Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 73 (2008) 201–211
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Preparation and characterization of chitosan microspheres for doxycycline delivery

S. Shanmuganathan [a,b,1], N. Shanumugasundaram [a,1], N. Adhirajan [a], T.S. Ramyaa Lakshmi [a], Mary Babu [a,*]

[a] *Biomaterials Division, Central Leather Research Institute, TICEL Biopark, Taramani Road, Taramani, Chennai 600 113, Tamil Nadu, India*
[b] *Department of Pharmaceutics, College of Pharmacy, Sri Ramachandra University, Porur, Chennai 600 116, Tamil Nadu, India*

Received 30 July 2007; received in revised form 5 November 2007; accepted 5 November 2007
Available online 2 February 2008

Abstract

Doxycycline-loaded chitosan microspheres were developed using a novel water-in-oil emulsion technique, involving oil phase ionic gelation. Microspheres were prepared by using 6% v/v of chitosan (3% w/v in acetic acid), soya oil–*n*-octanol oil mixture (1:2 v/v) as continuous phase and 5% span 80 as emulsifier. Doxycycline was entrapped by equilibrium swelling method with 8.4% total entrapment. The drug-loaded spheres were spherical with smooth surface morphology. The MTT assay showed that doxycycline-loaded microspheres were able to improve the percentage cell viability in comparison to the pure drug. *In vitro* release studies showed that a burst release of 42% in 6 h was achieved and maintained an equilibrium concentration of 72% in 24 h. Assessment of antibacterial activity showed that doxycycline was able to exhibit a minimum microbicidal concentration (MIC) of 16.5, 17.4, 11.2 and 98.3 μg against *Klebsiella pneumoniae* (ATCC 15380), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 9144) and *Pseudomonas aeruginosa* (ATCC 25619), respectively. Gelatin zymography studies revealed that it could inhibit MMP 2 and MMP 9 at sub-antimicrobial concentration. The present investigation provides scope for using doxycycline-loaded chitosan microspheres for healing infected wounds.

Keywords: Chitosan microspheres; Doxycycline; Biocompatibility; Fibroblasts; MMP

1. Introduction

Emergence of newer antibiotics to combat bacterial resistance has been effective in controlling pathogens. The broad spectrum of activity against various pathogens involves structural interaction with bacterial receptors or blocking the metabolic function of microbes. Doxycycline is a semi-synthetic antibiotic from *Streptomyces* species and possesses vicinal diols capable of binding with divalent ions and arresting the metabolic function. Doxycycline elicits its antimicrobial activity by preventing the addition of amino acids to growing peptide chains in bacteria (Lynn, 1996). Apart from antimicrobial properties doxycycline also acts as an inhibitor of matrix metalloproteinases (MMPs) (Nordstrom et al., 1998), which is attributed to its metal-binding property. Evidence from animal studies shows that treatment with doxycycline improves healing parameters, like increasing the tensile strength of rat intestinal anastomoses after surgery and reducing the incidence of ulceration in alkali-injured rabbit eyes (Siemonsma et al., 2003).

Doxycycline therapy at sub-antimicrobial dose has been shown to reduce periodontal disease activity by reducing MMPs and pro-inflammatory cytokines (Choi et al., 2004). Doxycycline inhibits host-derived MMPs by mechanisms independent of their antimicrobial properties. It is safe and effective at the dosages typically used in clinical practice and successfully tested in several conditions associated with elevated MMP activity. Doxycycline has been

* Corresponding author. Tel.: +91 44 22542490; fax: +91 44 24911589.
E-mail addresses: marybabu@hotmail.com, babumary2000@yahoo.com (M. Babu).
[1] These authors contributed equally to this work.

0144-8617/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2007.11.039

shown to reduce MMP activity in arthritis, periodontitis (Gapski et al., 2004), and aortic aneurysms (Manning, Cassis, & Daugherty, 2003). Adjunctive sub-antimicrobial dose doxycycline therapy can also improve the clinical parameters by controlling the level of MMP 8 in chronic adult periodontitis (Choi et al., 2004).

Recent reports have provided evidence that doxycycline acts as a potent inhibitor of MMPs in wound healing (Chin, Thigpin, Perrin, Moldawer, & Schultz, 2003), which increases the scope for it to be utilized as an antibiotic and also to modulate MMP levels. It is essential to deliver doxycycline at the intended therapeutic concentrations to the wound site to elicit its activity both during infection and healing stages. Recently, we have shown that the antibacterial agent, silver sulphadiazine, can be delivered in a controlled fashion at appropriate sites through microspheres developed with natural polymers (Shanmugasundaram, Sundaraseelan, Uma, Selvaraj, & Babu, 2006). In the current investigation we have chosen chitosan (poly (β-(1,4)-2-amino-2-deoxy-D-glucopyranose), a cationic polysaccharide, for its excellent mucoadhesive (Hirano, Seino, Akiyama, & Nonaka, 1988; Issa, Hoggard, & Arthurson, 2005) and biocompatibility properties. Chitosan is extensively used as a biomaterial to develop wound dressing in various forms like membranes, sponges and bi-layered dressings (Khan & Peh, 2003; Mi et al., 2001). These dressings are hydro-active, regulate oxygen transport, and prevent infection by delivering antibiotics (Aoyagi, Onishi, & Machida, 2007).

Chitosan spheres prepared using a covalent crosslinker like glutaraldehyde is available and widely used as a delivery system (Blanco, Gomez, Olmo, Muniz, & Teijon, 2000; Tahnoo, Sunny, & Jayakrishnan, 1992). Due to reported toxicity of glutaraldehyde, alternate methods of microsphere preparation like spray drying (Mi, Wong, Shyu, & Chang, 1999) and ionic gelation using Na_2SO_4 or tripolyphosphate are carried out (Berthod et al., 1994; Clavo, Vila-Jato, & Alonso, 1997).

In this study, chitosan microspheres were prepared by ionic gelation with KOH as crosslinking agent. The spheres were prepared by a w/o emulsion technique and are further utilized to encapsulate doxycycline. The drug-loaded microspheres were characterized for their morphological and biocompatibility properties. Release rate of doxycycline was analyzed using diffusion cell and its biocompatibility was assessed *in vitro* in human dermal fibroblasts.

2. Materials and methods

The following materials were obtained from Sigma Chemicals, USA: soya oil, chitosan (75–85% deacetylation, Mr: 90,000–100,000), span 80, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Collagenase (crude Type 1A), L-glutamine, Streptomycin, Penicillin, Gentamycin, Fungizone, α-naphthylamine, 4-Aminophenyl mercuricacetate (APMA), MMP 2 from human fibroblast (EC 3.4.24.24) and MMP 9 from human fibroblast (EC 3.4.24.35) as standard enzymes. Doxycycline hyclate and Calcein AM ester were obtained from Fluka, Switzerland and *n*-octanol was from Merck, Germany. Trypsin, Trypticase Soy Broth (TSB), Trypticase Soy Agar (TSA), Mueller Hinton Broth (MHB) and Mueller Hinton Agar (MHA) were obtained from HI-MEDIA, Mumbai, India. Microbial cultures *Pseudomonas aeruginosa* (ATCC 25619), *Staphylococcus aureus* (ATCC 9144), *Klebsiella pneumoniae* (ATCC 15380) and *Escherichia coli* (ATCC 25922) were obtained from IMTECH, Chandigarh, India. All other chemicals used in this work are of analytical grade.

