

Development and Evaluation of In-Situ Gel Containing Ornidazole Loaded Microspheres for Treatment of Periodontitis

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ABSTRACT

The present investigation aims at developing novel injectable in-situ gel containing ornidazole (ORDZ) loaded chitosan microspheres for treatment of periodontal disease. Microspheres were prepared by emulsification-ionic gelation method. Prepared microspheres were evaluated extensively for particle size, equilibrium swelling studies, bioadhesion, percentage drug release and antimicrobial activity. The mean diameter of the microspheres was found in the range of 29.1-52.65 μm . The microspheres showed good swelling properties. Percentage equilibrium swelling was dependent upon the amount of chitosan. The *in vitro* drug release and bioadhesion studies were dependent on the extent of crosslinking and chitosan concentration. The ORDZ-loaded microspheres showed drug encapsulation in the range of 11.02 \pm 0.98 - 32.45 \pm 0.62 % and sustained the release up to 5 days. The drug release from microspheres was diffusion controlled. The antimicrobial study indicated inhibition of growth of *Staphylococcus aureus* at all drug concentrations of *in vitro* release samples. In situ gel containing optimized microspheres extended the drug release up to 7 days. Results of the study demonstrated good bioadhesion of the in-situ gel containing microspheres as well as exhibited sustained release of drug. The in-situ gel containing ORDZ- loaded chitosan microspheres may be an efficient alternative to the other known delivery systems for treatment of Periodontitis.

INTRODUCTION

Periodontal diseases are commonly found in all groups, races and genders. They are recognized as one of the major public health problems (Chandur *et al.*, 2007). They are characterized by a presence of disease-causing bacteria, inflammation of the gums, and loss of bone around the teeth. Periodontitis is collection of inflammatory diseases affecting the tissues that surround and support the teeth. It involves progressive loss of the bone around teeth which may lead to loosening and eventual loss of teeth, if untreated (Rahman *et al.*, 2003). Systemic administration of antibiotics have shown

several 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 and high peak-plasma antibiotic concentrations which may be associated with various side effects. These obvious disadvantages have evoked an interest in the development of novel intra-pocket drug delivery systems for the treatment of periodontal diseases (Govender *et al.*, 2005). The periodontal pocket provides a natural reservoir, which is easily accessible for the insertion of a delivery device. The gingival crevicular fluid (GCF) provides a leaching medium for the release of a drug from the dosage form and for its distribution throughout the pocket. These features, together with the fact that the periodontal diseases are localized to the immediate environment of the pocket, make the periodontal pocket a natural site for treatment with local delivery systems (Goodson *et al.*, 1979).

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The intra-pocket delivery systems investigated so far are fibers (Sachdeva & Agarwal, 2011), strips (Tariq *et al.*, 2012), films (Gulzar *et al.*, 2009), gels (Rajashree *et al.*, 2009), microparticles (Pichayakorn & Boonme, 2013), nanoparticles (Piñón-Segundo *et al.*, 2005) and vesicular (Vyas *et al.*, 2001) systems. Fibers, strips and films used for intra-pocket delivery have limited acceptance because of intricacies of winding them into place, the need to retain these devices within the pocket and then their removal after seven to ten days (Jain *et al.*, 2008). Although the microspheres can overcome the problems associated with fibres, films and strips, they have to be dispersed into the saline prior to their administration. Such dispersion may ooze out of the pocket after administration. On other hand, plain gels cannot give the controlled release and have limited bioadhesive property (Park *et al.*, 2005).

Therefore, it is hypothesized that the bioadhesive microspheres containing thermoreversible gels could be effective in such situation. Chitosan is one of the widely used natural polymers in pharmaceutical industry. It is a polysaccharide composed of copolymers of glucosamine and N-acetylglucosamine (Kumar *et al.*, 2004). It is a biocompatible, biodegradable and mucoadhesive polymer of low toxicity suitable for microparticle based drug delivery systems (Nair *et al.*, 2009; Suresh & Dewangan, 2011).

Till date, the chitosan microparticles of many drugs have been prepared by the researchers in order to achieve bioadhesion and controlled release (Suresh & Dewangan, 2011; Pichayakorn & Boonme, 2013; Asane *et al.*, 2011; Swamy *et al.*, 2013). Govender *et al.*, (2005) have reported the application of chitosan microspheres for delivery of tetracycline to the periodontal pocket for treatment of periodontitis. However, the use of thermoreversible gels containing drug loaded chitosan microspheres for intrapocket delivery in periodontitis has not been explored. Pluronic flake 127 (a nonionic surfactant) solutions are known to exhibit the phenomenon of reverse thermal gelation. At low temperatures they remain as solutions and gel when the temperature is increased (Park *et al.*, 2003). This phenomenon may be helpful to retain the microspheres within the pocket for longer duration.

Ornidazole (ORDZ) is chemically 1-chloro-3-(2-methyl-5-nitro-1H-imidazol-1-yl) propan-2-ol. It is used in the treatment of hepatic and intestinal amoebiasis, giardiasis, trichomoniasis of urogenital tract and bacterial vaginosis. It is also used in the treatment and prophylaxis of susceptible anaerobic infections in dental and gastrointestinal surgery and in other mixed aerobic-anaerobic infections (Sweetman, 2005; Neil *et al.*, 2006).

