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Tetracycline release from tripolyphosphate–chitosan cross-linked sponge: a preliminary *in vitro* study

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Background and Objective: The aim of this study was to design a tripolyphosphate–chitosan cross-linked tetracycline-containing (TPP-TC) sponge that slowly releases tetracycline, for future periodontal applications.

Material and methods: Chitosan sponge was made by freezing and drying 2.5% chitosan solution. Tripolyphosphate-chitosan cross-linked (TPP) sponge was made by immersing the chitosan sponge in tripolyphosphate solution and air drying it. Tetracycline-containing chitosan (TC) sponge was prepared by freezing and drying a mixture of chitosan and tetracycline. TPP-TC sponge was made by immersing the TC sponge in tripolyphosphate solution. The weight, thickness and diameter of the four chitosan sponges were recorded. Their surface microstructures were inspected using scanning electron microscopy. The amount of tetracycline released from the sponges was analyzed by spectrophotometry. Antimicrobial activities of the residual sponges were tested against *Staphylococcus aureus* and *Escherichia coli*.

Results: The topography of the scaffolds was intact after the addition of tetracycline. However, increased surface irregularities were noted. In sponges with tripolyphosphate, intensified surface folding was observed. The weight of the sponges increased after tripolyphosphate and tetracycline were added, but their thicknesses and diameters decreased after cross-linking. Tetracycline was detected in the solution containing TPP-TC sponges until day 11. On day 7, the tetracycline released from TC sponges was less than that released from TPP-TC sponges. Bacterial growth was inhibited by sponges containing tetracycline. The inhibitory effect of the TPP-TC sponges was detectable until day 11.

Conclusion: Our data showed that TPP-TC sponge was suitable as a slow-release device for tetracycline and that it maintained antimicrobial effects against the bacteria tested for up to 11 d.

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It has long been recognized that bacteria are the primary etiological agents of chronic periodontitis. Effective bacterial control is the key to the prevention and treatment of the disease. The major method of eliminating pathogenic bacteria in periodontitis has been the mechanical removal of bacterial deposits (1). In addition, several antibacterial agents have been shown to produce bactericidal or bacteriostatic activity.

Consequently, they are employed as adjuncts to the mechanical treatment of periodontal diseases (2–9). Different methods of systemic and local antimicrobial delivery have been attempted, including mouth rinses, irrigation solution, fiber systems and subgingival medications (10–12). These strategies of periodontal therapy commonly involve the introduction of antibacterial agents into targeted sites at adequate concentrations for a sufficient amount of time to control the infection.

In recent years, functional biomaterial research has been directed towards the development of improved scaffolds and new drug delivery systems. In this regard, considerable attention has been given to chitosan-based materials (13). Chitosan is a de-acetylated derivative of chitin, the second most abundant natural biopolymer worldwide, and commonly found in shells of marine crustaceans and cell walls of fungi. Chitosan is a linear polysaccharide, composed of glucosamine and *N*-acetyl glucosamine linked in a β1–4 manner, with the glucosamine/*N*-acetyl glucosamine ratio being referred to as the degree of deacetylation. Depending on the source and preparation procedure, the molecular weight of chitosan may range from 300 to over 1000 kD with the degree of deacetylation ranging from 30 to 95%. In its crystalline form, chitosan is normally insoluble in aqueous solutions above pH 7; however, in diluted acids (pH < 6.0), the protonated free amino groups on glucosamine facilitate solubility of the molecule (14, 15).

One of the favorable properties of chitosan is that it can be molded into various forms (16). Chitosan possesses excellent ability to form porous structures. Porous scaffolds are generated by freezing and lyophilizing chitosan solutions (17) or by processes such as the 'internal bubbling process' where $CaCO_3$ is added to chitosan solution to generate chitosan–$CaCO_3$ gels (18). Ice removal by lyophilization generates a porous material, the pore size and orientation of which can be controlled by variation of the freezing rate, the ice crystal size and the geometry of thermal gradients during freezing. The obtained material can then be molded into porous membranes, blocks, tubes or beads. Pore size and orientation are shown to influence the mechanical properties of chitosan scaffolds (17).

Another useful property of chitosan is its bioadhesive nature, ensuring its good retention on oral surfaces (19, 20). Based on this unique quality, chitosan is suitable as a vehicle for the release of effective therapeutic agents for periodontal applications (20, 21). The aim of this pilot study was to test the efficacy of tetracycline release from a new formulation of chitosan sponge containing tetracycline, with tripolyphosphate cross-linking.