2.1. Preparation of chitosan microspheres

Chitosan microspheres (CSM) were prepared by a novel water-in-oil (w/o) emulsification process along with an ionic coacervation technique. A w/o emulsion of chitosan was prepared by slowly adding 6 ml of 3% w/v chitosan (in 0.5 M acetic acid) to a mixed oil phase containing 30 ml of soya oil and 60 ml of *n*-octanol. Span 80 (5% v/v) was used as an emulsifier. The mixture was emulsified using a magnetic stirrer and an overhead stirrer simultaneously. The stirring speed of the overhead stirrer was maintained at 1600–1700 rpm while a magnetic stirrer was maintained at 1000 rpm throughout the process of microsphere preparation. Stirring was continued for 1 h until a stable w/o emulsion was obtained. Ionic gelation was initiated by slow, dropwise addition of 1% w/v of KOH (1.5 ml/15 min for 4 h) in *n*-octanol. After the crosslinking reaction, the oil phase of the mixture containing chitosan microspheres was slowly decanted and the spheres were immediately added to 100 ml of acetone. The washing was repeated twice with acetone until discrete, oil-free spheres were obtained. The whole process was carried out at room temperature. The recovered spheres were dried in a vacuum desiccator. The microspheres were optimized according to the process variables listed in Table 1.

2.2. Determination of particle size distribution and swelling ratio

A particle size analyzer (Malvern Mastersizer E – Laser, UK) was used to determine the particle size distribution of

Table 1
Variables involved in preparation of chitosan microspheres

S. No.	Process parameters	Variables involved
(1)	Soya oil:*n*-octanol ratio	2:1, 1:1, 1:2, 1:3, 1:4 & 1:5
(2)	Percentage of emulsifier (Span 80)	3%, 4%, 5%, 6% and 7% v/v
(3)	Percentage v/v of 3% w/v chitosan in 0.5 M acetic acid.	4%, 5%, 6% and 7% v/v
(4)	Overhead stirrer speed	1600, 1800, 2000 and 2200 rpm
(5)	Percentage v/v of KOH in *n*-octanol	1%, 2% and 3%

chitosan microspheres. The particles were analyzed at focal length of 300 mm by using isopropyl alcohol as a non-dissolving and non-reacting dispersion medium. The samples were stirred constantly using a magnetic cell stirrer until completion of analysis in order to maintain homogenous and discrete dispersion of the spheres. The swelling ratio of chitosan microspheres was determined using Malvern diffraction particle size analyzer (Malvern Mastersizer E – Laser, UK). To determine the swelling capacity of CSM, a known amount of microspheres (25 mg) was dispersed in deionized water and change in the size of microspheres at appropriate time intervals was determined. The measurements were carried out at 15 min time intervals for 1 h, then at every 1 h until 6 h, and then 12, 24 and 48 h. The swelling ratio was calculated from the ratio of size of swollen particles at various time intervals to that of dry spheres.

2.3. Doxycycline entrapment

Doxycycline was entrapped in chitosan microspheres (Doxy-CSM) by equilibrium swelling method. To the aqueous solutions of doxycycline (5 mg/ml), various amount of CSM were added in ratio of 1:5, 1:10, 1:15, 1:20% w/w of doxycycline: CSM and allowed to swell under mild rocking condition for 90 min after which the supernatant solution was decanted and the residual spheres were collected and dried using a centrifugal drier (SpeedVac, SPD 111v, Thermo Savant, USA) until constant weight was obtained. Percentage entrapment of doxycycline was determined by the following equation,

$$\%\ \text{Drug entrapment} = \frac{\text{Amount of drug in known amount of spheres}}{\text{Initial drug load}} \times 100$$

The amount of doxycycline was assessed spectrophotometrically (Perkin-Elmer lamda 45) at the wavelength of 267 nm by dissolving a known weight of loaded microspheres in 1 ml of 6 N HCL at 60 °C for 1 h. Before determining the drug concentration photometrically, the assay mixture was centrifuged at 4000 rpm for 15 min and supernatant was separated out from residual chitosan.

2.4. Morphological analysis

The morphological features of CSM and Doxy-CSM were assessed by both light microscopic and scanning electron microscopic (SEM) techniques. The light microscopic images were taken using LEICA DMIRB microscope, Leica Wetzlar, Germany to observe the bulk morphology. The ultra-structural features were analyzed by JEOL JSM-5610 series scanning electron microscope, equipped with electron optical system (EOS) consisting of 0.5–30 kV capacity electron gun and an electron detector. Before the samples were analyzed they were sputter coated with gold using a JEOL JFC-1600 Autofine coater.

2.5. In vitro release studies

In vitro release studies of free doxycycline and Doxy-CSM were carried out using Franz diffusion model finite dosage apparatus (Franz, 1978). Type I collagen was isolated from calf skin and fabricated into a thin membrane using the protocol as given in our earlier work (Shanmugasundaram, Ravikumar, & Babu, 2004) and used as a semi-permeable membrane. Known amounts of both the free drug and Doxy-CSM were spread over the collagen membrane, allowed to swell in the release medium and the subsequent release of the drug into the release compartment was determined. The reservoir solution contained synthetic serum electrolytic solution (SSES composed of 0.601 g of sodium chloride, 0.235 g of sodium bicarbonate, 0.0283 g of disodium hydrogen phosphate and 0.0284 g of sodium sulphate/100 ml). The temperature was maintained at 37 °C with constant stirring using magnetic stirrer (Spinot, Torrson, Mumbai). Aliquots of sample were withdrawn at regular time intervals 6–72 h and analyzed for percentage release of doxycycline spectrophotometrically at 267 nm.

2.6. Biocompatibility

Biocompatibility of Doxy-CSM was evaluated by assessing their ability to support the growth of dermal fibroblasts. Human dermal fibroblasts were derived from skin biopsies collected from patients undergoing cosmetic surgery as per our previous protocol (Kumar, Shanmugasundaram, & Babu, 2003). The study was conducted in accordance with the rules and regulation set out by the Ethical Committee of CLRI, and K.K Child Trust Hospital to deal with human samples. Briefly, tissues were washed thrice with PBS containing antibiotics and kept in trypsin (0.5 M) overnight at 4 °C. Epidermal layer was removed and the dermal layer was cut into pieces, incubated overnight at 37 °C with collagenase to release the cells from the tissues. The cell suspension was then centrifuged and the collected cell pellet was dispersed in a flask containing DMEM supplemented with 10% FCS and incubated at 37 °C in a humidified 5% CO_2, 95% air atmosphere. The fibroblasts grown to confluency was subcultured and the passage 3–5 was used for the study.

