

Research Article

Physicochemical Characterization and Microbiological Evaluation of Tetracycline HCl Loaded Chitosan Beads for Periodontitis Treatment: A Preliminary Study

Rukiye Sevinç Özakar¹, Yeşim Aktaş^{2*}, Dilek Bilici³ and Ahmet Ayyıldız⁴

¹Department of Pharmaceutical Technology, Faculty of Pharmacy, Atatürk University, 25240 Erzurum, Turkey

²Department of Pharmaceutical Technology, Faculty of Pharmacy, Erciyes University, 38039 Kayseri, Turkey

³Department of Microbiology, Regional Training and Research Hospital, 25240 Erzurum, Turkey

⁴Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Atatürk University, 25240 Erzurum, Turkey

*Corresponding author: Yeşim Aktaş, Department of Pharmaceutical Technology, Faculty of Pharmacy, Erciyes University, 38039 Kayseri, Turkey, Tel: +90 3524380486; Fax: +90 3524379169; E-mail: yaktas@erciyes.edu.tr; aktasyesim@yahoo.com

Received Date: 21 August, 2017; Accepted Date: 14 September, 2017; Published Date: 29 September, 2017

Abstract

The aim of this study was to prepare and characterize tetracycline HCl loaded chitosan beads and to test their antimicrobial potential against different bacteria which are known to exist in periodontitis and susceptible to tetracycline HCl. Tetracycline HCl is a bacteriostatic and broad-spectrum antibiotic with low toxicity. Tetracycline HCl-loaded chitosan beads were prepared by ionotropic gelation method. Encapsulation efficiency, practical loading, yield, particle size and release characteristics of chitosan beads were determined. Finally, blank and tetracycline HCl loaded chitosan beads were evaluated for their antimicrobial activity against *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603. Spherical beads were formed spontaneously upon the incorporation of chitosan solution to the tripolyphosphate solution under magnetic stirring. Encapsulation efficiency and practical loading were well correlated with the initial tetracycline HCl concentration and the results are $85.121\% \pm 1.315\%$ and $45.957\% \pm 12.428\%$, respectively. The yield was found to be $35.860\% \pm 7.368\%$. Particle size of tetracycline HCl loaded chitosan beads ($1859.24 \mu\text{m} \pm 6.17 \mu\text{m}$ and $1134.27 \mu\text{m} \pm 14.7 \mu\text{m}$ for wet and dry beads, respectively) was significantly higher than that of the blank chitosan beads. Approximately 40% of the encapsulated tetracycline HCl was released in 12 hours. According to the zone diameter of growth inhibition for the bacteria, tetracycline HCl and chitosan displayed similar antimicrobial activity against

Pseudomonas aeruginosa and *Klebsiella pneumoniae* when the highest concentration of release samples was administered. *Staphylococcus aureus* and *Escherichia coli* were susceptible for tetracycline HCl loaded chitosan beads. Our data not only showed the suitability of the chitosan beads as a carrier for the continuous release of tetracycline HCl, but also they preserved their antimicrobial activity for a long time. This new tetracycline HCl loaded chitosan beads suggest a safe and effective therapy by means of providing an efficient drug concentration in a controlled manner.

Keywords

Chitosan Bead; Ionotropic Gelation; Periodontitis; Tetracycline HCl

Introduction

Dental diseases are recognised as one of the major and most common diseases afflicting humankind throughout the world. Dental diseases are common in all age groups, ethnicities, races, genders and socioeconomic level [1]. Periodontal disease has been considered as a possible risk factor in other systemic diseases such as cardiovascular disease, including coronary heart disease and stroke, diabetes and pre-term low birth weight infants [2,3]. Approximately \$5 billion is spent on treatment of periodontal diseases each year [4]. Periodontal disease is a collective term for a number of pathological conditions characterized by inflam-

mation and degeneration of the gums (Gingiva), supporting bone (Alveolar bone), periodontal ligament and cementum. Periodontitis is an inflammation of the supporting tissue surrounding teeth caused by anaerobic bacteria and in the diseased state, supporting collagen of the periodontium is destroyed and the alveolar bones begin to resorb. The epithelium of the gingiva migrates along the tooth surface forming 'periodontal pockets' that provide an ideal environment for the growth and proliferation of microbes. More severe stages of the disease lead to the loosening and ultimately loss of teeth. The importance of bacteria in the aetiology of periodontitis has been clearly established and the treatment is directed towards controlling the bacterial flora in the periodontal pocket [1,5].