Materials and methods

The preparations of control, tetracycline-containing, tripolyphosphate cross-linked, and cross-linked tetracycline-containing chitosan sponges

Chitosan (Primex Ingredients AS, Avaldenes, Norway), with a molecular weight of 450,000 and a degree of deacetylation of > 90%, was selected and used. The freshly prepared solvent consisted of vitamin C solution (Merck, Darmsatadt, Germany) at a concentration of 20 mg/mL in distilled deionized water. The stock chitosan solution was made by adding 0.25 g of chitosan powder to 10 mL of vitamin C solution. Four types of sponges were prepared in this study. The control chitosan sponge was made by freezing and drying 1 mL of 2.5% chitosan solution in a mold with 1.5 cm internal diameter. The second type, tetracycline-containing chitosan (TC) sponge, was prepared from 1 mL of distilled water with 10 mg of tetracycline (Sinphar, Taipei, Taiwan) and 0.025 g of chitosan. After filtration, freezing and drying for 24 h, the sponge was stored at room temperature until use. The third type, tripolyphosphate-chitosan cross-linked (TPP) sponge, was made by immersing the chitosan sponge into 10 mL of 5% tripolyphosphate (Acros Organics, Morris Plains, NJ, USA) solution for 30 min followed by air drying for 3 d (22). The fourth type was tripolyphosphate-chitosan cross-linked tetracycline-containing (TPP-TC) sponge, which was made by immersing the second-type/TC sponge in 10 mL of 5% tripolyphosphate solution, as described for the tripolyphosphate sponges of the third type. The weights, thicknesses and diameters of the chitosan sponges were recorded. After the sponges were coated with a film of gold–palladium (Ion Suptter E-1010; Hitachi, Berkshire, UK) under vacuum, their surface details were examined by scanning electron microscopy (S-3500N; Hitachi).

Determination of tetracycline release and the antimicrobial effects of the sponges

To determine the tetracycline release from the sponges at specific intervals, 56 sponges (28 each of TC and TPP sponges) were immersed in 50-mL glass bottles containing 10 mL of distilled water. The solution was collected from every sponge-containing bottle (four bottles for each type) on days 0, 1, 3, 5, 7, 9 and 11 and the amount of tetracycline released from the sponges (Thermo Spectormic, Rochester, NY, USA) was determined spectrophotometrically. Because the tetracycline-containing chitosan sponges had disintegrated by day 9, measurements made on days 9 and 11 were not included in this study. The immersed sponges were also collected afterwards for antimicrobial tests. Two commercially available tetracycline-sensitive bacteria, *Staphylococcus aureus* ACTT 25923 and *Escherichia coli* ACTT 25922 (Sancordon Co., Taipei, Taiwan), were used in this study to determine the antimicrobial effects of the chitosan sponges. The tested sponges were placed on agar plates seeded with *S. aureus* and *E. coli* and incubated in a CO_2 incubator at 37°C for 24 h, according to the instructions of the manufacturer. The inhibitory effects were determined from the diameters of the inhibition rings. Four sponges from each of TC and TPP-TC sponges were tested. A commercially available tetracycline disc (Sensi-Disc™; Becton-Dickinson and Co., Sparks, MD, USA) was used as the positive control.

Statistical analysis

One-way analysis of variance was used to compare the weights, thicknesses and diameters of the four types of chitosan sponges and the tetracycline release from the two tetracycline-containing (TC and TPP-TC) sponges during the observation periods. At each observation interval, a comparison of tetracycline release from the sponges, with and without cross-linking, was analyzed using a *t*-test.

Results

Porous scaffolds were observed in the chitosan control sponge when viewed under scanning electron microscopy (Fig. 1A,E). In the chitosan sponges containing tetracycline, the porous macrostructure was intact. However, irregularities in the scaffold surfaces, as well as thin projections, were observed (Fig. 1B,F). After the addition of tripolyphosphate, a folding phenomenon of the membranous surface of the scaffold structures was observed in sponges both with and without tetracycline. Irregular surfaces were only present on the sponges containing tetracycline (Fig. 1C–D,G–H).

The weight of the chitosan sponges increased after the addition of tripolyphosphate and tetracycline relative to that of the control sponge (Fig. 2A). The thickness of the sponges with added tetracycline also increased. However, the thickness and diameter of the sponges decreased when tripolyphosphate, the cross-linking agent, was added (Fig. 2B, C).

Release of tetracycline from sponges

The tetracycline content of the TPP-TC sponges was detectable from the optical density of the sponge-containing solution until day 11. Significantly more tetracycline was detected between days 5 and 11 than on days 1 and 3, and the highest level was observed on day 9 (2.29 ± 0.006) (Fig. 3). However, the highest level of tetracycline in the TC sponges was detected on day 5, and the mean level had decreased on day 7, to a level lower than that in the TPP-TC sponges ($p = 0.06$).