Fibroblasts were seeded (2×10^4 cells/well) into 24-well plate with 1 ml of culture medium and incubated (Thermoforama, USA) overnight for attachment of cells at 37 °C in 5% CO_2 and 95% air atmosphere. Doxy-CSM equivalent to the concentration of 100–500 μg of the drug and same concentration of doxycycline were added to the respective wells and incubation continued for 48 h. After incubation, 50 μl of MTT (3-3-(4,5-dimethylthiozole-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) was added to each well and incubated for 4 h at 37 °C (Mosmann, 1983). After which medium containing microspheres was removed gently and 500 μl of dimethylsulfoxide (DMSO) was added to solubilize the formazan complex

and read in a microplate reader (BIORAD, Model 680, USA) at 570 nm. The percent cell viability relative to control was calculated by [A] test/[A] control × 100. Control was maintained same as above but omitting doxycycline and Doxy-CSM. Values reported are the means of three determinations.

CSM was further evaluated for its ability to act as a scaffold for fibroblasts by allowing the cells to migrate into the microspheres and viewing the cells by calcein AM fluoresecent dye. CSM were added in duplicates to a 24-well plate containing fibroblasts (2×10^4 cells/ well) with 1 ml of culture medium and incubated for 96 h in 5% CO_2 and 95% air atmosphere. After which, to each well, 0.2 μM of calcein AM was added and incubated for 20 min. CSM were transferred into Eppendorf tube and excess of calcein AM was removed by washing with 500 μl of PBS. Few drops of CSM suspension in PBS was placed in cavity slide and viewed under phase contrast fluorescent microscope (Leica DMIRB, USA). Photomicrographs were taken to visualize the metabolically active cells inside the CSM using blue filter.

2.7. In vitro antibacterial activity of Doxy-CSM

Antibacterial activity of the drug-loaded spheres was examined against *P. aeruginosa* (ATCC 25619), *S. aureus* (ATCC 9144), *K. pneumoniae* (ATCC 15380) and *E. coli* (ATCC 25922). Initial inoculums of these strains were prepared by inoculating five colonies from fresh cultures (overnight cultured) in TSB for *S. aureus, K. pneumoniae* and *E. coli,* and in MHB for *P. aeruginosa*, incubated at 35 °C till logarithmic growth phase. From this culture, 100 μl of the sample was transferred to 10 ml of respective media and incubated at 35 °C to attain exponential logarithmic phase (≡0.5 McFarland). The cultures were appropriately diluted to produce 5×10^5 cfu/ml and used as primary inoculum (Lim & Wan, 1998; Prasitsilp, Jenwithisuk, Kongsuwan, Damrongchai, & Watts, 2000). Different amounts of Doxy-CSM containing drug concentration equivalent to 10–100 μg per ml were introduced into the flasks containing various cultures and incubated at 35 °C for 72 h. Samples were withdrawn at different time intervals to determine the minimal bactericidal concentration (MBC) (99.9% kill from initial inoculums) and mean number of survivors at MBC.

2.8. Evaluation of doxycycline as MMP inhibitor by gelatin zymography

Post-burn granulation tissue (Day 7) was collected from human burn patients after prior consent, complying with the ethical guidance of Central Leather Research Institute (CLRI) and Kilpauk Medical College and Hospital (KMCH), Chennai. Tissue was washed four times with water and then with 20 mM HEPES buffer (pH 7.5) to remove adhering blood clot and other debris. The tissue was then homogenized for 2 min in buffer solution (50 mM Tris–HCl, pH 7.5) on ice bath, centrifuged at 10,000 rpm (Sigma 3K30, USA) for 15 min and the supernatant was collected, aliquoted and stored at −70 °C.

Granulation tissue lysate (containing 20 μg of protein) was incubated along with various concentrations of doxycycline for 15 h at 37 °C. The incubated samples were mixed with non-reducing Laemmli's sample buffer and electrophoresed using a 7.5% PAGE containing 0.1% gelatin substrate. Standard MMP 2 and MMP 9 activated by 4-aminophenylmercuric acetate (APMA) buffer (0.5 mM APMA, 50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.005% Triton X-100), were simultaneously electrophoresed. After electrophoresis, the gel was washed with 2.5% Triton X-100 for 1 h and then incubated with enzyme buffer (50 mM of Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$ and 0.05% sodium azide) at 37 °C for 20 h to allow reactivation of MMPs. Gels were then stained with 0.5% Coomassie Brilliant Blue R-250 (Madlener, Parks, & Werner, 1998) and destained with 10% v/v of acetic acid containing 30% v/v of methanol. The MMPs were visualized as clarified bands corresponding to zones of digestion of substrate gelatin.

3. Results

3.1. Influence of process variables on microspheres size and morphology

The variables involved in this process and their effects on morphology and size of microspheres are presented in Table 2. Chitosan microspheres were prepared by w/o emulsification process with simultaneous ionic coacervation technique. In this process a mixture of oil phase (soya oil and *n*-octanol) was used since it provided continuous phase of optimal relative hydrophilic lipophilic balance (RHLB) to form uniform aqueous phase chitosan micelles, which eventually transform into rigid spheres, without any agglomeration. The oil mixtures of various ratios were used for different batches of microsphere preparation. Oil mixture ratio of 2:1, 1:1, (soya oil:*n*-octanol) caused spheres of irregular morphology. In these ratios, chitosan spheres obtained were discrete, but the high viscosity gradient of soya oil had certain impact on surface morphology. The spheres appeared sickle due to leaching of soya oil through the pores during recovery. However oil mixture ratio of 1:2 (soya oil:*n*-octanol) resulted in formation of porous microspheres without affecting the morphology of microspheres. Further enhancement of *n*-octanol resulted in the aggregation of spheres.

Due to low RHLB of oil phase mixture, Span 80 with low HLB (4.7) was used. A 5% v/v of span 80 was found to be optimal to form microspheres of uniform surface morphology with the size range of 200–225 μm, below which aggregation of spheres occurred. Span 80 at 6% and 7% v/v causes discrete spherical microspheres with the size range of 150–200 μm and 75–180 μm, respectively. The most critical step in the preparation of microsphere is percentage w/v of chitosan used for preparation of spheres.