Tetracycline HCl (TC) is a bacteriostatic and broad-spectrum antibiotic with a low toxicity that is produced by *Streptomyces rimosus* [6,7]. As they are effective on many periodontopathogens and inhibit collagenase enzyme, tetracyclines are widely used for the treatment of periodontal diseases [8]. They differ from other antibiotics since their concentration in gingival fluid is 2-10 folds compared to blood. Additionally, tetracyclines chelate with calcified tissues and this allows a sustained drug release which is very advantageous for antimicrobial activity in periodontal site and help bone regeneration by the inhibition of alveolar bone resorption [9].

Systemic antibiotic administration for the treatment of periodontitis have some drawbacks including: inadequate antibiotic concentration at the site of the periodontal pocket; a rapid decline of the plasma antibiotic concentration to sub-therapeutic levels; the development of microbial resistance due to sub-therapeutic drug levels and peak-plasma antibiotic concentrations which may be associated with various side effects, such as hypersensitivity, gastrointestinal intolerance and drug interactions with alcohol. All these disadvantages have provoked researchers to develop localised drug delivery systems to provide an effective antibiotic concentration at the site of action for the duration of the treatment with minimal side effects. Since the pathogen-specific drug can be placed directly in the periodontal pocket achieving effective concentrations, the local drug administration is considered to be more effective compared to systemic drug delivery in periodontology. Additionally undesired side effects caused by high systemic doses or resistance development can be reduced. For effective elimination of pathogenic bacteria, the antibiotic agent has to be available in the periodontal pocket in adequate concentrations for a sufficiently long period of time. It is therefore necessary to use local delivery systems that control the release of their agents and guarantee lasting drug concentrations in the pocket in spite of high sulcular fluid rates [10]. Localised controlled release drug delivery system for insertion into and/or around the periodontal pocket include some advantages such as increased local drug concentration at the periodontal site to maintain an effective concentration of antibiotic; a decrease in superfluous distribution of the drug to non-target organs, a decrease in the amount of administered dose, a subsequent decrease in side effects, a decrease in manufacturing costs, the maintenance of drug levels in a therapeutic range for an extended period of time. All these advantages can lead to improved patient compliance due to the reduction in the frequency of

administration of doses and decreased side effects. A pre-requisite for drug delivery systems for localised periodontal therapy is therefore retention on the mucosal surface and controlled drug release at the site of action [1,11,12].

Chitosan (CS) is a wide spectrum of antimicrobial agent against Gram-positive and Gram-negative bacteria whereas it has lower toxicity toward mammalian cells. This antimicrobial activity varies according to some factors; microorganism specie and cell age; intrinsic factors of chitosan, including positive charge density, molecular weight, concentration, hydrophilic/hydrophobic characteristic and chelating capacity; physical state, namely water-soluble and solid state of chitosan; and environmental factors, involving ionic strength in medium, pH, temperature and reactive time [13]. CS is the N-deacetylated derivative of chitin. Chitin is the second abundant and the second most important natural polysaccharide after cellulose [14,15]. It is the principal component of protective cuticles of crustaceans such as crabs, shrimps, prawns, lobsters and cell walls of some fungi such as *Aspergillus* and *Mucor* [15,16]. Chitin is a straight homopolymer composed of β -(1,4)-linked N-acetyl-glucosamine units with a three-dimensional α -helical configuration [14,15]. Partial deacetylation of chitin results in the production of CS, which is a polysaccharide comprising copolymers of glucosamine and N-acetylglucosamine. CS is a polysaccharide comprising copolymers of glucosamine (β -(1-4)-linked 2-amino-2-deoxy-D-glucose) and N-acetylglucosamine (2-acetamido-2-deoxy-D-glucose) and can be derived by partial deacetylation of chitin [17]. Properties such as biodegradability, low toxicity and good biocompatibility make it suitable for use in biomedical and pharmaceutical formulations [15]. Besides its favorable properties, CS has attracted attention in dental applications due to its antimicrobial activity and capacity of extended retention time on the oral mucosa [18,19]. Chitosan based systems have previously been investigated for local delivery to the oral cavity of therapeutics including tetracycline, chlorhexidine, triclosan and fluoride [11,20-22]. Like other CS-based biomaterials, CS beads provide desirable concentrations of drugs locally for a prolonged period of time. The use of complexation between the oppositely charged macromolecules to prepare CS beads (or microspheres) as controlled drug release formulation, especially for instable molecules has attracted much attention because this process is very simple and mild [23]. In addition, reversible physical cross-linking by electrostatic interaction instead of chemical cross-linking is applied to avoid possible toxicity of reagents and other undesirable effects [23-26].