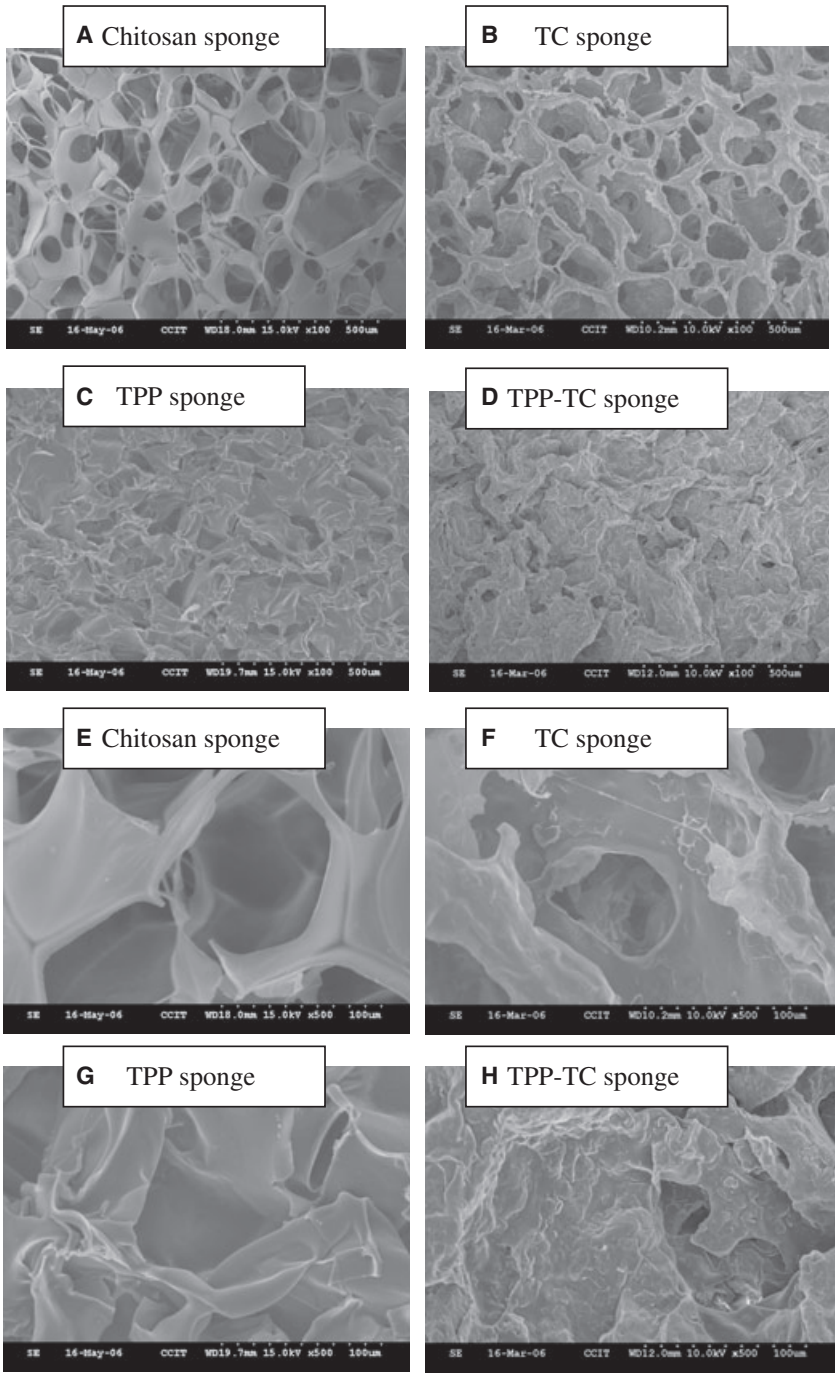

Fig. 1. The microtopography of the control chitosan sponge (A and E), tetracycline-containing chitosan sponge (B and F), tripolyphosphate-chitosan cross-linked sponge (C and G), and tripolyphosphate–chitosan cross-linked tetracycline-containing sponge (D and H). Original magnification, ×100 (A–D) or ×500 (E–H).

Antibiotic activities of chitosan sponges

There were no inhibitory effects around the control or tripolyphosphate cross-linked sponges on the bacterial agar plates (Fig. 4, left plates). However, clear inhibition zones were observed around the chitosan sponges containing tetracycline (TC and TPP-TC) (Fig. 4, right plates). Although the zones of inhibition were smaller on day

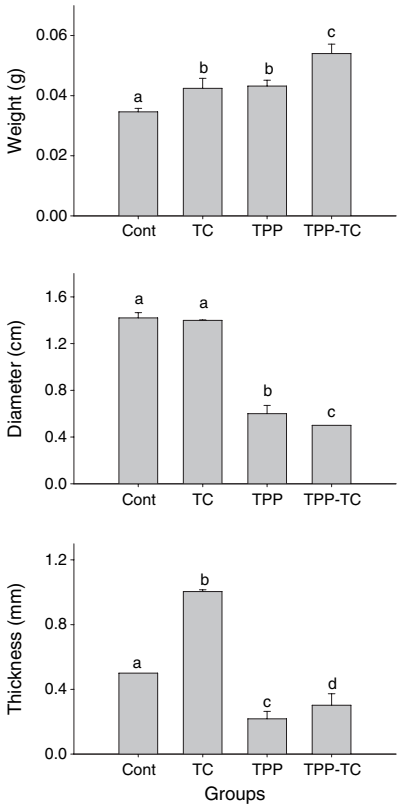

Fig. 2. The means and standard deviations of the weights, thicknesses and diameters of the four types of chitosan sponges (Cont, control chitosan sponge; TC, tetracycline-containing chitosan sponge; TPP, tripolyphosphate–chitosan cross-linked sponge; and TPP-TC, tripolyphosphate–chitosan cross-linked tetracycline-containing sponge). The letter a, b, c or d indicates the subgroups according to the *post hoc* analysis after a significant difference is obtained by one-way analysis of variance.