Recent clinical studies suggest that ORDZ is effective systemically as well as locally for periodontitis (Patel *et al.*, 2014; Patil *et al.*, 2013). As periodontal disease is an infectious disease caused by several pathogenic anaerobic microfloras, ORDZ has been used for the local delivery in this treatment (Park *et al.*, 2005). In present work, an attempt was made to formulate in situ gel containing microspheres for easy insertion directly to the site and maintain its stay for longer duration giving controlled release.

MATERIALS AND METHODS

Materials

Ornidazole, Chitosan and Pluronic Flake 127 were obtained as a gift sample from Blue-cross Laboratories Ltd., (Nashik, India), Mahtani Chitosan Pvt. Ltd., (Veraval, India) and Cipla Mfg. Division, (Patalganga, India) respectively. Acetic acid, sodium hydroxide, span 80, hexane and petroleum ether were purchased from Loba Chemie (Mumbai, India).

Preparation of ORDZ microspheres

The microspheres were prepared by emulsification and ionotropic gelation using sodium hydroxide (NaOH) as gelling agent (Lim *et al.*, 1997). The drug was dispersed in a solution containing 40 ml of 2% w/v chitosan in 2.5% v/v aqueous acetic acid (table 1). The solution was dispersed in 200 ml hexane solution containing 2% (w/v) span 80 using mechanical stirrer (Remi motors, Mumbai, India) at 2000 rpm. The resultant emulsion was stirred for 30 minutes. Fifteen ml of 2 N NaOH was added slowly at the rate of 5 ml/min. Stirring was continued for 2.5 h. The microspheres were separated by filtration, washed with petroleum ether, followed by distilled water and then air dried. To optimize the preparation of microspheres, the formulation variables like drug concentration, polymer concentration, and concentration of crosslinking agent were changed.

Table 1: Formula for different batches of ORDZ-loaded chitosan microspheres.

Formulation Variables	Formulation Code						
	A1	A2	A3	B1	B2	C1	C2
ORDZ (g)	0.8	1.6	2.4	0.8	0.8	0.8	0.8
Chitosan (% w/v)	2	2	2	2.5	3	2	2
Sodium hydroxide solution (N)	2	2	2	2	2	2.5	3

Characterization of ORDZ microspheres

Compatibility study

The prepared microspheres were subjected to compatibility study by infrared spectroscopy (IR) and differential scanning calorimetry (DSC) analysis in order to ensure the unusual interaction between ORDZ and polymers.

Percentage yield

The yields of production were calculated as the weight percentage of the final product after drying, with respect to the initial amount of drug and chitosan used for preparations (Yadav and Mote, 2008).

Particle size determination

The particle size of the microspheres was determined using optical microscope (Lawrence and Mayo, Mumbai, India) fitted with an ocular micrometer and a stage micrometer. The particle diameters of more than 300 microspheres were measured randomly by optical microscope. The average particle size was determined by using Edmondson's equation (Sinko and Sing, 1996),

$$D_{\text{mean}} = \sum nd / \sum n \quad (1)$$

Where,

- n = number of microspheres observed
- d = mean size range.

Surface morphology

The surface morphology of the microspheres was studied using scanning electron microscopy (SEM), (JSM-6360, JEOL Ltd., Tokyo, Japan). Microspheres were sprinkled on to double sided tape, sputter coated with platinum and examined under the microscope at 20 kV.

Percentage drug encapsulation

The encapsulation was determined by taking known quantity of microspheres (50 mg) in 50 ml volumetric flask. Sufficient quantity of isotonic phosphate buffered saline (IPBS) pH 7.2 was added to make volume. The suspension was shaken vigorously for 15 min and then left for 24 h at room temperature with intermittent shaking. The resultant solution was filtered, diluted with IPBS and ORDZ concentration in the samples was determined by using UV visible spectrophotometer at 318.5 nm (Patel DM *et al.*, 2012). Efficiency of drug entrapment for each batch was calculated in the term of percentage drug entrapment (PDE) as per the following formula (Dias *et al.*, 2015),

$$\% \text{ Drug entrapment (PDE)} = \frac{\text{Actual drug content}}{\text{Theoretical drug content}} \times 100 \quad \dots 2$$

Equilibrium swelling studies

The water absorption capacity of chitosan microspheres was determined using the earlier reported method (Mi *et al.*, 1997). One hundred milligrams of the microsphere sample was placed in 25 ml water for 2.5 h. The microspheres were filtered, blotted with filter paper to remove excess water on the surface and weighed immediately on an electronic balance (Adventurer™ Ohaus, NJ, USA). Percent swelling of microspheres at equilibrium was calculated as;

$$E_{sw} = \frac{W_e - W_0}{W_0} \times 100 \quad \dots 3$$

Where,

- E_{sw} = the percent swelling of microspheres at equilibrium
- W_e = the weight of microspheres at equilibrium swelling
- W_0 = the initial weight of microspheres.