In ionotropic gelation, CS is dissolved in acetic acid or water and CS cations are formed. CS solutions are extruded dropwise through a needle into different concentrations of aqueous solutions of magnetically stirred TPP. The beads are removed from the counter ion solution by filtration, washed with distilled water, dried by an air jet and further air dried at ambient temperature [14,15]. Tripolyphosphate (TPP) is a non-toxic and multivalent polyanion which can interact with CS via electrostatic forces to form ionic cross-linked networks. It can form a gel by ionic interaction with the positively charged amino groups of CS. TPP can be used for the preparation of CS beads and microspheres because of its quickly gelling ability [27,28].

Main objective of this study was to minimize the administration frequency and the dose of TC in order to increase patient compliance. We aimed to prepare CS beads for intraperiodontal administration which could potentially act as a reservoir for TC. Thus, the aim of this study was to prepare Tetracycline HCl-loaded Chitosan (TC-CS) beads which provide a controlled drug release in periodontal site and to identify optimal formulation parameters.

Materials and Methods

Materials

Chitosan (Protasan UP CL-213, FMC Biopolymers, NORWAY), tripolyphosphate (Sigma Chemical Co., USA), and Mueller-Hinton Agar I (Oxoid, UK) were purchased. Tetracycline HCl was a generous gift from ADEKA İlaç San. ve Tic. A.Ş., TURKEY. Ultrapure water (Barnstead, USA) was used throughout the studies. All other chemicals used were of analytical or pharmaceutical grade.

Preparation of chitosan beads

CS beads were prepared according to the ionotropic gelation process [15,29]. For the preparation of CS (blank) beads, CS was

dissolved in deionized water and bubble free CS solution (2%) was added dropwise into TPP solution (2%) by 26-G syringe under magnetic stirring at 700 rpm. The formation of the beads was a result of the interaction between the negative groups of TPP and the positively charged amino groups of CS. The concentrations of the CS and TPP solutions for bead formation were established according to the preliminary studies. 10 mL of TPP solution was used whereas the volume of the CS solution varied for some formulations in order to obtain spherical beads. The same procedure was applied for TC-CS beads except the incorporation of TC into the CS solution prior to the addition into the TPP solution. Variable amounts of TC were incorporated to the CS solution prior to the formation of beads in order to investigate the effect of the initial TC concentration on the bead characteristics and *in vitro* release profiles. Bead suspension was incubated for 25 minutes for a complete interaction between CS and TPP. Then, beads were filtered and dried. Formulation variables of the beads are shown in table 1.

Characterization of beads

Particle size distribution: Mean diameter [$\mu\text{m} \pm$ Standard Deviation (SD)] values of the CS and TC-CS beads, were determined by Quasi-elastic light scattering technique using Malvern Master-

Formulation code	CS solution (mL)	TC amount (mg)	Stirring time for CS+TC (minutes)	Stirring time for CS+TPP (minutes)
F1	1	10	85	60
F2	1	20	90	60
F3	1	40	85	60
F4	1	50	120	60
F5	1	60	120	60
F6	1.8	90	120	60
F7	2	100	120	60

Table 1: Formulation variables of the beads.

sizer (Malvern Instruments, Worcestershire, UK). Particle size measurements were performed for freshly prepared beads both in wet and dried form as well as after the release studies.

Drug quantity of beads: Determination of the entrapped drug quantity was performed by separation of beads from the aqueous suspension by filtration and centrifugation of the filtrate at +4°C and 10,000 rpm for 20 minutes to obtain a clear solution containing the free TC which is not entrapped. The amount of free TC in the supernatant was determined spectrophotometrically ($\lambda_{\text{max}}=357$ nm, calibration curve equation is $y=0.0325x + 0.0005$ and determination coefficient is $R^2=1$). Encapsulation Efficiency (EE) values were calculated according to the following equation ($n=30$): $EE (\%)=100 \times (\text{Total TC} - \text{Free TC})/\text{Total TC}$. As well as, practical loading and yield results were calculated.