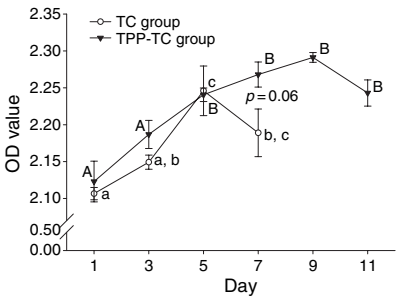

Fig. 3. Tetracycline released from the tetracycline-containing (TC) and tripolyphosphate-chitosan cross-linked tetracycline-containing (TPP-TC) sponges, measured by spectrophotometry (means and standard deviations are shown). The letters indicate the subgroups according to *post hoc* analysis after obtaining a significant difference by one-way analysis of variance: a–c for the TC sponges, and A–B for the TPP-TC sponges. OD, optical density.

11 than on day 0, the inhibitory effects of the sponges could still be seen on day 11 in the antibiotic-containing sponges with cross-linking (Fig. 5, right plates) (Table 1).

Discussion

This study demonstrated that a steady release of tetracycline occurred from chitosan sponges after they were cross-linked with tripolyphosphate. A study of the prolonged release of minocycline from chitosan microcapsules for 7 d has previously been reported (23). The injectable microspheres were prepared by creating an outer chitosan layer and an inner alginate layer. In the present study, a cross-linked scaffold was constructed instead, to prolong the release of tetracycline. Our results demonstrated tetracycline release from the chitosan scaffold for 7 d, whereas at least 11 d of drug release was achieved after cross-linking. The dimensions of the sponges decreased after the addition of the cross-linking agent, although their thickness increased after they were combined with tetracycline (Fig. 1A,E). Folding of the membranous surfaces of the scaffolds was observed (by scanning electron microscopy) after tripolyphosphate, the cross-linking agent, had been added to the sponges (Fig. 1C,G) (24). The release of tetracycline from TPP-TC sponges was recorded up to day 11, whereas decreasing levels of tetracycline were observed from day 7 from sponges without cross-linking, attributed to the disintegration of cross-linking after day 7 (Fig. 3). Prolonged release of tetracycline may be more suitable for periodontal use because a 2-wk duration of local or systemic tetracycline periodontal therapy has been recom-

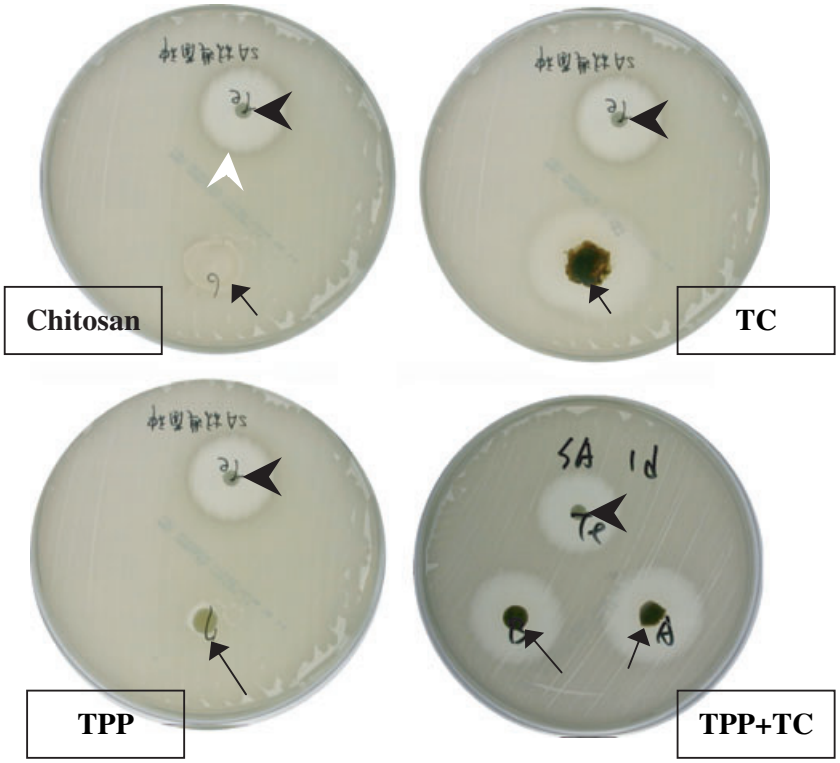

Fig. 4. Antibacterial activities of the four types of sponges, after immersion for 24 h, against *Staphylococcus aureus*. Chitosan, control chitosan sponge; TC, tetracycline-containing chitosan sponge; TPP, tripolyphosphate-chitosan cross-linked sponge; TC + TPP, tripolyphosphate–chitosan cross-linked tetracycline-containing sponge. The black and white arrowheads indicates the standard tetracycline disc and its inhibition ring, respectively; and the arrow indicates the inhibition ring or the sponge itself.