Table 2
Influences of process variables on size range and morphology of chitosan microspheres

S. No	Variables studied	General aspects observed	Analyzed size range (μm)
1.	*Oil mixture ratio soya oil:n-octanol*		
	2:1 and 1:1	Spheres did not have uniform morphology. Though they were discrete, 90% were oversized	380–400 (90%) and 55–100 (10%)
	1:1.5	Spheres had better surface morphology but they were found to be of wide range.	120–350
	1:2	Spheres were discrete and of desired size range	200–225
	Beyond 1:3	Aggregated spheres	75–150
2.	*Percentage emulsifier (Span 80, %v/v)*		
	3%	Micelles of chitosan were not obtained. The polymer matrix settled down as a gel. Spheres formed with aggregates and >90% oversized	–
	4%	Spheres with good morphological features. Lesser aggregates than observed with lower % v/v	⩾300
	5%	Spheres of desired size and shape	200–225
	6%	Lesser aggregates than observed with lower % v/v	150–200
	7%	Discrete spheres with good morphology	150–200
3.	3% w/v (in 0.5 M acetic acid	Spheres formed were rigid and uniform in shape (>90%), hence volume ratio with respect to continuous phase was optimized	
	4% v/v	Coacervates with slightly weak structures	75–150
	5% v/v	Discrete Spheres with good morphology	80–200
	6% v/v	Microspheres with ideal size and shape and ⩾95% were discrete	200–225
	7% v/v	Ten percent of spheres were found to be aggregated, while 90% were uniformly sized	200–350
4.	*Overhead stirrer speed (rpm)*		
	<1600	Bigger particles with heavy agglomerates	>300
	1600	⩾90% uniform and discrete	200–225 μm
	1800	Discrete spheres were obtained but 50% of spheres varied in size	⩽150 μm
	2000	Spheres were discrete but heavily undersized	<100 μm
	2200	90% undersized and more than 25% aggregates	<100 μm
5.	*KOH in n-octanol* [a,b]		
	1% w/v	Matrix congealing in a uniform manner with the >95% recovery of spheres	200–225 (>95%)
	2%w/v	More than 80% of spheres obtained were of good shape but with more than 25% of irreversible aggregates. Total recovery was 85%	200–250μm (>85%)
	3% w/v	spheres adhered to the vessel walls and more than 25% polymer settled down as jelly matrix	–

[a] All batch experiments were carried out with KOH dissolved in *n*-octanol by sonication with 40% amplitude for 3 min (9/4 s – on/off pulse mode).
[b] Rate of addition of crosslinking agent was uniform for all batches – 1.5 mL/15 min.
[c] Magnetic stirrer speed was maintained at 1000 rpm for all batch experiments.

Earlier studies (Janes, Fresneau, Marazuela, Fabra, & Alonso, 2001) showed that concentration of chitosan below 3% w/v results in coacervates of weak structures, which are susceptible to breakage during the process of agitation. Hence, 3% w/v of chitosan prepared in 0.5 M acetic acid was used in various concentrations. The 6% v/v of chitosan solution was found to yield spheres of desirable size (200–225 μm) and morphology. When volume of chitosan was increased to 7% v/v, the spheres aggregated and strongly bound to each other, with the size range of 200–350 μm. Below 6% v/v, discrete spheres were obtained, but they were found to be fragile and some of the spheres disintegrated into smaller particles. Moreover, with lower concentrations of chitosan the size of the spheres varied widely. To obtain microspheres of uniform size and sphericity, different batches of spheres were prepared at various speeds. The spheres prepared with 1600 rpm was >95% uniformly sized and discrete. Microspheres prepared with 1800 rpm resulted in formation of discrete spheres with >50% size variation. Batch experiments carried out with speed >2000 rpm resulted in heavily undersized (<100 μm), while those <1600 rpm yielded agglomerated and oversized spheres (>300 μm). Hence the speed range 1600–1700 rpm was used for further optimization and microsphere preparation.

Crosslinking of microspheres was carried out using potassium hydroxide. In order to improve the crosslinking it is essential that the anions move uniformly from the core of the spheres to surface of matrix. In most of the cases aqueous anionic solutions are used, but this resulted in crosslinking of surface at faster rate than the core accounting for uneven crosslinking and formation of fragile structures. Hence in this process KOH was dissolved in *n*-octanol at various concentrations by sonication and batch experiments were carried out individually. With 3% w/v of KOH, spheres adhered to the vessel walls and more than 25% polymer settled down as jelly matrix. Batches prepared with 2% w/v of KOH resulted in much better yield of spheres (>85% recovery), but surface aggregation was inevitable. Hence the process was validated with 1% w/v

of KOH, which resulted in matrix congealing in a uniform manner with the >95% recovery of spheres in 200–225 μm size. The rate and volume of addition of KOH was also considered equally important. Slow crosslinking by dropwise addition at the rate of 1.5 ml/min of 1% KOH at every 15 min time interval was found to be optimal. Total time consumed to obtain rigidized spheres was 4 h and final process of collection involves slow and complete recovery of microspheres from oil phase using acetone. The spheres with above conditions were found to be ideal and the spheres obtained were of uniform size with spherical morphology and were completely rigidized.

3.2. Particle size distribution and swelling ratio

The particle size distribution curve (Fig. 1A) shows sharp distribution range of microspheres, with 90% of spheres in size range of 200–225 μm with average particle size of 208 μm and only 10% were oversized. To assess the ability of chitosan microspheres to swell, 25 mg was placed in deionized water and increase in size was assessed through particle size analysis at regular time intervals. A plot of the swelling ratio with respect to time (Fig. 2) shows that the microspheres were able to swell 1.59-fold than the original size in 1 h and remained in equilibrium for 6 h. After which, only a slight increase (≈2%) in size occurred for 48 h. Fig. 1B shows the size distribution shift, analyzed using particle size analyzer with respect to time, towards higher size range after swelling for 48 h. The horizontal bar between (A) and (B) represents the size shift that occurred after swelling (≈125 μm increase from its original size). The spheres retained their morphology throughout the study without any disruptions.

3.3. Doxycycline entrapment

Maximum percentage entrapment of doxycycline observed at 1:10% w/w Doxycycline: CSM and it was found to be 8.4% w/w. At higher concentration (as detailed in Section 2) there was no significant increase in percentage entrapment. Higher amount of CSM with respect to drug did not show any significant improvement in entrapment efficiency.

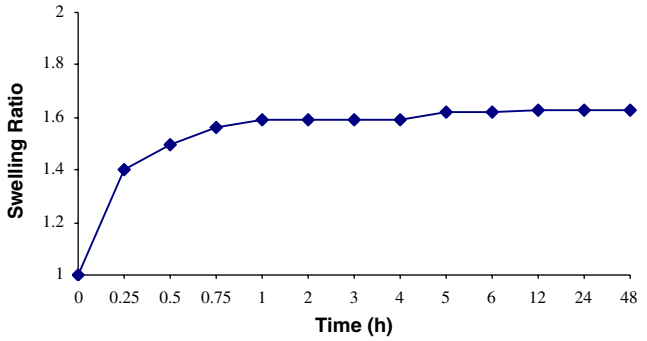

Fig. 2. Swelling ratio of chitosan microspheres determined by Malvern particle size analyzer.

3.4. Morphological features of CSM and Doxy-CSM

The chitosan microspheres exhibited typical characteristic features of an ionically crosslinked coacervated matrix. Fig. 3.1 shows the light microscopic images, of spheres with uniform size and shape. The SEM images (Fig. 3.2 and 3.3) further confirmed the uniformity and also show the porous nature of the microspheres due to interconnecting network of chitosan. Light microscopic image of Doxy-CSM (Fig. 3.4) indicates that the spheres did not loose their morphology after entrapment. SEM images confirmed further that the porous nature is retained after drug loading (Fig. 3.5 and 3.6).