In vitro TC release from beads: An incubation method was used for the investigation of TC release from beads. The *in vitro* release studies of TC-CS beads were carried out at 37°C and 50 rpm for 12 hours by using a shaking water bath. Dry bead formulations were resuspended in 50 mL of pH 7.4 Phosphate Buffer Saline solution (PBS) as the release medium under sink conditions. At

predetermined time intervals (10, 20, 30, 45, 60,...720 minutes), 1 mL of samples were taken and 1 mL of fresh medium was added after each sampling. All samples were assayed spectrophotometrically. The amount of released drug was calculated by using calibration curve of TC ($n=4$).

Antimicrobial activity of beads: For the antimicrobial activity study, CS and TC-CS beads release samples and free TC solution were tested against *Staphylococcus aureus* ATCC 29213 (*S. aureus*), *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*), *Escherichia coli* ATCC 25922 (*E. coli*) and *Klebsiella pneumonia* ATCC 700603 (*K. pneumonia*) using agar well diffusion method [30]. Release samples were collected from the *in vitro* release study of CS and TC-CS beads at different time intervals (10, 20, 30, 45, 60,...720 minutes). TC solution was prepared in a concentration of 30 $\mu\text{g}/\text{mL}$. Suspensions of each bacterium equal to 0.5 McFarland turbidity was streaked on to Muller-Hinton agar, then 80 μL of each solutions were added to each well of 5 mm diameter that were cut into the agar equally spaced each other. The plates were then incubated for 24 hours at $37 \pm 0.5^\circ\text{C}$. After the incubation period, the diameter (mm) of zone of growth inhibition surrounding each well was measured [31].

Results and Discussion

Ionotropic gelation method is a simple process that involves the interaction of an ionic polymer with oppositely charged cross-linkers like TPP to form spherical beads. The use of aqueous solvents while the preparation of beads is eliminated environmental problems associated with organic solvents. Natural polymers and their derivatized products is successfully used for various pharmaceutical applications. Compared with many other natural polymers, chitosan is positively charged and have spontaneous mucoadhesive property [32].

In the preformulation studies, spherical beads were formed spontaneously upon the incorporation of CS solution to the TPP

solution under magnetic stirring (Figure 1). However, some formulations had a tendency to loose there. It was seen that the F4-coded beads had the most stable form when the bead structure was evaluated. In F6 and F7-coded beads, the chitosan amount was increased and then the structure was deformed. This may be attributed to chitosan amount that the TPP amount was low and there was not enough interaction with chitosan. In Govender et al.'s study [33], they used sodium sulphate as a cross-linking agent, resulting in a weak interaction with chitosan and deformation in the microsphere's structure. Our cross-linking time was 60 minutes for bead formation. In Shu and Zhu's study [25], chitosan beads were prepared with sodium sulphate, sodium citrate and TPP and cross-linking time for sodium sulphate and sodium citrate was 30 minutes and 60 minutes for TPP [23].