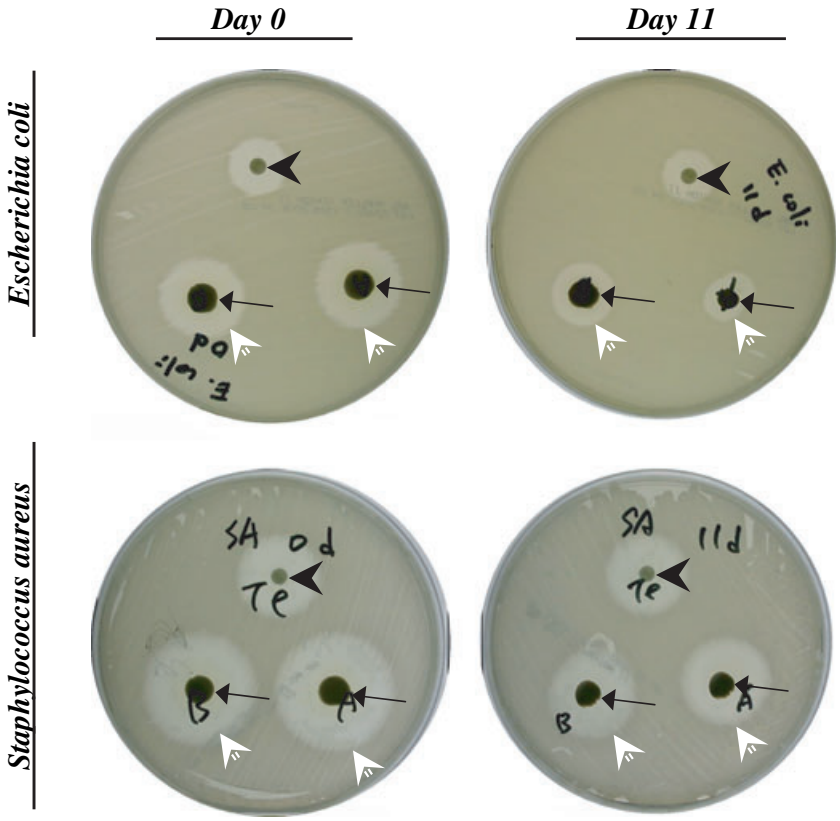

Fig. 5. Antibacterial activities of tripolyphosphate–chitosan cross-linked tetracycline-containing (TPP-TC) sponges against *Staphylococcus aureus* and *Escherichia coli* at days 0 and 11. The black arrowhead indicates the standard tetracycline disc; and the white arrowhead and black arrow indicate the inhibition ring linked and the TPP-TC sponge, respectively.

Table 1. The diameters (in mm) of the rings of inhibition for *Staphylococcus aureus* and *Escherichia coli* in the four chitosan sponges tested and in positive-control tetracycline discs (commercially available)

		Noncross-linked sponges		Cross-linked sponges	
	Positive-control discs	Chitosan	Chitosan + TC	Chitosan + TPP	Chitosan + TC + TPP[a]
Staphylococcus aureus ACTT 25923					
Day 0	24	0	45	0	35, 32
Day 1	25	–	31	–	29, 28
Day 3	25	–	31	–	32, 31
Day 5	25	–	41	–	27, 30
Day 7	25	–	25	–	27, 32
Day 9	25	–	DI	–	27, 32
Day 11	24	–	DI-	–	27, 28
Escherichia coli ACTT 25922					
Day 0	20	0	35	0	30, 30
Day 1	20	–	30	–	27, 27
Day 3	21	–	30	–	18, 27
Day 5	20	–	29	–	22, 26
Day 7	20	–	28	–	23, 24
Day 9	19	–	DI	–	19, 23
Day 11	19	–	DI	–	17, 22

[a]Two sponges were tested at each time point.
–, not done; DI, disintegration of the sponge; TC, tetracycline; TPP, tripolyphosphate.

mended, rather than single-dose therapy, for the removal of pathogens from the gingival sulcus (25).

In the present study, tripolyphosphate (instead of glutaraldehyde) was selected as the cross-linking agent for the tetracycline-containing chitosan sponges. Being one of the most commonly accepted cross-linking reagents, glutaraldehyde has been used in the preparation of bioprosthetic heart valves (26, 27), as well as in a dermal collagen for guided periodontal regeneration (28). It has been shown that the glutaraldehyde–chitosan cross-linked microspheres possess a long-acting biodegradable ability suitable for the controlled delivery of many drugs (29–31). However, glutaraldehyde is highly cytotoxic, which may impair the biocompatibility of the cross-linked biomaterials (32, 33). Thus, the reversible physical cross-linking by electrostatic interaction, instead of chemical cross-linking, is applied to avoid possible toxicity of reagents and other undesirable effects (34, 35).

Because of the anionic property of tripolyphosphate, it can interact with cationic chitosan by electrostatic forces (36, 37). As the tripolyphosphate–chitosan complex can be prepared easily, many researchers have investigated its potential pharmaceutical usage (38–42). The electrostatic forces between cross-linked tripolyphosphate and chitosan lead to decreased intermolecular spaces and consequently result in suspension of the release of tetracycline, as well as prolonged disintegration of sponges. On the other hand, the disintegration of noncross-linked sponges may be attributed to the gradual release of residual mild acids (including vitamin C and tetracycline HCl itself) from the sponges. Nevertheless, the exact mechanism of the slow release of tetracycline from sponges after cross-linking with tripolyphosphate, as observed in this study, is still uncertain and needs further investigation.