In vitro bioadhesion

The *in vitro* bioadhesion of microspheres was determined using earlier method (Ranga Rao and Buri, 1989) with certain modification. Microspheres (50 mg) were placed on sheep cheek membrane (2 cm) and kept for 20 min in a humidity temperature control cabinet (Remi Instruments, Mumbai, India) at 75% relative humidity and temperature of $25 \pm 2^\circ\text{C}$ to allow hydration of the microspheres. This was followed by thorough washing of the

mucosal lumen with isotonic phosphate buffered saline pH 7.2. The washings were then dried at 70°C in a hot air oven and weighed. Percent bioadhesion was determined by the following formula:

$$\% \text{ bioadhesion} = \frac{W_0 - W_d}{W_0} \times 100 \quad \dots 4$$

Where,

- W_0 = weight of applied microspheres
- W_d = Weight of detached microspheres.

In vitro drug release

The *in vitro* drug release from different batches of microspheres was evaluated in IPBS, pH 7.2. Microspheres (equivalent to 1 mg drug) were dispersed in 4 ml of IPBS pH 7.2 at 37°C and shaken at 3 rpm. At predetermined time intervals, the entire buffer medium was withdrawn and replenished with fresh IPBS (pH 7.2). The amount of ORDZ released into the buffer was measured at 318.5 nm (Chandur *et al.*, 2007; Haerdi-Landerer *et al.*, 2008).

In vitro antimicrobial activity

In vitro antimicrobial activity was carried out by using *Staphylococcus aureus* which is one of the isolates usually found in periodontitis patients (Saini *et al.*, 2003). For this purpose, the samples collected from *in vitro* release study of blank microspheres, drug-loaded microspheres and ORDZ alone were tested against *S. aureus*, at different time intervals (24, 48, 72, 96 and 120 h) (Govender *et al.*, 2005; Mastiholmath *et al.*, 2006). The nutrient agar medium was prepared and sterilized by autoclaving under aseptic condition and transferred to sterile petriplates. After solidification of nutrient agar medium, lawn was made with 0.1 ml microorganism i.e. *S. aureus* in petri plates. Wells were made at equidistant from one another and the samples collected from *in vitro* drug release were filled into it. Samples were allowed to diffuse for 1 h in refrigerator and finally plates were kept for incubation in incubator at $37 \pm 5^\circ\text{C}$ for 24 h (Friedman and Steinberg, 1998).

Statistical analysis

Statistical analysis of data was performed using one-way ANOVA followed by Dunnett's multiple comparison tests. The *P* value less than 0.05 were considered statistically significant.

Optimization of thermoreversible PF-127 aqueous solution

Thermoreversible gel was prepared according to the cold method (Schmolka, 1972). In brief, sufficient quantity of distilled water was cooled to 4°C and slowly the known amount of PF-127 (16-20%, w/v) was added with continuous stirring. The mixture was kept overnight at 4°C and immediately gelation temperature was recorded in triplicate. 1% w/v drug equivalent optimized microspheres were dispersed in prepared PF-127 aqueous solutions and gelation temperature was recorded. Finally, the

concentration of PF-127 in aqueous solution containing optimized microspheres which gave gelation temperature in the range of 25 to 32 °C, was selected.

Preparation of PF-127 aqueous solution containing optimized microspheres

Sufficient quantity of distilled water was cooled to 4°C and slowly the optimized concentration of PF-127 was added with continuous stirring. The mixture was kept overnight at 4°C. 1% w/v drug equivalent optimized microspheres were dispersed in prepared PF-127 aqueous solution. The prepared formulation was immediately subjected to surface pH, gelation temperature, viscosity and bioadhesion within 24 h.

Characterization of in-situ gel containing ORDZ microspheres Gelation temperature

The gelation temperature of aqueous solution of PF-127 was measured by using procedures reported by Choi *et al.*, 1998. A 20 ml transparent vial containing 10g aqueous solution of PF-127 was heated at an increasing rate of 2°C /min with constant speed of 100 rpm on a magnetic stirrer (Remi, Mumbai, India). The temperature at which rotation of bar (15x6 mm) stopped was taken as the gelation temperature.

Surface pH

A digital glass electrode pH meter was used to measure the surface pH. The pH was noted by bringing the electrode near the surface of the formulation and allowing it to equilibrate for one minute (Bottenberg *et al.*, 1991).

Viscosity measurement

The viscosities of various formulations were determined by using cone and plate viscometer (Brookfield viscometer, Model Cap 2000 + 2, USA). A sample solution of 0.5 ml was applied to the lower plate of the viscometer using glass rod. The viscosity was recorded using spindle no. 3, at speed of 10 rpm with increase in temperature.

Determination of bioadhesive force

The bioadhesive potential of formulation was determined by measuring the force required to detach the formulation from sheep cheek mucosa by using a modified chemical balance. A section of cheek mucosa was instantly fixed with mucosal side out onto each glass vial using a rubber band. The vials with cheek mucosa were stored at 37°C for 10 minutes. Then first vial with a section of mucosa was connected to the balance in inverted position while second vial was placed on a height adjustable pan. Fixed amount of sample was placed onto the tissue mucosa of second vial. The height of second vial was adjusted so that mucosal surfaces of both vials come in intimate contact. Two minutes contact time was given to ensure intimate contact between tissues and the sample. The weight was kept rising in the pan until vials get detached. The bioadhesive force was determined from the

minimal weights that detached the tissues from each other. Following formula was used to determine bioadhesive force in dyne/cm² (Choi *et al.*, 1998).

$$\text{Bioadhesive force (dyne/cm}^2\text{)} = (m) \times (g/A) \dots(5)$$

Where,

m = Weight required for detachment of two membrane in grams

g = Acceleration due to gravity (980 cm²/s)

A = Area of tissue exposed (cm²).