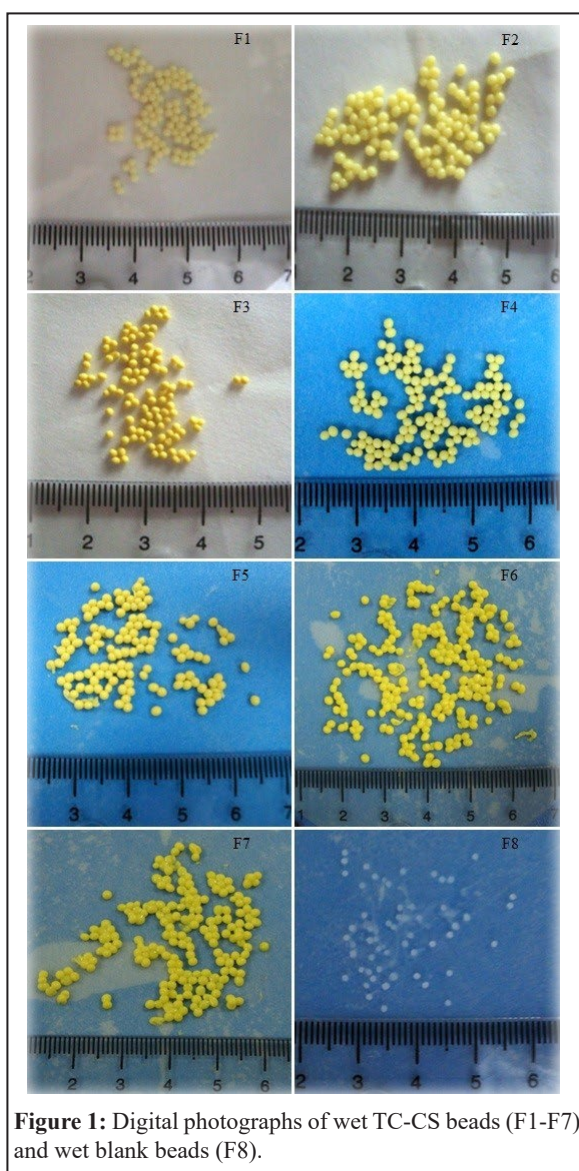


Figure 1: Digital photographs of wet TC-CS beads (F1-F7) and wet blank beads (F8).

Particle size of the TC-CS beads was significantly higher than that of the blank CS beads. Thus, it can be concluded that the size of beads increased, when TC was encapsulated as a result of an interaction between CS and TC. It is noteworthy that the particle size decreased significantly after the drying process of the beads due to the high swelling capacity of CS. The mean diameter of blank

beads was about $85.63 \mu\text{m} \pm 1.62 \mu\text{m}$ and $33.99 \mu\text{m} \pm 0.24 \mu\text{m}$, respectively in wet and dry form; whereas the mean diameter of the drug loaded beads was $1859.24 \mu\text{m} \pm 14.70 \mu\text{m}$ and $1134.27 \mu\text{m} \pm 6.17 \mu\text{m}$, respectively and $1372.67 \mu\text{m} \pm 17.78 \mu\text{m}$ after the in vitro release studies suggesting a possible erosion from the bead surface table 2. A narrow size distribution was achieved in all formulations.

Blank		Drug loaded		
Wet	Dry	Wet	Dry	After release
85.63 ± 1.62	33.99 ± 0.24	1859.24 ± 14.70	1134.27 ± 6.17	1372.67 ± 17.78

Table 2: Particle size of blank and drug loaded beads (F4-coded beads, $\mu\text{m} \pm$ standard deviation).

EE and practical drug loading were well correlated with the initial TC concentration. The increase in the incorporated TC amount resulted in higher encapsulation efficiency and drug loading as seen in table 3. Our results were in inconsistency with the findings

of Bodmeier et al., [29], Govender et al., [33], Srinatha et al., [34] and Avadi et al., [35]. On the other hand, as the incorporated TC amount increased above 100 mg, a fast deformation in the beads was observed.

Formulation code	Theoretical drug loading (mg/mL)	Practical drug loading (mg/mL)	EE (%)
F1	0.909	0.415	45.69
F2	1.818	1.215	66.81
F3	3.636	2.987	82.15
F4	4.545	3.879	85.36
F5	5.454	4.783	87.7
F6	7.627	6.724	88.15
F7	8.333	7.471	89.66

Table 3: Theoretical drug loading, practical drug loading and EE values of various bead formulations.

Particle size and morphology of the beads are crucial for the application in the periodontal environment. Spherical shape of the beads is dependent on the viscosity of CS solution and the concentration of TPP solution. Avadi et al., [35] reported that, when a 1% CS solution was used, no beads were formed. However, smooth beads were obtained upon dropping 1.5 and 2% CS solution into different concentrations of TPP solutions. Similar results were observed by Alsarra et al., [36]. The values for EE, practical loading and yield of F4-coded beads are summarized in table 4.

EE (%)	Practical loading (%)	Yield (%)
85.121	45.957	35.86
1.315	12.428	7.368

Table 4: EE, practical loading and yield values of F4-coded beads (mean \pm standard deviation).

As seen in (Figure 2), approximately 40% of the encapsulated TC was released in 12 hours. This release profile was similar with the data of Govender et al., [11]. TC is a freely soluble drug, its release behaviour is strongly affected by bead structure and swelling degree. Our TC-CS beads did not display significant swelling after the 12 hours release study table 2. After a period of 12 hours, the release medium and the TC-loaded beads started to turn brown indicating a chemical degradation of TC. This is supported with the stability testing on TC carried out by Wu et al., [37].