In this study, reduced optical density values were observed in both TC and TPP-TC sponges immediately before the disintegration of the sponges (Fig. 3). This might be a result of interference from disinte-

grated particles. For the same reason, the nonsignificant difference in optical density levels ($p = 0.06$) between the TPP-TC and TC sponges on day 7 might also be caused by disintegration of the tetracycline-containing chitosan sponges.

In the present study, tetracycline was selected because of its wide application, both locally and systemically, in the treatment of periodontal diseases. Tetracycline has been shown to be effective against many of the common periodontopathic bacteria, in particular against *Prevotella intermedia* and *Porphyromonas gingivalis* (43–45). In the present study, two commercially available bacterial strains were selected to meet the preliminary goal of whether the activity of antibiotics can be preserved after incorporation into preparations. Our results showed that the cross-linked chitosan sponges were able to deliver active antibiotic for 11 d; however, the effectiveness has yet to be evaluated against potential periodontal pathogens. Because periodontitis is a localized inflammation of the periodontal pocket caused by bacterial infection, current microbiological treatment strategies involve are either the use of systemic antibiotics or a localized delivery system incorporating an antibiotic.

Chitosan sponges with porous scaffolds were designed in this study as carriers for antibiotics and showed a steady release of the medication. Three-dimensional chitosan matrices have been shown to be excellent tissue-engineering scaffolds for cell attachment and growth. Chitosan has a scalloped structure and has been used in tissue engineering to culture hepatocytes, fibroblasts and cartilage cells because of its ability to promote cell attachment and growth (46–55). Periodontal regeneration with chitosan has been observed after the implantation of chitosan in one-wall alveolar bony defects in dogs (56). In this study, chitosan was selected as the carrier for tetracycline, mainly because it can both carry and deliver the medication, but also because it has other useful bioactivities such as anti-inflammatory properties (50, 57).

In conclusion, a steady slow release of tetracycline, while maintaining antibiotic effects against the tested bacteria, for at least 11 d was shown from chitosan sponges cross-linked with tripolyphosphate. Based on our results, we conclude that the cross-linked chitosan sponge is a suitable carrier for tetracycline to be slow-released. Within the limitations of the study design, our results suggest that chitosan sponge cross-linked with tripolyphosphate is a suitable biomaterial for periodontal applications. However, future investigations are necessary to validate this hypothesis.

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References

1. Goodson JM, Haffajee A, Socransky SS. Periodontal therapy by local delivery of tetracycline. *J Clin Periodontol* 1979;**6:**83–92.
2. Goodson JM, Cugini MA, Kent RL *et al*. Multicenter evaluation of tetracycline fiber therapy: I. Experimental design, methods, and baseline data. *J Periodont Res* 1991; **26:**361–370.
3. Goodson JM, Cugini MA, Kent RL *et al*. Multicenter evaluation of tetracycline fiber therapy: II. Clinical response. *J Periodont Res* 1991;**26:**371–379.
4. Newman MG, Kornman KS, Doherty FM. A 6-month multi-center evaluation of adjunctive tetracycline fiber therapy used in conjunction with scaling and root planing in maintenance patients: clinical results. *J Periodontol* 1994;**65:**685–691.
5. Soskolne WA, Heasman PA, Stabholz A *et al*. Sustained local delivery of chlorhexidine in the treatment of periodontitis: a multi-center study. *J Periodontol* 1997; **68:**32–38.
6. van Steenberghe D, Bercy P, Kohl J *et al*. Subgingival minocycline hydrochloride ointment in moderate to severe chronic adult periodontitis: a randomized, double-blind, vehicle-controlled, multicenter study. *J Periodontol* 1993;**64:**637–644.
7. Ainamo J, Lie T, Ellingsen BH *et al*. Clinical responses to subgingival application of a metronidazole 25% gel compared to the effect of subgingival scaling in adult periodontitis. *J Clin Periodontol* 1992; **19:**723–729.
8. Jeffcoat MK, Bray KS, Ciancio SG *et al*. Adjunctive use of a subgingival controlled-release chlorhexidine chip reduces probing depth and improves attachment level compared with scaling and root planing alone. *J Periodontol* 1998;**69:**989–997.
9. Garrett S, Johnson L, Drisko CH *et al*. Two multi-center studies evaluating locally delivered doxycycline hyclate, placebo control, oral hygiene, and scaling and root planing in the treatment of periodontitis. *J Periodontol* 1999;**70:**490–503.
10. Tonetti M, Cugini MA, Goodson JM. Zero-order delivery with periodontal placement of tetracycline-loaded ethylene vinyl acetate fibers. *J Periodont Res* 1990;**25:**243–249.
11. Greenstein G. Effects of subgingival irrigation on periodontal status. *J Periodontol* 1987;**58:**827–836.
12. Rethman M, Greenstein G. Oral irrigation in the treatment of periodontal diseases. *Curr Opin Periodontol* 1994:99–110.
13. Khor E, Lim LY. Implantable applications of chitin and chitosan. *Biomaterials* 2003;**24:**2339–2349.
14. Athanasiou KA, Shah AR, Hernandez RJ, LeBaron RG. Basic science of articular cartilage repair. *Clin Sports Med* 2001; **20:**223–247.
15. Dornish M, Kaplan D, Skaugrud O. Standards and guidelines for biopolymers in tissue-engineered medical products: ASTM alginate and chitosan standard guides. American Society for Testing and Materials. *Ann NY Acad Sci* 2001;**944:**388–397.
16. Hu Q, Li B, Wang M, Shen J. Preparation and characterization of biodegradable chitosan/hydroxyapatite nanocomposite rods via in situ hybridization: a potential material as internal fixation of bone fracture. *Biomaterials* 2004;**25:**779–785.
17. Risbud MV, Bhonde RR. Polyacrylamide-chitosan hydrogels: in vitro biocompatibility and sustained antibiotic release studies. *Drug Deliv* 2000;**7:**69–75.
18. Chow KS, Khor E. Novel fabrication of open-pore chitin matrixes. *Biomacromolecules* 2000;**1:**61–67.
19. Senel S, Ikinci G, Kas S, Yousefi-Rad A, Sargon MF, Hincal AA. Chitosan films and hydrogels of chlorhexidine gluconate for oral mucosal delivery. *Int J Pharm* 2000;**193:**197–203.
20. Needleman IG, Smales FC, Martin GP. An investigation of bioadhesion for periodontal and oral mucosal drug delivery. *J Clin Periodontol* 1997;**24:**394–400.
21. Lin DM, Kalachandra S, Valiyaparambil J, Offenbacher S. A polymeric device for delivery of anti-microbial and anti-fungal drugs in the oral environment: effect of temperature and medium on the rate