3.5. Biocompatibility

The biocompatibility of the doxycycline and Doxy-CSM was demonstrated by performing MTT assay. The free doxycycline showed dose-dependent inhibition of fibroblast growth and the drug concentration beyond 200 μg, was found to be toxic to the cells (Fig. 4). The drug-loaded microspheres did not show any toxicity even at 400 μg.

The fluoroprobe Calcein AM was used to indicate the ability of CSM to act as a scaffold for fibroblasts. Calcein AM is a membrane permeant, non-fluorescent mole-

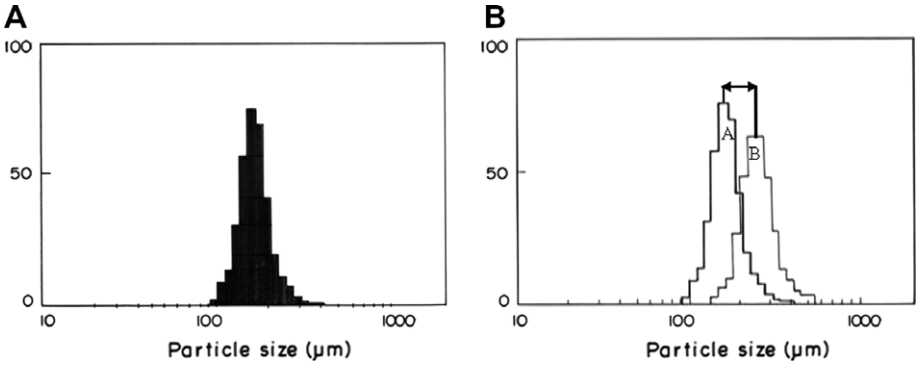

Fig. 1. (A) Particle size distribution of chitosan microspheres, (B) distribution curve showing the shift in size range of chitosan microspheres after swelling.

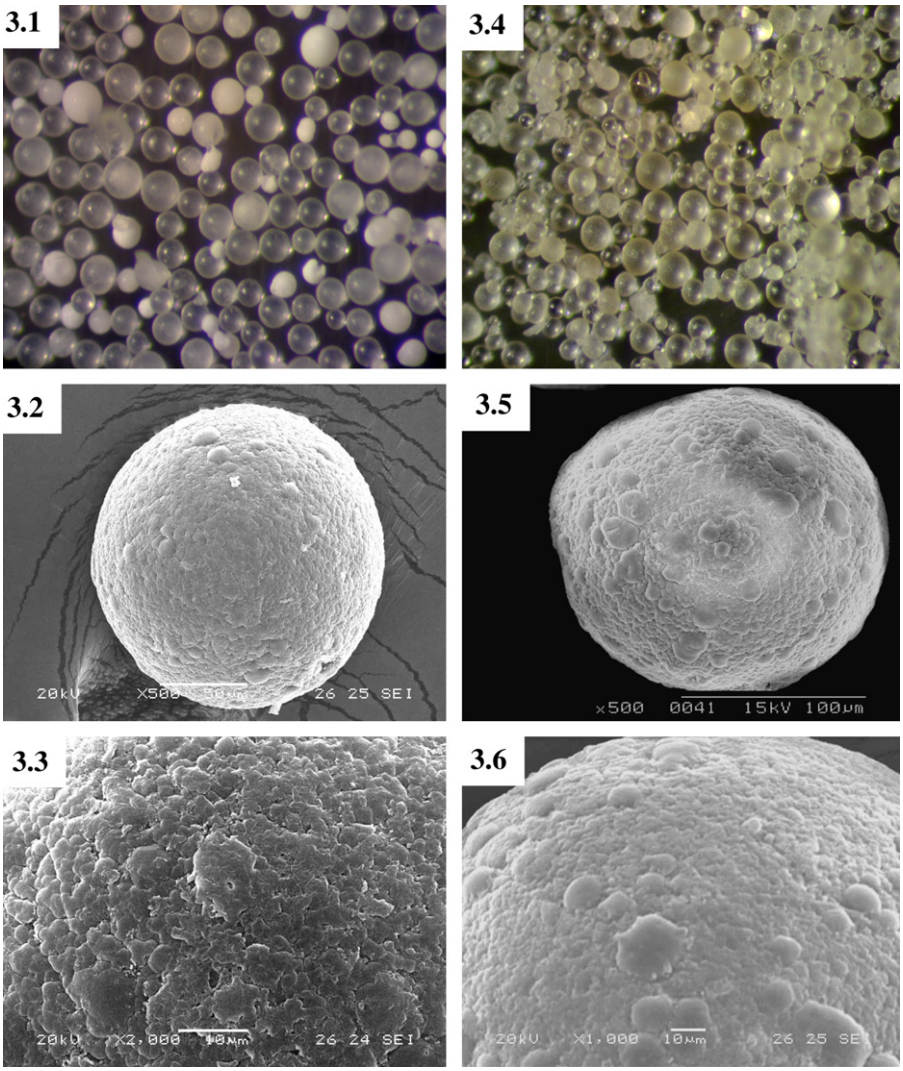

Fig. 3. Morphological features of chitosan microspheres and doxycycline-loaded chitosan microspheres. Photomicrograph 3.1 (20×) shows uniformly sized spheres with smooth surface. The SEM images 3.2 and 3.3 show the porous nature of the chitosan microspheres. Photomicrograph 3.4 is light micrographic images of Doxy-CSM taken at 20×. SEM images of Doxy-CSM (3.5 and 3.6) show that the morphological features of the microspheres are maintained after entrapment.

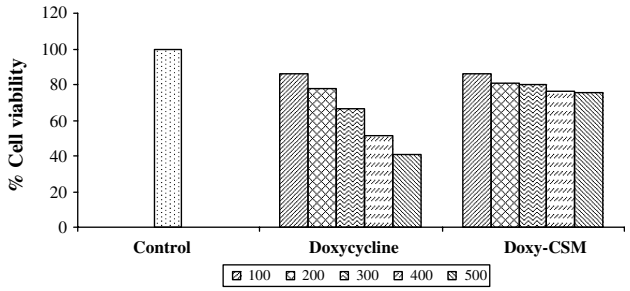

Fig. 4. MTT assay of human dermal fibroblast, treated for 48 h with free doxycycline and doxycycline-loaded chitosan microspheres.

cule, once inside the cells it is hydrolyzed by endogenous esterases into green fluorescent calcein and retained in the cytoplasm of the live cells (Fig. 5a). During the study the cells were observed to migrate towards the microspheres and penetrate through the pores of the microspheres rather than spreading over the entire culture surface. The fluorescent photomicrographs (Fig. 5b) of CSM exposed to the fibroblasts for 96 h, showed deeply embedded metabolically active cells in CSM. The study reveals that the developed CSM is a good substratum and can be utilized as a scaffold for various biomedical applications.