Although TC is well known to be effective against many Gram-positive and Gram-negative bacteria [7], bacteria may become resistant

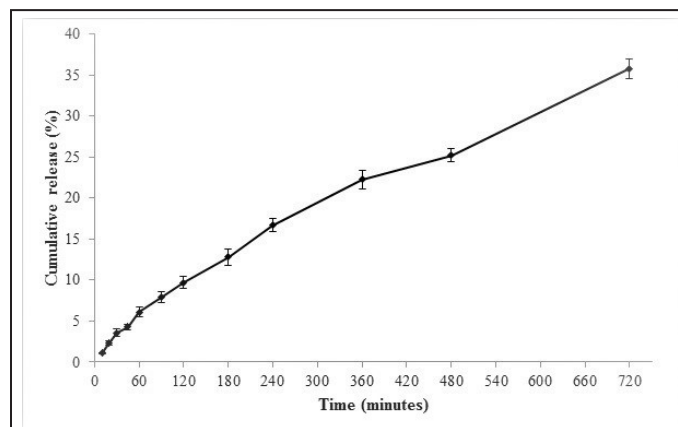


Figure 2: Release profile of F4-coded beads.

by the time. In this study, antimicrobial activity of encapsulated TC against *S. aureus*, *P. aeruginosa*, *E. coli* and *K. pneumonia* were evaluated. *S. aureus* is reported to exist in aggressive and chronic periodontitis patient. *S. aureus* and *E. coli* are known to be susceptible to TC [38-40]. In Reddy et al.'s study [40], the CaSO_4 -TC nanoparticles-composite beads displayed antibacterial activity against both gram-positive *S. aureus* as well as gram-negative *E. coli*. The main mechanism of action of TC in *S. aureus* and *E. coli* corresponds to the immediately widespread impairment of protein synthesis by interfering with the 30S ribosomal subunit of these bacteria. In addition, bacterial cell division also stopped in the presence of TC [40]. de Oliveira et al., [41] isolated *P. aeruginosa* from many periodontitis patients. According to the

diameters (mm) of zone of growth inhibition for the bacteria (Table 5) TC and CS displayed similar antimicrobial activity against *P. aeruginosa* and *K. pneumonia* when the highest concentration of release samples was administered. *S. aureus* and *E. coli* were susceptible to TC released from the

CS beads obtained from the *in vitro* release study, indicating that a continuous release of TC was obtained for 12 hours. Further studies are needed to evaluate antibacterial activity against specific periopathogens such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*.

Release Sampling Time (minutes)	Zone Diameter			
	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumonia</i>
10	13 ± 1.73	0 ± 0	0 ± 0	0 ± 0
20	14.7 ± 1.53	0 ± 0	0 ± 0	0 ± 0
30	16.3 ± 1.16	5 ± 8.66	0 ± 0	0 ± 0
45	18.7 ± 1.16	5 ± 8.66	0 ± 0	0 ± 0
60	20 ± 1	13.3 ± 2.08	0 ± 0	0 ± 0
90	22 ± 1	17.3 ± 1.53	0 ± 0	0 ± 0
120	22 ± 1	18.7 ± 1.16	0 ± 0	0 ± 0
180	23 ± 0	20.3 ± 1.53	0 ± 0	0 ± 0
240	23.7 ± 0.58	21.3 ± 2.31	0 ± 0	0 ± 0
360	24.3 ± 0.58	22.3 ± 2.08	3.7 ± 6.35	0 ± 0
480	24.7 ± 0.58	23 ± 2.65	4 ± 6.93	0 ± 0
720	25.7 ± 0.58	26 ± 2.65	11 ± 9.64	4 ± 6.93

Table 5: Median inhibition zone diameters of the antibacterial activity of test samples against four different bacteria (n=3, mm ± standard deviation).

As a conclusion, a polymeric system intended for the periodontal delivery of TC was developed and characterized for various parameters, including particle size, EE and practical drug loading. The beads displayed a sustained release profile up to 12 hours. TC has been used in the treatment of periodontal diseases for a long time and its short residence time is an important drawback. This new tetracycline loaded-chitosan beads suggest a safe and effective therapy for periodontitis by means of providing an efficient drug concentration in a controlled manner. However, *in vivo* data is needed for the demonstration of antimicrobial activity in periodontitis cases. Our data not only showed the suitability of the CS beads as a carrier for the continuous release of TC, but also that they preserved its antimicrobial activity for a long time. This new tetracycline loaded-chitosan beads suggest a safe and effective therapy for periodontitis by means of providing an efficient drug concentration in a controlled manner. However, *in vivo* data is needed for the demonstration of antimicrobial activity in periodontitis cases.