In vitro drug release

Release of drug from gel containing optimized formulation of ORDZ microspheres was studied employing the permeation apparatus. A glass cylinder with both ends open, 10 cm height, 3.7 cm outer diameter and 3.1 cm inner diameter was used as donor compartment. Cellophane membrane was tied to one end of donor compartment. 1 mg drug equivalent formulation was taken in donor compartment and the cell was immersed in a beaker (receptor compartment) containing 40 ml of isotonic phosphate buffered solution (IPBS), pH 7.2. The cell was immersed to a depth of 1 cm below the surface of IPBS in the receptor compartment. Receptor compartment was stirred at 100rpm by a magnetic stirrer and temperature was maintained at 37 ± 1°C throughout the study. Aliquots of 5 ml were withdrawn periodically at intervals of 1 day for a period of 7 days. Sink condition was maintained. The amount of drug release was estimated using UV spectrophotometer at 318.5 nm (Rajashree *et al.*, 2009).

RESULTS AND DISCUSSION

Characterization of ORDZ microspheres

Compatibility study

The IR spectrum of ORDZ shows characteristic absorption peaks at 3174.10 cm⁻¹ (O-H stretching), 3112.94 cm⁻¹ and 3090.98 cm⁻¹ (C-H stretching), 1538.10 cm⁻¹ (asymmetric NO₂ stretching), 1363.13 cm⁻¹ and 1271.72 cm⁻¹ (symmetric NO₂ stretching), 1149.83 cm⁻¹ (C-O stretching), 829.31 cm⁻¹ (C-N, NO₂ stretching). The IR spectrum of chitosan shows prominent peaks at 3390.13 cm⁻¹ (O-H stretching), 2922.15 cm⁻¹ (C-N stretching) and 1654.48 cm⁻¹ (carbonyl, C=O-NHR). In the formulation, the peaks of ORDZ at 3174.10, 3112.94, 3090.98, 1149.83 and 829.31 cm⁻¹ completely disappeared due to their masking by the corresponding peaks of PF-127 and chitosan. As can be seen from figure 1, the carbonyl peak of chitosan from 1654.48 cm⁻¹ was shifted to 1642.30 cm⁻¹. This may be due to the hydrogen bonding interaction between -OH group of ORDZ and Carbonyl group of chitosan. These differences were due to the interactions taking place between the carbonyl group of chitosan with the nitro group of ORDZ (Papageorgiou *et al.*, 2009).

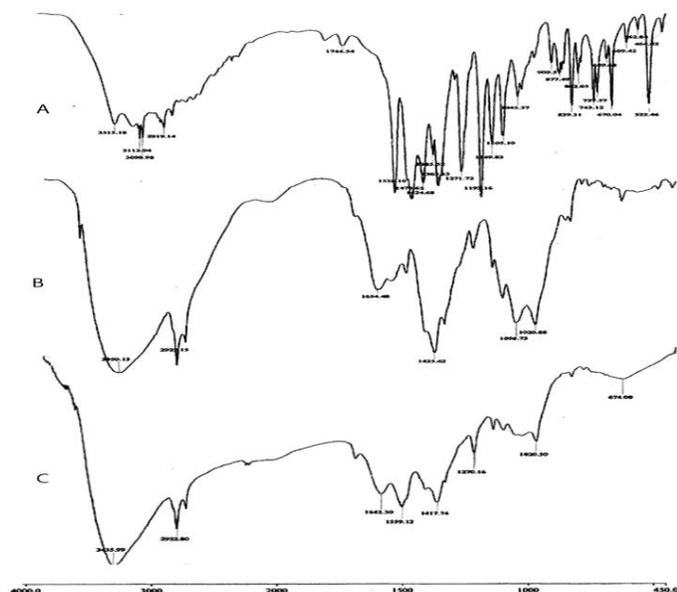


Fig. 1: IR analysis of ORDZ (A), chitosan (B) and drug loaded chitosan microspheres (C).

The DSC thermogram of physical mixture of drug and polymer showed intensity of peak at 87.29°C which represents characteristic endothermic peak of pure ORDZ at 86.66°C, which is equivalent to the melting point of ORDZ (figure 2). The examination of thermogram of drug loaded microspheres revealed disappearance of ORDZ endothermic peak which indicates molecular dispersion of drug in microspheres (Dias *et al.*, 2015). The overall results of the compatibility study indicated lack of unusual interactions between drug and excipients.