of drug release. *Dent Mater* 2003;**19:**589–596.
22. Mi FL, Shyu SS, Chen CT, Schoung JY. Porous chitosan microsphere for controlling the antigen release of Newcastle disease vaccine: preparation of antigen-adsorbed microsphere and in vitro release. *Biomaterials* 1999;**20:**1603–1612.
23. Park YJ, Lee JY, Yeom HR *et al*. Injectable polysaccharide microcapsules for prolonged release of minocycline for the treatment of periodontitis. *Biotechnol Lett* 2005;**27:**1761–1766.
24. Chung TW, Lu YF, Wang SS, Lin YS, Chu SH. Growth of human endothelial cells on photochemically grafted Gly-Arg-Gly-Asp (GRGD) chitosans. *Biomaterials* 2002;**23:**4803–4809.
25. Flemmig TF, Weinacht S, Rudiger S, Rumetsch M, Jung A, Klaiber B. Adjunctive controlled topical application of tetracycline HCl in the treatment of localized persistent or recurrent periodontitis. Effects on clinical parameters and elastase-alpha1-proteinase inhibitor in gingival crevicular fluid. *J Clin Periodontol* 1996; **23:**914–921.
26. Odim J, Laks H, Allada V, Child J, Wilson S, Gjertson D. Results of aortic valve-sparing and restoration with autologous pericardial leaflet extensions in congenital heart disease. *Ann Thorac Surg* 2005; **80:**647–653; discussion 644–653.
27. Myken PS. Seventeen-year experience with the St Jude medical biocor porcine bioprosthesis. *J Heart Valve Dis* 2005;**14:**486–492.
28. Lu HK, Lee SY, Lin FP. Elastic modulus, permeation time and swelling ratio of a new porcine dermal collagen membrane. *J Periodont Res* 1998;**33:**243–248.
29. Jameela SR, Jayakrishnan A. Glutaraldehyde cross-linked chitosan microspheres as a long acting biodegradable drug delivery vehicle: studies on the in vitro release of mitoxantrone and in vivo degradation of microspheres in rat muscle. *Biomaterials* 1995;**16:**769–775.
30. Jameela SR, Kumary TV, Lal AV, Jayakrishnan A. Progesterone-loaded chitosan microspheres: a long acting biodegradable controlled delivery system. *J Control Release* 1998;**52:**17–24.
31. Jameela SR, Misra A, Jayakrishnan A. Cross-linked chitosan microspheres as carriers for prolonged delivery of macromolecular drugs. *J Biomater Sci Polym Ed* 1994;**6:**621–632.
32. Nishi C, Nakajima N, Ikada Y. In vitro evaluation of cytotoxicity of diepoxy compounds used for biomaterial modification. *J Biomed Mater Res* 1995;**29:**829–834.
33. Speer DP, Chvapil M, Eskelson CD, Ulreich J. Biological effects of residual glutaraldehyde in glutaraldehyde-tanned collagen biomaterials. *J Biomed Mater Res* 1980;**14:**753–764.
34. Liu LS, Liu SQ, Ng SY, Froix M, Ohno T, Heller J. Controlled release of interleukin-2 for tumor immunotherapy using alginate/chitosan porous microspheres. *J Control Release* 1997;**43:**65–74.
35. Polk A, Amsden B, De Yao K, Peng T, Goosen MF. Controlled release of albumin from chitosan-alginate microcapsules. *J Pharm Sci* 1994;**83:**178–185.
36. Kawashima Y, Handa T, Kasai A, Takenaka H, Lin SY. The effects of thickness and hardness of the coating film on the drug release rate of theophylline granules coated with chitosan-sodium tripolyphosphate complex. *Chem Pharm Bull (Tokyo)* 1985;**33:**2469–2474.
37. Kawashima Y, Handa T, Kasai A, Takenaka H, Lin SY, Ando Y. Novel method for the preparation of controlled-release theophylline granules coated with a polyelectrolyte complex of sodium polyphosphate-chitosan. *J Pharm Sci* 1985;**74:**264–268.