Acknowledgements

This research was supported by the Atatürk University Research Council (Project No: 2009/313).

References

- Vyas SP, Sihorkar V, Mishra V (2000) Controlled and targeted drug delivery strategies towards intraperiodontal pocket diseases. J Clin Pharm Ther 25: 21-42.
- Otomo-Corgel J, Pucher JJ, Rethman MP, Reynolds MA (2012) State

of the science: chronic periodontitis and systemic health. J Evid Based Dent Pract 12: 20-28.

- Southard GL, Godowski KC (1998) Subgingival controlled release of antimicrobial agents in the treatment of periodontal disease. Int J Antimicrob Agents 9: 239-253.
- Bonito AJ, Lux L, Lohr KN (2005) Impact of local adjuncts to scaling and root planing in periodontal disease therapy: a systematic review. J Periodontol 76: 1227-1236.
- Schwach-Abdellaoui K, Vivien-Castioni N, Gurny R (2000) Local delivery of antimicrobial agents for the treatment of periodontal diseases. Eur J Pharm Biopharm 50: 83-99.
- MacDougall C, Chambers HF (2011) Protein Synthesis Inhibitors and Miscellaneous Antibacterial Agents. In: Brunton LL, Chabner BA, Knollmann BC (eds.). Goodman-Gilman's The Pharmacological Basis of Therapeutics. (12th edn), The McGraw-Hill Companies. USA. Pg no: 1522-1527.
- [No Authors Listed] (2012) Antibacterials. In: Sweetman SC (ed.). Martindale: The Complete Drug Reference. (36th edn), The Pharmaceutical Press. UK. Pg no: 347-350.
- Sapadin AN, Fleischmajer R (2006) Tetracyclines: nonantibiotic properties and their clinical implications. J Am Acad Dermatol 54: 258-265.
- Etienne D (2003) Locally delivered antimicrobials for the treatment of chronic periodontitis. Oral Dis 9: 45-50.
- Reise M, Wyrwa R, Müller U, Zylinksi M, Völpe A, et al. (2012) Release of metronidazole from electrospun poly(L-lactide-co-D/L-lactide) fibers for local periodontitis treatment. Dent Mater 28: 179-188.
- Govender S, Pillay V, Chetty DJ, Essack SY, Dangor CM, et al. (2005) Optimisation and characterisation of bioadhesive controlled release tetracycline microspheres. Int J Pharm 306: 24-40.