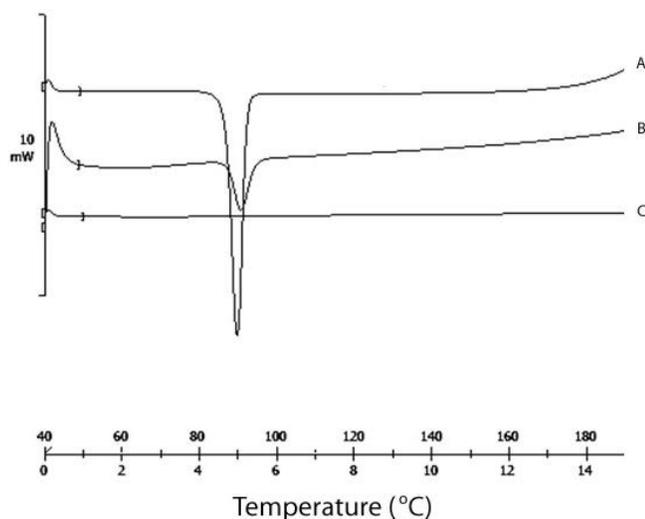


Fig. 2: DSC analysis of ORDZ (A), drug + polymer physical mixture (B) and drug loaded chitosan microspheres (C).

Morphology, yield and size distribution

The SEM analysis revealed that the formulated microspheres exhibited spherical shape before drug loading. Drug

loading led to the formation of irregular microspheres with rough surface. This may be due to presence of pores on surface due to entrapment of drug. The surface roughness of the microspheres was found to be increased with increase in drug concentration. A microphotograph of blank and drug loaded microsphere is shown in figure 3.

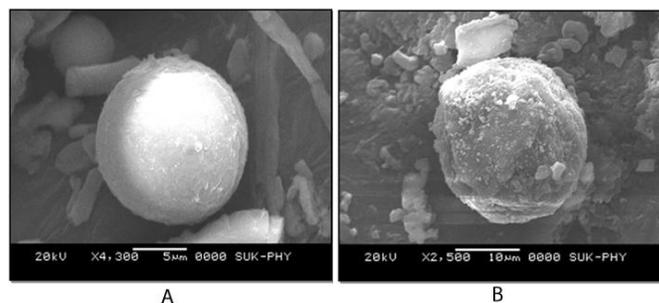


Fig. 3: SEM analysis of chitosan microspheres. Surface morphology of blank microsphere (A), surface morphology of drug loaded microsphere (B).

The percentage yield of formulated microspheres was 60-82% (table 2). The particle size of the microspheres was found in the range of 29.1-52.65µm. Particle size of microspheres showed inverse relation with the stirring speed. It was found that increasing the speed up to 2200 rpm resulted in decline in particle size and number of spherical particles. Clumping of particles and large size particles were observed at lower stirring speed of 1700 rpm. In present study, stirring speed was optimized to 2000 rpm to obtain the microspheres in the range of 25-55 µm.

An increase in the concentration of drug increased the particle size which may be attributed to increase in the viscosity of the droplets formed during emulsification, which may have caused an increase in the interfacial tension. A significant increase in the particle size ($P < 0.05$) observed with increase in polymer concentration may be due to the fact that increase in the concentration of polymer increases the crosslinking and hence the matrix density of microspheres further resulting into increase in particle size (Patil and Murthy, 2006). However, the concentration of crosslinking agent (NaOH) did not affect the particle size.

Percentage drug encapsulation

The percentage drug encapsulation was found to be directly proportional to polymer and drug loading. The significant ($P < 0.05$) increase in the drug encapsulation with increase in drug loading may be due to increase in the drug concentration in polymer solution. Similar behavior with an increase in the polymer concentration can be due to increased viscosity, which results in the formation of larger microspheres, thus increasing the drug encapsulation significantly ($P < 0.05$) (Dubey and Parikh, 2004; Mali *et al.*, 2010). As the concentration of crosslinking agent increases, there is marginal decrease in the drug encapsulation. The percent drug encapsulation was found to be in the range of 11.02-32.45% (table 2).

Table 2: Physical characteristics of prepared ORDZ-loaded chitosan microspheres.

Batch code	Percent yield (% w/w)	Particle size (μm) (mean \pm SD)	Percent drug encapsulation (% w/w* \pm SD)	Percent Equilibrium Swelling (E_{sw}) (%* \pm SD)	Percent <i>in vitro</i> bioadhesion (%* \pm SD)
A1	81.96	29.1 \pm 1.3	13.38 \pm 0.18	73.53 \pm 0.55	73.86 \pm 4.27
A2	68.41	35.46 \pm 0.56	23.99 \pm 0.56	68.33 \pm 0.81	69.06 \pm 1.66
A3	60.68	43 \pm 0.98	32.45 \pm 0.62	63.06 \pm 1.35	65.46 \pm 2.66
B1	74.93	51.35 \pm 0.75	17.07 \pm 0.19	74.43 \pm 0.72	81.46 \pm 2.75
B2	71.57	52.65 \pm 0.30	18.48 \pm 0.95	76.4 \pm 0.7	85.73 \pm 3.40
C1	79.04	38.9 \pm 0.5	12.56 \pm 0.54	73.06 \pm 0.60	72.13 \pm 1.28
C2	74.90	39.16 \pm 0.68	11.02 \pm 0.98	72.43 \pm 0.85	69.33 \pm 2.11

* Mean of three readings, SD- Standard deviation.

