 4, bFGF was released more rapidly from the hydrogel than at pH 6.8 and about 60% of the bFGF was released in the first 24 h. At pH 6.8, about 45% was released in the first 24 h and 77% was released in the following 48 h.

Fig. 2. In vitro bFGF release profiles from CS-HTCC/GP thermosensitive hydrogels with different (a) concentrations of bFGF and (b) pH values of the artificial saliva buffer.

2.8.4 MTT assay
The MTT assay was used to evaluate the cytocompatibility of the CS-HTCC/GP-bFGF thermosensitive hydrogel to HPDLCs as shown in Fig. 3. The concentration-dependent study (Fig. 3 a) showed the incubation of the cells with different concentrations of extract for 1, 3, and 5 days resulted in significant differences compared with the controls (group 1). The CS-HTCC/GP-bFGF thermosensitive hydrogel promoted HPDLC proliferation with various concentrations of CS-HTCC/GP extracts (group 2, 3, 4) at 1, 3, and 5 days, respectively. On the same culture day, there was a significant increase in the number of HPDLCs for group 4 compared with group 1 (P < 0.001). At 3 d, compared with the control group (group 1), group 3 had significantly increased proliferation of HPDLCs (P < 0.01). Fig. 3 (b) shows the time-dependent effect of the CS-HTCC/GP- bFGF thermosensitive hydrogel to HPDLCs. In groups 3 and 4, HPDLC proliferation was significantly increased over 5 d compared with 1 day (P < 0.001).

Fig. 3. MTT assay of (a) concentration-dependent and (b) time-dependent effects of the CS-HTCC/GP-bFGF thermosensitive hydrogel extract on HPDLCs. The data were depicted as mean±SD (n=6).

*P < 0.05, **P < 0.01, ***P < 0.001: significant difference compared with the controls (group 1) over the same culture time.
#P < 0.05, ## P < 0.01, ### P < 0.001: significant difference compared with 1 d with the same hydrogel extract concentration.

2.8.5 ALP activity

Fig. 4 outlines the results of the ALP activity assay in HPDLCs affected by the CS-HTCC/GP-bFGF thermosensitive hydrogel. The CS-HTCC/GP-bFGF thermosensitive hydrogel noticeably improved the ALP activity in HPDLCs. The ALP activity was improved at days 1, 3 and 5 in all 4 groups, with significant differences between groups 1 and 4 at days 3 and 5 (P < 0.001) (Fig. 4 a). When comparing days 1 and 5, a significant difference was found between groups 4 (P < 0.001) and 3 (P < 0.05) (Fig. 4 b).

Fig. 4. (a) Concentration-dependent and (b) time-dependent effects of ALP activity in HPDLCs due to the CS-HTCC/GP-bFGF thermosensitive hydrogel extract as measured with an ALP kit. The data depict the mean± SD (n=3).

*P < 0.05, **P < 0.01, ***P < 0.001: significant difference compared with the controls (group 1) over the same culture time.

#P < 0.05, ## P < 0.01, ### P < 0.001: significant difference compared with 1 d with the same hydrogel extract concentration.

2.9 Discussion

CS has a cationic nature that makes it an inhibitor of mRNA synthesis within bacteria [29,30]. The cationic nature also modulates the activity of several growth factors and cytokines, as has been demonstrated in bone and cartilage regeneration [31]. CS has been used widely in regenerative medicine and tissue engineering, and its role in drug delivery is also documented [18]. bFGF can regulate vascularization, bone formation and remodeling. In another study, bFGF was reported to stimulate early bone formation in cranial regeneration in a murine model [32]. However, it is highly unstable in physiological environments [11]. The sustained-release system (CS-HTCC/GP-bFGF) with CS as carrier enhanced the effect of bFGF.

CS-HTCC/GP-bFGF was used as an in situ and injectable, CS-based thermosensitive hydrogel. The aqueous solution of the hydrogel was administered more easily and adapted to the anatomical complexities of periodontal pockets or the pocket size. Hydrogels are bio-adhesive to the mucosa in the dental pocket, are biodegradable, and allow controlled release of drugs. Application of the presented formulation provides an effective drug concentration for an ideal period in the periodontal pocket, makes direct contact with the subgingival flora, and suppress or destroys microbial growth. It is physically crosslinked, no chemical cross agents are used in the formulation, and it exhibits good thermosensitivity. Furthermore, the sol–gel transition process can be achieved within 8–10 min. As a result, the porous substructure of the hydrogel allows small molecules and cells to move in the network easily, suggesting their potential as scaffolds for cell infiltration and growth.

In the present investigation, the initial rate of release of bFGF was rapid and then slowed after several hours. In vitro, bFGF was released from the CS-HTCC/GP thermosensitive hydrogel over 48 h in artificial saliva with a pH of 6.8. The release of bFGF was retarded more effectively. Protein release may be due to a mixture of diffusion- and degradation-controlled mechanisms. First, bFGF on the
surface of hydrogels diffuses rapidly owing to surface and bulk erosion. The remaining bFGF in hydrogels cannot be released until hydrogels are completely degraded or dissolved in the release medium. Then, protein is released slowly from the CS-HTCC/GP-bFGF hydrogels. Both CS and HTCC have a positive charge, resulting in poor interaction with bFGF molecules that are also positively charged because of their isoelectric point (pI of 9.6). This can partially explain the initial burst release of bFGF. Another study showed that HA is superior to the affinity to bFGF because of the charge. Therefore, a further study should be designed to improve the release kinetics of the CS-HTCC/GP-bFGF hydrogels.

According to the results of the release profile, the pH of the medium buffer as well as the drug concentration will alter protein release rates. It is therefore possible to control protein release rates to a relative desired value. Additionally, the release may be strongly influenced by protein type, polymer chemistry, water sorption and degradation. Though bFGF was released from the CS-HTCC/GP thermosensitive hydrogel at a relatively higher level and with an initial burst release, it in turn provided sufficient concentrations to be effective at the site.

PDL cells are primarily fibroblastic cells, but they differ from gingival fibroblasts, with their protein expressions related to osteogenic differentiation and mineralized tissue forming proteins such as ALP, osteocalcin, bone sialoprotein, and osteonectin. Evidence suggests that PDL cells have the capacity to differentiate and produce the mineralized tissues of the periodontium as well as PDL fibers. ALP is an enzyme belonging to a group of membrane-bound glycoproteins. Expression and activity of ALP are widely used as markers for osteoblastic cells. In this study, a CS-HTCC/GP-bFGF thermosensitive hydrogel significantly enhanced proliferation and ALP activity in HPDLCs with good cytocompatibility. Antibacterial activity on periodontal pathogens, implant histocompatibility and in vivo toxicity of CS–HTCC/GP thermosensitive hydrogel were determined in our previous studies. The results of these experiments may provide useful data for the tissue engineering of periodontal tissue lost to periodontitis, trauma, or other causes.

CS-HTCC/GP-bFGF can be a potential strategy for periodontal regeneration. More biological characteristics of CS-HTCC/GP-bFGF should be studied in future for clinical applications.

3. Conclusion

The results indicate that the CS-HTCC/GP-bFGF thermosensitive hydrogel is a highly suitable candidate as a local protein delivery system for periodontal tissue regeneration.

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References