12. İkinci G, Şenel S, Akıncıbay H, Kaş S, Erciş S, et al. (2002) Effect of chitosan on a periodontal pathogen *Porphyromonas gingivalis*. *Int J Pharm* 235: 121-127.
13. Kong M, Chen XG, Xing K, Park HJ (2010) Antimicrobial properties of chitosan and mode of action: a state of the art review. *Int J Food Microbiol* 144: 51-63.
14. Kas HS (1997) Chitosan: properties, preparations and application to microparticulate systems. *J Microencapsul* 14: 689-711.
15. Sinha VR, Singla AK, Wadhawan R, Kaushik R, Kumria R, et al. (2004) Chitosan microspheres as a potential carrier for drugs. *Int J Pharm* 274: 1-33.
16. Kumar MN, Muzzarelli RA, Muzzarelli C, Sashiwa H, Domb AJ (2004) Chitosan chemistry and pharmaceutical perspectives. *Chem Rev* 104: 6017-6084.
17. Baldrick P (2010) The safety of chitosan as a pharmaceutical excipient. *Regul Toxicol Pharmacol* 56: 290-299.
18. Uraz A, Boynueğri D, Özcan G, Karaduman B, Uç D, et al. (2012) Two percent chitosan mouthwash: A microbiological and clinical comparative study. *J Dent Sci* 7: 342-349.
19. Hayashi Y, Ohara N, Ganno T, Yamaguchi K, Ishizaki T, et al. (2007) Chewing chitosan-containing gum effectively inhibits the growth of cariogenic bacteria. *Arch Oral Biol* 52: 290-294.
20. Giunchedi P, Juliano C, Gavini E, Cossu M, Sorrenti M (2002) Formulation and *in vivo* evaluation of chlorhexidine buccal tablets prepared using drug-loaded chitosan microspheres. *Eur J Pharm Biopharm* 53: 233-239.
21. Kockisch S, Rees GD, Young SA, Tsibouklis J, Smart JD (2003) Polymeric microspheres for drug delivery to the oral cavity: an *in vitro* evaluation of mucoadhesive potential. *J Pharm Sci* 92: 1614-1623.
22. Keegan GM, Smart JD, Ingram MJ, Barnes LM, Burnett GR, et al. (2012) Chitosan microparticles for the controlled delivery of fluoride. *J Dent* 40: 229-240.
23. Shu XZ, Zhu KJ (2002) Controlled drug release properties of ionically cross-linked chitosan beads: the influence of anion structure. *Int J Pharm* 233: 217-225.
24. Kawashima Y, Handa T, Kasai A, Takenaka H, Lin SY, et al. (1985) Novel method for the preparation of controlled-release theophylline granules coated with a poly-electrolyte complex of sodium polyphosphate chitosan. *J Pharm Sci* 74: 264-268.
25. Shu XZ, Zhu KJ (2000) A novel approach to prepare tripolyphosphate/chitosan complex beads for controlled release drug delivery. *Int J Pharm* 201: 51-58.
26. Kawashima Y, Handa T, Kasai A, Takenaka H, Lin SY (1985) 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* 33: 2469-2474.
27. Anal AK, Stevens WF (2005) Chitosan-alginate multilayer beads for controlled release of ampicillin. *Int J Pharm* 290: 45-54.
28. Mi FL, Sung HW, Shyu SS, Su CC, Peng CK (2003) Synthesis and characterization of biodegradable TPP/genipin co-crosslinked chitosan gel beads. *Polymer* 44: 6521-6530.
29. Bodmeier R, Oh KH, Pramart Y (1989) Preparation and evaluation of drug-containing chitosan beads. *Drug Dev Ind Pharm* 15: 1475-1494.
30. Balouiri M, Sadiki M, Ibensouda SK (2016) Methods for *in vitro* evaluating antimicrobial activity: A review. *J Pharm Anal* 6: 71-79.
31. Holder IA, Boyce ST (1994) Agar well diffusion assay testing of bacterial susceptibility to various antimicrobials in concentrations non-toxic for human cells in culture. *Burns* 20: 426-429.
32. Racoviță Ș, Vasiliu S, Popa M, Luca C (2009) Polysaccharides based on micro and nanoparticles obtained by ionic gelation and their applications as drug delivery systems. *Revue Roumaine de Chimie* 54: 709-718.
33. Govender S, Lutchman D, Pillay V, Chetty DJ, Govender T (2006) Enhancing drug incorporation into tetracycline-loaded chitosan microspheres for periodontal therapy. *J Microencapsul* 23: 750-761.
34. Srinatha A, Pandit JK, Singh S (2008) Ionic cross-linked chitosan beads for extended release of ciprofloxacin: *In vitro* characterization. *Indian J Pharm Sci* 70: 16-21.
35. Avadi MR, Ghassemi AH, Sadeghi AMM, Erfan M, Akbarzadeh A, et al. (2004) Preparation and characterization of theophylline-chitosan beads as an approach to colon delivery. *Iran J Pharm Res* 2: 73-80.
36. Alsarra IA, Neau SH, Howard MA (2004) Effects of preparative parameters on the properties of chitosan hydrogel beads containing *Candida rugosa* lipase. *Biomaterials* 25: 2645-2655.
37. Wu Y, Fassihi R (2005) Stability of metronidazole, tetracycline HCl and famotidine alone and in combination. *Int J Pharm* 290: 1-13.
38. Fu J-X, Wang H-J, Zhou Y-Q, Wang J-Y (2009) Antibacterial activity of ciprofloxacin-loaded zein microsphere films. *Mater Sci Eng C* 29: 1161-1166.
39. Shen EC, Wang C, Fu E, Chiang CY, Chen TT, et al. (2008) Tetracycline release from tripolyphosphate-chitosan cross-linked sponge: a preliminary *in vitro* study. *J Periodontol Res* 43: 642-648.
40. Reddy NS, Sowmya S, Bumgardner JD, Chennazhi KP, Biswas R, et al. (2014) Tetracycline nanoparticles loaded calcium sulfate composite beads for periodontal management. *Biochim Biophys Acta* 1840: 2080-2090.
41. de Oliveira LF, Jorge AO, Dos Santos SS (2006) *In vitro* minocycline activity on superinfecting microorganisms isolated from chronic periodontitis patients. *Braz Oral Res* 20: 202-206.