Percent equilibrium swelling and bioadhesion

Estimation of percent equilibrium swelling (E_{sw}) revealed the swelling behavior of microspheres (table 2). The increase in the drug concentration caused decrease in the E_{sw} significantly ($P < 0.05$). This may be due to increase in the drug concentration with respect to the polymer concentration. On other hand, increase in the polymer concentration led to significant ($P < 0.05$) increase in E_{sw} , which may be because of swelling property of chitosan. The increase in the concentration of crosslinking agent caused negligible decrease in the E_{sw} . Low E_{sw} values on increasing the concentration of crosslinking agent may be due to introduction of numerous cross-links in the hydrogel structure (Hamdi and Ponchel, 1999). The microspheres showed bioadhesion in the range of 65.46 – 85.73%. The percent *in vitro* bioadhesion showed significant increase ($P < 0.05$) with increase in polymer concentration. This may be due to availability of more polymer chains for entanglement with the mucus. However, addition of drug in microspheres as well as increase in concentration of crosslinking agent (NaOH) decreased its bioadhesive property due to reduction in number of polymer chains available for entanglement with the mucus (batch A1, C1, C2).

In vitro drug release

The *in vitro* drug release profile of ORDZ from microspheres is shown in figure 4, 5 and 6. The formulations exhibited slow release. The batches (A1, B1, B2, C1, C2) released almost 50% of drug within 48 h except the batches A2 and A3.

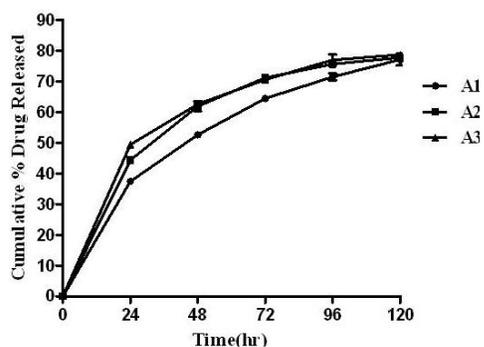


Fig. 4: In-vitro release profile of ORDZ in IPBS, pH 7.2 from chitosan microspheres containing different concentrations of drug.

The microspheres from batch B1, B2, C1 and C2 extended the release up to 120 h (figure 4). As the amount of drug

was increased from 0.8 gm to 2.4 gm (batch A1, A2 and A3) the rate and extent of release was found to be increased. A decrease in the rate and extent of drug release was observed with the increase in polymer concentration in microspheres of batch A1, B1 and B2 (figure 5).

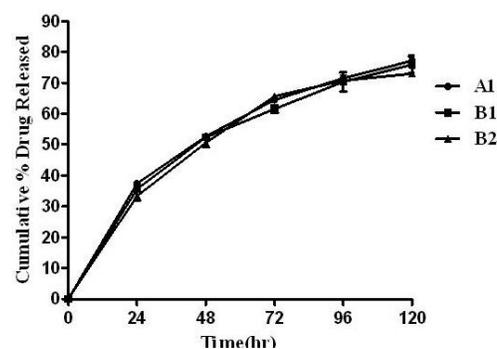


Fig. 5: In-vitro release profile of ORDZ in IPBS, pH 7.2 from chitosan microspheres containing different concentrations of chitosan.

This can be attributed to an increase in the density of polymer matrix and the diffusional path length that the drug has to traverse. The initial rapid release was reduced with an increase in polymer concentration which can be due to decrease in the amount of surface-associated drug with increase in the encapsulation efficiency. Further it was observed that the rate and extent of drug release was decreased with increase in concentration of sodium hydroxide (figure 6). This may be due to increased crosslinking of polymer which forms more compact structure resulting in less diffusion of drug from microspheres.

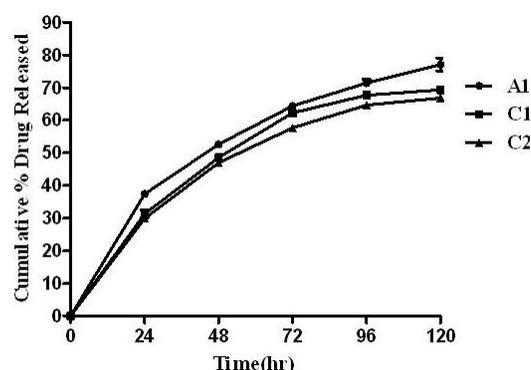


Fig. 6: In-vitro release profile of ORDZ in IPBS, pH 7.2 from chitosan microspheres containing different concentrations of sodium hydroxide.

The drug release kinetics and mechanism were examined by fitting the release data into the models representing zero-order, first-order and Higuchi's square root of time. The linear regression analysis is summarized in table 3. The coefficient of determination (R^2) values were much closer to 1 for the Higuchi kinetics, thus indicating that drug release from the microspheres followed diffusion controlled mechanism.

Table 3: Kinetics of *in vitro* ORDZ release from drug loaded chitosan microspheres.