3.6. In vitro release of doxycycline

The release of doxycycline follows a swelling-controlled release mechanism. The Franz diffusion model is used in order to define the dosage system pertaining to percutaneous drug absorption. Doxy-CSM (Fig. 6A) showed ≈42% burst release, after which the release was faster nearly 72% by 24 h. Whereas the free drug (Fig. 6B) showed 97% release by 24 h. It is noteworthy that though percentage entrapment was lower, it exhibited a controlled release

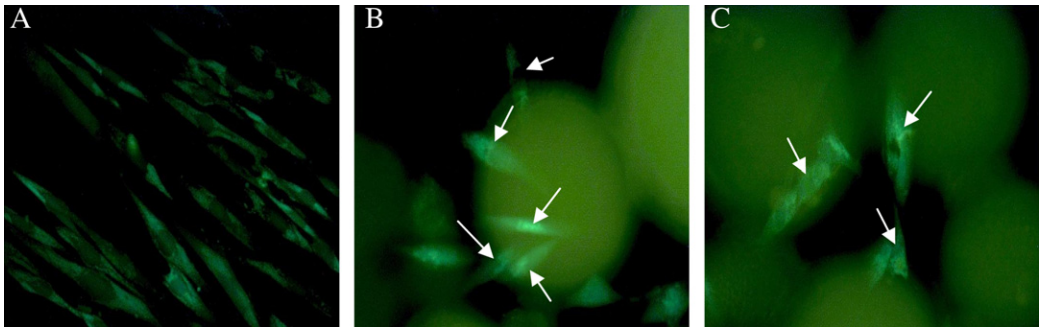

Fig. 5. Fluorescent photomicrograph of fibroblast cultured over cover slip as well as with chitosan microspheres. (A) Shows the green fluorescence of the cytoplasm of the spindle shaped viable cells after treatment with calcein AM (2 μM) for 20 min. (B and C) Photomicrograph showing fibroblasts as fluorescence in chitosan microspheres (indicated by arrows).

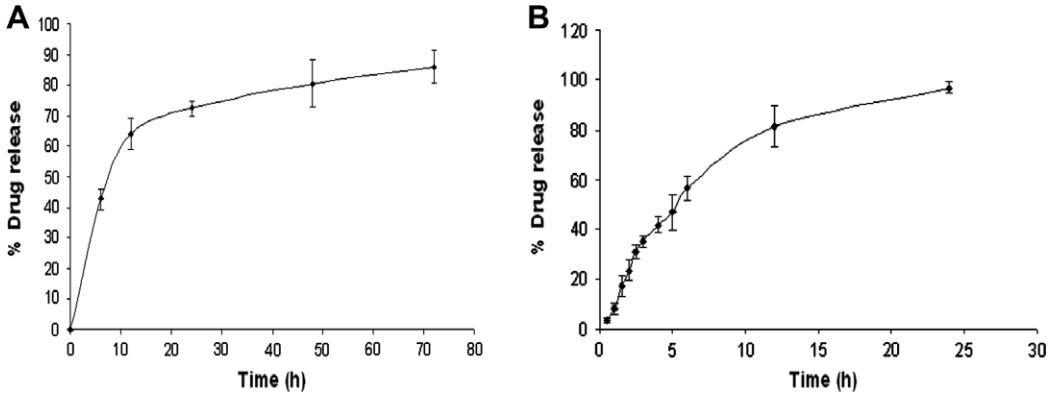

Fig. 6. In vitro release profile of doxycycline (A) from chitosan microspheres, (B) as free drug in synthetic serum electrolyte solution (SSES), pH 7.4, at 37 °C. The results are means of three individual experiments with standard deviation.

pattern indicating the nature of chitosan microspheres to release the drug. Moreover, chitosan is a hydrophilic polymer, which swells enormously by absorbing large quantity of aqueous solution hence a high percentage of drug release could be observed (86% at 72 h).

3.7. Antibacterial activity

Antibacterial efficiency of the Doxy-CSM was assessed by determining the MBC by standard tube dilution method against four standard pathogenic strains (ATCC) in mid-logarithmic phase cultures. The MBC was found to be nearly equal for *K. pneumoniae* (16.5 μg/ml) and *E. coli* (17.4 μg/ml). *P. aeruginosa* required higher drug concentrations (98.3 μg/ml), while *S. aureus* exhibited susceptibility (MBC) with lesser concentration levels (11.2 μg/ml). Though the number of survivors observed varied with different time points, they were found to be well within the susceptibility limits (20–300 cfu).

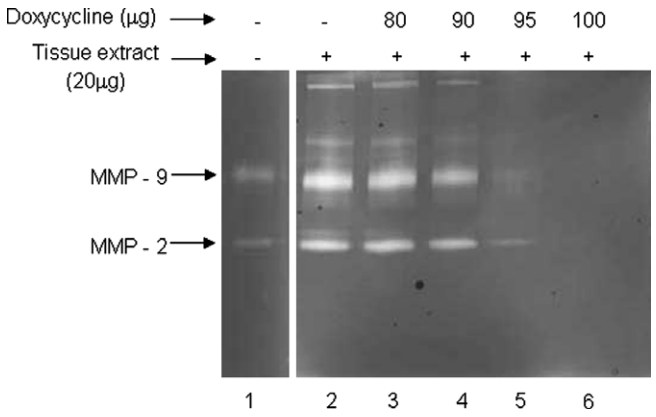

Fig. 7. Gelatin zymogram showing MMP inhibitory effect of doxycycline with extracts of human post-burn granulation tissue. Lane 1 indicates the standard enzymes, MMP 2 and MMP 9. Lane 2 shows the MMPs expressed in the granulation tissue extracts in which active forms of MMP 2 and MMP 9 are major enzymes expressed. Lane 3–6 show the inhibition of MMPs with increasing concentrations of doxycycline 80, 90, 95 and 100 μg, respectively.

3.8. MMP inhibition activity of doxycycline

Gelatin zymography was carried out with extracts of human post-burn granulation tissue, as a source of MMPs. Lane 1 in Fig. 7 is the standard enzymes of human MMP 2 and MMP 9 activated by APMA. Lane 2 shows the MMPs in the granulation tissue extracts, in which active form of MMP 2 and MMP 9 are major enzymes expressed. It can be clearly seen that there is a concentration-dependent inhibition of MMPs (lane 3–6) by doxycycline and complete

inhibition (lane 5) was achieved at 95 μg concentration of doxycycline.

4. Discussion

Pathogens responsible for inducing severe infection also possess the ability to degrade host tissues. They secrete exotoxins and endotoxins, which in turn trigger a chain of host response including inflammatory reaction and secretion of enzymes. Most of the antibiotics are designed to cover broad spectrum of susceptibility towards pathogenic microbes. Due to bacterial resistance mechanism to antibiotics there is a necessity to identify newer antibiotics. Doxycycline a tetracycline-based antibiotic was primarily used toward prophylaxis and treatment of malaria (Mehta, 1998). Later on, its potential benefits against standard pathogens have prompted the use doxycycline for treating various pathologies. Due to its low dose therapeutic limits, design of a controlled delivery system has become very essential.