38. Aydin Z, Akbuga J. Chitosan beads for the delivery of salmon calcitonin: preparation and characteristics. *Int J Pharm* 1996; **131:**101–103.
39. Calvo P, Remunan-Lopez C, Vila-Jato JL, Alonso MJ. Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. *Pharm Res* 1997;**14:**1431–1436.
40. Calvo P, Remuñán-López C, Vila-Jato JL, Alonso MJ. Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. *J Appl Polym Sci* 1998; **63:**125–132.
41. Sezer AD, Akbuga J. Controlled release of piroxicam from chitosan beads. *Int. J. Pharm*. 1995;**121:**113–116.
42. Shu XZ, Zhu KJ. A novel approach to prepare tripolyphosphate/chitosan complex beads for controlled release drug delivery. *Int J Pharm* 2000;**201:**51–58.
43. Baker PJ, Evans RT, Slots J, Genco RJ. Susceptibility of human oral anaerobic bacteria to antibiotics suitable for topical use. *J Clin Periodontol* 1985;**12:**201–208.
44. Gordon J, Walker C, Hovliaras C, Socransky S. Efficacy of clindamycin hydrochloride in refractory periodontitis: 24-month results. *J Periodontol* 1990;**61:** 686–691.
45. Miyake Y, Tsuruda K, Okuda K, Widowati, Iwamoto Y, Suginaka H. In vitro activity of tetracyclines, macrolides, quinolones, clindamycin and metronidazole against periodontopathic bacteria. *J Periodont Res* 1995;**30:**290–293.
46. Ishihara M, Ono K, Sato M *et al*. Acceleration of wound contraction and healing with a photocrosslinkable chitosan hydrogel. *Wound Repair Regen* 2001;**9:**513–521.
47. Guo JF, Jourdian GW, MacCallum DK. Culture and growth characteristics of chondrocytes encapsulated in alginate beads. *Connect Tissue Res* 1989;**19:**277–297.
48. Kawase M, Michibayashi N, Nakashima Y, Kurikawa N, Yagi K, Mizoguchi T. Application of glutaraldehyde-crosslinked chitosan as a scaffold for hepatocyte attachment. *Biol Pharm Bull* 1997;**20:**708–710.
49. Koyano T, Minoura N, Nagura M, Kobayashi K. Attachment and growth of cultured fibroblast cells on PVA/chitosan-blended hydrogels. *J Biomed Mater Res* 1998;**39:**486–490.
50. Lee YM, Park YJ, Lee SJ *et al*. Tissue engineered bone formation using chitosan/tricalcium phosphate sponges. *J Periodontol* 2000;**71:**410–417.
51. Zhang Y, Zhang M. Three-dimensional macroporous calcium phosphate bioceramics with nested chitosan sponges for load-bearing bone implants. *J Biomed Mater Res* 2002;**61:**1–8.
52. Zhao F, Yin Y, Lu WW *et al*. Preparation and histological evaluation of biomimetic three-dimensional hydroxyapatite/chitosan-gelatin network composite scaffolds. *Biomaterials* 2002;**23:**3227–3234.
53. Anitua E. Plasma rich in growth factors: preliminary results of use in the preparation of future sites for implants. *Int J Oral Maxillofac Implants* 1999;**14:**529–535.
54. Klokkevold PR, Vandemark L, Kenney EB, Bernard GW. Osteogenesis enhanced by chitosan (poly-N-acetyl glucosaminoglycan) in vitro. *J Periodontol* 1996; **67:**1170–1175.
55. Mizuno K, Yamamura K, Yano K *et al*. Effect of chitosan film containing basic fibroblast growth factor on wound healing in genetically diabetic mice. *J Biomed Mater Res* 2003;**64A:**177–181.
56. Park JS, Choi SH, Moon IS, Cho KS, Chai JK, Kim CK. Eight-week histological analysis on the effect of chitosan on surgically created one-wall intrabony defects in beagle dogs. *J Clin Periodontol* 2003; **30:**443–453.
57. Chou TC, Fu E, Shen EC. Chitosan inhibits prostaglandin E2 formation and cyclooxygenase-2 induction in lipopolysaccharide-treated RAW 264.7 macrophages. *Biochem Biophys Res Commun* 2003;**308:**403–407.