Batch code	Zero order		First order		Higuchi	
	k (mg h ⁻¹)	R	k (h ⁻¹)	R	K (mg h ^{-1/2})	R
A1	0.00595	0.936	-0.0119	-0.989	0.0716	0.936
A2	0.00586	0.887	-0.0123	-0.958	0.0733	0.887
A3	0.00576	0.873	-0.0123	-0.956	0.0730	0.978
B1	0.00586	0.940	-0.0115	-0.990	0.0704	0.998
B2	0.00585	0.933	-0.0112	-0.976	0.0704	0.993
C1	0.00559	0.933	-0.0101	-0.973	0.0672	0.992
C2	0.00535	0.937	-0.0093	-0.977	0.0641	0.995

k- release rate constant, R- coefficient of correlation.

To understand the mechanism of diffusion controlled release of ORDZ from microspheres, the results were further analysed according to equation (Sankar and Mishra, 2003)

$$Q(t) = at^n \quad (6)$$

Where Q(t) is the fraction of drug released after time t and a denotes a coefficient, n is a release exponent. When $n \leq 0.5$, this indicates a quasi-Fickian diffusion mechanism, for $n > 0.5$, an anomalous non-Fickian solute diffusion is observed, where as $n = 1$ indicates zero-order kinetics. Values of a and n are listed in table 4. The values of n were in the range of 0.29-0.51, which is further indication of the diffusion controlled drug release.

Table 4: Coefficient and exponent of ORDZ release from drug-loaded chitosan microspheres

Batch code	Equation coefficient (a)	Release exponent (n)	R ²
A1	1.59	0.45	0.997
A2	1.17	0.35	0.980
A3	1.28	0.29	0.994
B1	0.91	0.47	0.996
B2	0.84	0.50	0.983
C1	0.80	0.51	0.982
C2	0.78	0.51	0.986

R²– Coefficient of determination

Batch B2 exhibited optimum particle size 52.65 μm , 85.73 \pm 3.40 % bioadhesion and extended release of drug for 120 h. The smaller size of the microspheres is not preferable as the smaller microspheres less than 50 μm diameter can be removed from the periodontal pocket due to a high flow rate of gingival fluid (20 $\mu\text{l h}^{-1}$) (Park *et al.*, 2005). Therefore, batch B2 was taken as optimized formulation for further studies.

In vitro antimicrobial activity

The concentration of the drug in the samples remained well above the minimum inhibitory concentration (0.8 $\mu\text{g/ml}$) of

the drug for a period of 120 h (figure 7). After 24h, pure drug showed the maximum zone of inhibition (27.15mm). We found that the medium containing blank chitosan microspheres lacked antimicrobial activity. This may be due to the absence of ORDZ within the microspheres and insolubility of chitosan at pH 7.2. The *in vitro* antimicrobial activity demonstrated satisfactory antimicrobial profile for all the microspheres against microorganism i.e. (*Staphylococcus aureus*).

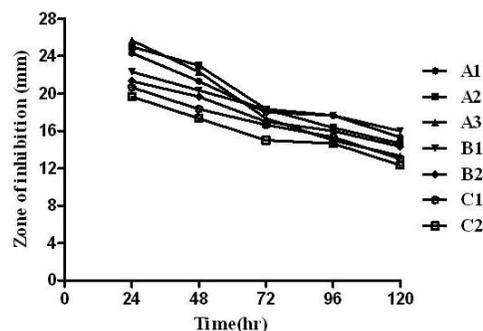


Fig. 7: *In vitro* antimicrobial activity of microspheres containing ORDZ.

Characterization of in-situ gel containing ORDZ microspheres Optimization of concentration of PF-127

Gelation temperature of plain PF-127 gels was observed for the concentration range of 16-20% w/w of PF-127 and it was found that gelation temperature of plain PF-127 gels decreased with increasing concentration of PF-127. When specified quantity of optimized formulation of microspheres based on the dose of ORDZ was added into PF-127 gels, the gelation temperature of formulation was found to be decreased significantly ($P < 0.05$). It was found that only 17% of PF-127 gel with optimized formulation of microspheres showed ability to form gel in the range of 25 to 32°C (table 5). Thus, 17% w/w concentration of PF-127 was used for further studies.

Table 5: Gelation temperature of poloxamer solutions (16-20% w/w)

Composition	Gelation Temperature (°C)	
	Mean	S.D.
PF-127 (16%)	43.3	0.45
PF-127 (16%) + Optimized formulation	39.43	0.64
PF-127 (17%)	31	0.62
PF-127 (17%) + Optimized formulation	27.9	0.45
PF-127 (18%)	24.03	0.77
PF-127 (18%) + Optimized formulation	20.93	1.00
PF-127 (19%)	23.66	0.90
PF-127 (19%) + Optimized formulation	19.56	0.70
PF-127 (20%)	22.3	0.55
PF-127 (20%) + Optimized formulation	18.93	0.68

PF-127- Pluronic flake 127.

PF-127 was selected due to its thermogelling property. In addition to this, it exhibited desirable gelation temperature range (25 to 32°C) in comparison to PF-188.

Gelation temperature

Gelation temperature for optimized plain PF-127 solution was observed at 30.36°C. When optimized formulation (batch B2)

of ORDZ microspheres was added into PF-127 gel, it was found that gelation temperature of formulation decreased (table 6).