Chitosan was selected as the carrier material to deliver doxycycline, since it is a unique polycationic polymer with excellent biocompatibility and biodegradable characteristics. Though many natural polymers like alginate, dextran and sodium carboxy methyl cellulose are reported, chitosan stands apart due to its better mucoadhesive, bacteriostatic and hydrogel forming property. Chitosan and its ability to hasten healing has been well accounted (Azad, Sermsintham, Chandrkrachang, & Stevens, 2004) and more importantly acts as cell-mediatory tool, inducing better healing. The free electropositive groups permit interaction between chitosan and cells *in vitro* and the NH^{3+} ions support cell attachment and migration (Prasitsilp et al., 2000). Chitosan microspheres prepared using covalent crosslinkers like glutaraldehyde and formaldehyde were reported to be toxic upon degradation. Hence alternative methods of microsphere preparation like spray drying (Mi et al., 1999) and ionic gelation using Na_2SO_4 or tripolyphosphate are carried out (Berthod et al., 1994; Clavo et al., 1997).

Chitosan forms films and gels with strong and integrated matrix when it reacts with anionic/polyanionic compounds. Anions like citrates and tripolyphosphates (TPP) are effective crosslinkers as they carry more than two negatively charged sites, but microspheres possess a fragile matrix (Shu & Zhu, 2002). In the present study chitosan was ionically crosslinked using KOH (dissolved in *n*-octanol by sonication), which enables the ions to reach the core of the micelles, without affecting their shape. A network is formed in the presence of negatively charged groups resulting in formation of bridges between $–NH^{3+}$ in chitosan. This interaction between oppositely charged ions is the major factor for inducing network. In addition to positively charge $–NH^{3+}$ interaction, $–COO^-$ ion contributes to electrostatic interaction with K+ ions, resulting in reduction in repulsion of oppositely charged ions. This result the heteropolysaccharide chains of chitosan to form intramolecular crosslinks.

The oil phase component, *n*-octanol, does not cause instability to emulsion throughout the process. Rather they enable the ions to get deposited throughout the micellar sites and get distributed in the continuous phase. Usage of oil phase crosslinking also obviates immediate surface crosslinking, which otherwise results in fragile matrix. Slow crosslinking is induced, thus resulting in uniform matrix crosslinking from core to the surface. The morphological features observed by SEM analysis provides evidence for the above fact which also proves that by adopting the present protocol spheres of uniform size can be obtained with high percentage recovery (>95%). The process variables like speed of emulsification, crosslinking rate and % w/v of chitosan used are key variables in obtaining spheres of desired range. Amongst all variables, concentration of chitosan is extremely important and low % of chitosan causes weak structures. A 6% v/v of chitosan was found to provide microspheres of stable porous structure with uniform size (>90% between 200 and 225 μm) and shape. Chitosan microspheres swelled ≈1.5-fold of their original size and maintained spherical morphology indicating the intact nature of crosslinked matrix.

Doxycycline possesses the ability to bind with divalent metal ions to cross the tissue barrier. This phenomenon finds application to inhibit MMPs by sequestering Zn^{2+} (Ryan, Usman, Ramamurthy, Golub, & Greenwald, 2001). This is an important reason to select doxycycline as an antibiotic especially in cases of chronic wounds. Previous reports show that doxycycline reduces proteolytic degradation of substrate in a dose-dependent manner, giving a clear indication of its ability to elicit therapeutic efficiency in a narrow range (Vachon & Yager, 2006). The % entrapment of the doxycycline was found to be 8.4%. The low entrapment is attributed to the low molecular weight of the drug, which highly influences the entrapment efficiency (Jameela & Jayakrishnan, 1995). Moreover the % entrapment can be increased by reducing the size of the microspheres. Effort of reducing the particle size was not attempted, since Doxy-CSM prepared were intended to use them for appropriate wound dressing wherein it is appreciable for a topically applied microspheres to be >150 μm, which will reduce bolus entry of microspheres (Shanmugasundaram et al., 2006)

The current investigation demonstrated that doxycycline when entrapped in CSM, could give clear demarcation in susceptibility concentration. It should be noted that the MIC levels against *K. pnuemoniae, E. coli*, *P. aeruginosa,* and *S. aureus* were calculated with respect to the amount of total drug present in microspheres, which is expected to be released in a controlled fashion, and hence the MIC levels are on the higher side of the concentration range. The actual amount of released drug would be dependent on the rate of release and equilibrium concentration of the drug at the wound site. Taking into account that dressing is intended to remain at wound site for at least 3 days; the experiment was designed and validated to determine the survivor count till 72 h.

It has been reported that doxycycline exhibits MBC of 32 μg (Fass & Barnishan, 1979) for *Pseudomonas aeruginosa*, where as controlled release requires a total drug concentration of 98.3 μg. Though the total drug concentration is higher, 42% burst release in 6 h and sustained equilibrium for 72 h provides advantage in developing formulations for wound healing application. This will enable the less frequent dressing change as well as reduce trauma due to repeated changes.

Another important aspect in controlling the release of the drug is to reduce the host cell toxicity. It is evident from MTT assay that controlled release of doxycycline induces less percentage of toxicity. Doxycycline when used as pure drug caused significant decrease in cell viability beyond 200 μg whereas equimolar concentration in Doxy-CSM does not induce toxicity. Moreover fluorescent assay shows CSM to act as template in inducing cell migration and proliferation. Hence the present study provides scope for using doxycycline at intended therapeutic range for both MMP inhibition as well as controlling infection without inducing any host tissue damage. Previous reports demonstrated that doxycycline induces positive modulation of MMP at sub-antimicrobial concentration (Choi et al., 2004). This is important in cases of chronic diseases where elevated enzyme levels are seen. Doxy-CSM may provide moist environment for healing as well as deliver the drug at required concentration to control infection and further release of doxycycline may be reduced or arrested when exudation decreases. The MMP inhibition study further confirms that doxycycline concentration required to kill pathogens would be sufficient to control MMPs. This would enable the system to prevent matrix degradation and induce positive healing. Further, the system developed provides wider scope to control the pathogens involved in infection and excess matrix degradation.

5. Conclusions

The current investigation well demonstrates that doxycycline can elicit both antimicrobial and MMP inhibition activities, which is advantageous in wound healing application. Since chronic infected wounds require a therapy to arrest infection and matrix degradation in a controlled fashion, the chitosan microspheres-loaded doxycycline would be an effective tool to develop therapeutic dressing encompassing both antimicrobial and MMP modulating ability. Further investigations are underway to develop Doxy-CSM-based wound dressing for treating chronic wounds.

Acknowledgements

Authors express their sincere thanks to Dr. A.B. Mandal, Director, CLRI, and Director Research, Sri Ramachandra University, for giving us the opportunity and encouragement to carry out the work.

References

Aoyagi, S., Onishi, H., & Machida, Y. (2007). Novel chitosan wound dressing loaded with minocycline for the treatment of severe burn wounds. *International Journal of Pharmaceutics, 330*, 138–145.

Azad, A. K., Sermsintham, N., Chandrkrachang, S., & Stevens, W. F. (2004). Chitosan membrane as a wound-healing dressing: Characterization and clinical application. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 69*, 216–222.