Surface pH

Surface pH of plain gel and the gel with optimized formulation was found in the range of 5.9 to 6.2 which was nearer to the oral pH. It suggests prepared formulations having pH near to neutral pH and could improve the biocompatibility (table 6).

Table 6: Gelation temperature, surface pH, in-vitro bioadhesive force of plain and microspheres containing gel.

Formulation Code	Gelation temperature (°C) (Mean ± SD)	Surface pH (Mean ± SD)	Bioadhesive Force (dyne/cm ² × 10 ²) (Mean ± S.D)
Plain gel	31 ± 0.62	6.2 ± 0.1	49.04 ± 1.68
Optimized formulation	27.9 ± 0.45	5.96 ± 0.20	75.15 ± 3.82

Viscosity measurement

The plots of viscosity versus temperature studies of plain PF-127 gel and gel containing optimized ORDZ microspheres are shown in figure 8. There was no considerable change in viscosity up to the point of gelation temperature. Near the point of gelation temperature the viscosity was found to be increased, followed by sudden increase in viscosity at transition temperature. It was found that gel containing optimized ORDZ microspheres remained in liquid state at room temperature and converted into gel at oral physiological temperature.

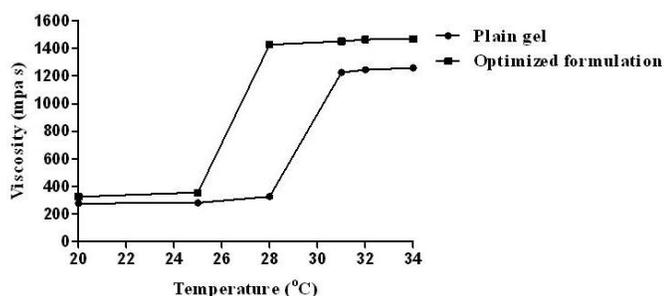


Fig. 8: Effect of temperature on viscosity of plain PF-127 gel and gel containing optimized ORDZ microspheres.

Determination of bioadhesive force

Bioadhesive force of plain gel and gel containing optimized formulation was found to be 49.04×10^2 and 75.15×10^2 dyne/cm² respectively (table 6). Addition of microspheres containing bioadhesive polymer (chitosan) into the gel produced further increase in the bioadhesive force. This may be due to interactions between the positively charged amino groups of chitosan and the negatively charged mucosa resulting into strong bonding.

In vitro drug release

In-vitro release studies were carried out by process containing donor compartment and receptor compartment using IPBS, pH 7.2. It was observed that the release rate of ORDZ was controlled for a period of 7 days (figure 9). This may be attributed

to combined effect of viscous nature of gel that increased contact period and prolongation of drug release due to chitosan microspheres.

The drug release kinetics and mechanism was examined by fitting the release data into the models representing zero-order, first-order and Higuchi's square root of time. The linear regression analyses are summarized in table 7. The coefficient of correlation (R^2) value was much closer to 1 for the Higuchi kinetics, thus indicating that drug release from the microspheres followed diffusion controlled mechanism. The data obtained was further analysed for 'n' values (table 8). The value of n was 0.57, indicating that the mechanism of drug release was diffusion controlled.

Table 7: Kinetics of *in vitro* ORDZ release from gel containing optimized chitosan microspheres.

Batch code	Zero order		First order		Higuchi	
	k (mg h ⁻¹)	R	k (h ⁻¹)	R	k (mg h ^{-1/2})	R
Optimized formulation	0.0043	0.948	-0.0087	-0.990	0.062	0.995

k- release rate constant, R- coefficient of correlation

Table 8. Coefficient and exponent of ORDZ release from gel containing optimized chitosan microspheres

Batch code	Equation coefficient (a)	Release exponent (n)	R ²
Optimized formulation	1.43	0.57	0.991

R² – Coefficient of determination.

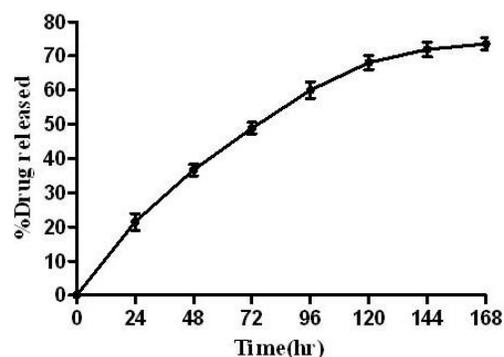


Fig. 9: *In-vitro* release study of gel containing optimized ORDZ microspheres in IPBS, pH 7.2.

CONCLUSION

Microspheres containing ORDZ with appropriate physical features and drug content was successfully prepared by using chitosan which provided good bioadhesion of the microspheres as well as exhibited sustained release of drug. With the help of in situ gel, microspheres can be easily administered into periodontal pocket and release of drug can also be extended. The developed formulation may be proposed as a promising system for delivery of ORDZ for local treatment of periodontitis. It may help to achieve adequate drug concentration in periodontal pocket and control its release. Further, *in vivo* studies are required to confirm the efficacy of the formulation.

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