Berthod, F., Saintigny, G., Chretien, F., Hayek, D., Collombel, C., & Damour, O. (1994). Optimization of thickness, pore size and mechanical properties of a biomaterial designed for deep burn coverage. *Clinical Materials, 15*, 259–265.

Blanco, M. D., Gomez, C., Olmo, R., Muniz, E., & Teijon, J. M. (2000). Chitosan microspheres in PLG films as devices for cytarabine release. *International Journal of Pharmaceutics, 202*, 29–39.

Chin, G. A., Thigpin, T. G., Perrin, K. J., Moldawer, L. L., & Schultz, G. S. (2003). Treatment of chronic ulcers in diabetic patients with a topical metalloproteinase inhibitor, doxycycline. *Wounds, 15*, 315–323.

Choi, D. H., Moon, I. S., Choi, B. K., Paik, W., Kim, Y. S., Choi, S. H., et al. (2004). Effects of sub-antimicrobial dose doxycycline therapy on cervicular fluid MMP-2, and gingival tissue MMP-9, TIMP-1 and IL-6 levels in chronic periodontitis. *Journal of Periodontal Research, 39*, 20–26.

Clavo, P., Vila-Jato, J. L., & Alonso, M. J. (1997). Evaluation of cationic polymer coated nanocapsules as ocular drug carriers. *Journal of Pharmaceutics, 153*, 41–50.

Fass, R. J., & Barnishan, J. (1979). Minimal inhibitory concentrations of 34 antimicrobial agents for control strains Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853. *Antimicrobial Agents and Chemotherapy, 16*, 622–624.

Franz, T. J. (1978). The finite dose technique as a valid in vitro model for the study of percutaneous absorption in man. *Current Problems in Dermatology, 7*, 58–68.

Gapski, R., Barr, J. L., Sarment, D. P., Layher, M. G., Socransky, S. S., & Giannobile, W. V. (2004). Effect of systemic matrix metalloproteinase inhibition on periodontal wound repair: A proof of concept trial. *Journal of Periodontology, 75*, 441–452.

Hirano, S., Seino, H., Akiyama, Y., & Nonaka, I. (1988). Biocompatibility of chitosan by oral and intravenous administrations. *Polymeric Materials: Science and Engineering, 59*, 897–901.

Issa, M. M., Hoggard, P. K., & Arthurson, P. (2005). Chitosan and the mucosal delivery of biotechnology drugs. *Drug Discovery Today: Technologies, 2*, 1–6.

Jameela, S. R., & Jayakrishnan, A. (1995). 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, 16*, 769–775.

Janes, K. A., Fresneau, M. P., Marazuela, A., Fabra, A., & Alonso, M. J. (2001). Chitosan nanoparticles as delivery systems for doxorubicin. *Journal of Controlled Release, 73*, 255–267.

Khan, T. A., & Peh, K. K. (2003). A preliminary investigation of chitosan film as dressing for punch biopsy wounds in rats. *Journal of Pharmacy and Pharmaceutical Sciences, 6*, 20–26.

Kumar, T. R., Shanmugasundaram, N., & Babu, M. (2003). Biocompatible collagen scaffolds from a human amniotic membrane: Physicochemical and in vitro culture characteristics. *Journal of Biomaterials Science, Polymer Edition, 14*, 689–706.

Lim, L. Y., & Wan, L. S. C. (1998). Effect of magnesium stearate on chitosan microspheres pepared by an emulsification – coacervation technique. *Journal of Microencapsulation, 15*, 319–333.

Lynn, S. F. (1996). Tetracycline and doxycycline applications. *Primary Care Update for Ob/Gyns, 3*, 224–227.

Madlener, M., Parks, W. C., & Werner, S. (1998). Matrix metalloproteinases (MMPs) and their physiological inhibitors (TIMPs) are differentially expressed during excisional skin wound repair. *Experimental Cell Research, 242*, 201–210.

Manning, M. W., Cassis, L. A., & Daugherty, A. (2003). Differential effects of doxycycline, a broad spectrum matrix metalloproteinase inhibitor, on angiotensin II-induced atherosclerosis and abdominal aortic aneurysms. *Arteriosclerosis, Thrombosis and Vascular Biology, 23*, 483–488.

Mehta, D. (1998). *British national formulary. Number 34*. London: British Medical Association and the Royal Pharmaceutical Society of Great Britain.

Mi, F. L., Wong, T. B., Shyu, S. S., & Chang, S. F. (1999). Chitosan microspheres: Modification of polymeric chem-physical properties of spray-dried microspheres to control the release of antibiotic drug. *Journal of Applied Polymer Science, 71*, 747–759.

Mi, F. L., Shyu, S. S., Wu, Y. B., Lee, S. T., Shyong, J. Y., & Huang, R. N. (2001). Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing. *Biomaterials, 22*, 165–173.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*, 55–63.

Nordstrom, D., Lindy, O., Lauhio, A., Sorsa, T., Santavirta, S., & Kontttinen, Y. T. (1998). Anti-collagenolytic mechanism of action of doxyclycline treatment in rheumatoid arthritis. *Rheumatology International, 17*, 175–180.

Prasitsilp, M., Jenwithisuk, R., Kongsuwan, K., Damrongchai, N., & Watts, P. (2000). Cellular response to chitosan in vitro: The importance of deacetylation. *Journal of Materials Science: Materials in Medicine, 11*, 773–778.

Ryan, M. E., Usman, A., Ramamurthy, N. S., Golub, L. M., & Greenwald, R. A. (2001). Excessive matrix metalloproteinase activity in diabetes: Inhibition by tetracycline analogues with zinc reactivity. *Current Medicinal Chemistry, 8*, 305–316.

Shanmugasundaram, N., Ravikumar, T., & Babu, M. (2004). Comparative physico-chemical and in vitro properties of fibrillated scaffolds from different sources. *Journal of Biomaterials Application, 18*, 247–264.

Shanmugasundaram, N., Sundaraseelan, J., Uma, S., Selvaraj, D., & Babu, Mary (2006). Design and delivery of silver sulfadiazine from alginate microspheres-impregnated collagen scaffold. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 77B*, 378–388.

Shu, X. Z., & Zhu, K. J. (2002). Controlled drug release properties of ionically crosslinked chitosan beads: The influence of anion structure. *International Journal of Pharmaceutics, 233*, 217–225.

Siemonsma, M. A., De Hingh, I. H. J. T., De Man, B. M., Lomme, R. M. L. M., Verhofstad, A. A. J., & Hendriks, T. (2003). Doxycycline improves wound strength after intestinal anastomosis in the rat. *Surgery, 133*, 268–276.

Tahnoo, B. C., Sunny, M. C., & Jayakrishnan, A. (1992). Crosslinked chitosan microspheres: Preparation and evaluation as a matrix for the controlled release of pharmaceuticals. *Journal of Pharmacy and Pharmacology, 44*, 283–286.

Vachon, D. J., & Yager, D. R. (2006). Novel sulfonated hydrogel composite with the ability to inhibit proteases and bacterial growth. *Journal of Biomedical Materials Research, 76A*, 35–43.