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Optimisation and characterisation of bioadhesive controlled release tetracycline microspheres

S. Govender [a], V. Pillay [b], D.J. Chetty [c], S.Y. Essack [a], C.M. Dangor [a], T. Govender [a,*]

[a] *School of Pharmacy and Pharmacology, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa*
[b] *Department of Pharmacy and Pharmacology, University of the Witwatersrand, 7 York Road, Parktown 2193, Johannesburg, South Africa*
[c] *GlaxoSmithKline, 1500 Littleton Road, Parsippany, NJ 0705, USA*

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Abstract

A Box-Behnken experimental design was employed to statistically optimise the formulation parameters of a tetracycline microsphere preparation for maximum bioadhesivity and controlled drug release. The quantitative effect of the formulation parameters at different levels on bioadhesion and drug release could be predicted using polynomial equations. A formulation comprising of 3% (w/w) chitosan, 10% (w/w) tetracycline HCl and 9% (w/v) tripolyphosphate was identified for maximising bioadhesivity and obtaining controlled drug release. The optimal microsphere preparation was subsequently characterised in terms of hydration dynamics, release kinetics, antimicrobial activity, thermal properties, morphology and surface pH. Kinetic models revealed that drug release followed Fickian diffusion while textural analysis showed minimal hydration over the test period. Antimicrobial studies showed that the drug concentrations in the in vitro release samples were above the minimum concentration of drug required for inhibition of *Staphylococcus aureus* growth. Thermal analyses showed a possible interaction between the drug and polymer. Scanning electron microscopy confirmed the integrity of the microspheres and identified the morphological changes following drug release. Surface pH of the microspheres was similar to salivary pH and did not show extremes in changes over the test period.

Keywords: Bioadhesion; Microspheres; Periodontal therapy; Experimental design; Physicochemical characterisation

1. Introduction

Dental diseases are recognised as one of the major and most common diseases afflicting mankind throughout the world (Uzunoglu et al., 2000). Periodontitis is

* Corresponding author. Tel.: +27 31 260 7358; fax: +27 31 260 7792.
E-mail address: govenderth@ukzn.ac.za (T. Govender).

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a general term, which encompasses several conditions, such as chronic periodontitis, aggressive periodontitis, systemic disease-associated periodontitis and necrotising periodontitis in which the supporting structures of the teeth are affected (Schwach-Abdellaoui et al., 2000). Maximising drug therapy for this condition may prove beneficial where systemic drugs are currently used, e.g. localised juvenile periodontitis, refractory periodontitis and periodontitis with secondary involvement, e.g. HIV periodontitis. Oral health care forms an integral component of patient management and promoting adherence to antiretroviral treatment. Systemic administration of antibiotics for the treatment of periodontitis have shown several drawbacks including: inadequate antibiotic concentration at the site of the periodontal pocket (Pitcher et al., 1980; Vanderkerchove et al., 1997); a rapid decline of the plasma antibiotic concentration to sub-therapeutic levels (Gates et al., 1994); 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 (Slots and Rams, 1990; Mombelli and Van Winkelhoff, 1997).

These obvious disadvantages have evoked an interest in the development of localised drug delivery systems that can provide an effective concentration of antibiotic at the periodontal site for the duration of the treatment with minimal side effects. The potential advantages of a localised controlled release drug delivery system for insertion into and/or around the periodontal pocket include the following: increased local drug concentration at the periodontal site to maintain an effective concentration of antibiotic; a decrease in superfluous distribution of the drug to other body organs with a subsequent decrease in side effects; a decrease in the amount of administered dose and hence a decrease in manufacturing costs; the maintenance of drug levels in a therapeutically desirable range over an extended period of time which may lead to improved patient compliance due to the reduction in the frequency of administration of doses and decreased side effects (Gates et al., 1994; Bromberg et al., 2000; Uzunoglu et al., 2000). 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.

A prolonged retention at the mucosal surface provides intimate contact between the dosage form and absorbing tissues that results in a prolonged period of drug exposure to the region. Therefore, an increased retention time is a desirable property of bioadhesive drug delivery systems. Retention time has been shown to be increased with an increase in the bioadhesivity measurement of the system (Yun et al., 1999; Yong et al., 2001). Maximising the bioadhesive forces of these systems therefore remains a significant goal in the development phase of bioadhesive drug delivery systems.

In addition to bioadhesivity, controlling the release of a drug from the dosage form is also desirable. Controlled drug delivery systems should provide a continuous delivery of drugs at predictable and reproducible kinetics for a predetermined period. The potential advantages of this concept include the minimisation of drug-related side effects (due to controlled therapeutic blood levels instead of oscillating blood levels) and improved patient compliance (due to reduced frequency of dosing). Therefore, a periodontal delivery system with controlled drug release, good retention in and around the periodontal pocket, ease of delivery and biodegradability is desirable. It is therefore clear that the challenge in the formulation of novel systems for bioadhesive drug delivery is to identify technologies and formulation excipients to simultaneously optimise both the bioadhesivity and drug release kinetics. Thus far in the literature, very few studies have focused on a systematic approach (experimental designs) to optimise microparticulate formulations for bioadhesivity and drug release kinetics. Rather, formulations were considered suitable by virtue of demonstrating a controlled drug release profile and displaying some bioadhesivity. Clearly, for suitable therapeutic outcomes, these two properties need to be optimised in a delivery system, to achieve maximal prolonged retention times and specified release kinetics. While tetracycline HCl-chitosan microspheres have recently been prepared (Hejazi and Amiji, 2004), to date, there exists a lack of studies regarding a statistical optimisation of formulation parameters to enhance both the bioadhesivity and drug release of chitosan microspheres prepared by the ionotropic gelation method. While the effect of formulation parameters on drug release from chitosan microspheres has previously been investigated (Lim et al., 1997; Gupta and Ravi Kumar, 2000; Dini et al., 2003),

there remains a need for studies investigating the quantitative effects of these parameters on the bioadhesivity of chitosan microspheres. The aim of this study was therefore to identify optimal formulation parameters for a tetracycline microsphere preparation with maximum bioadhesion and controlled drug release. Also a more detailed physicochemical/mechanical characterisation and mechanistic understanding of bioadhesive tetracycline microspheres is essential for optimisation of this system. Therefore, another aim was to undertake a physicochemical/mechanical characterisation of the optimal microsphere formulation in terms of hydration dynamics, release kinetics, thermal properties, antimicrobial activity, morphology and surface pH.

2. Materials and methods

2.1. Materials

Chitosan, with a M_w of 110,000 Da and a deacetylation degree of 98%, was purchased from Primex Ingredients (ASA, Norway) and used as obtained. Tetracycline HCl was purchased from Frankel Chemicals (SA). Porcine mucin and sodium tripolyphosphate (TPP) were purchased from Sigma–Aldrich (UK). Lactic acid was obtained from BDH Lab Supplies (UK). Distilled deionised water was used throughout the studies. All other chemicals used were of pharmaceutical grade.

2.2. Methods

2.2.1. Preparation of microspheres

The starting procedure was as follows: chitosan (6 g) and tetracycline HCl (1.5 g), equivalent to a theoretical loading of 25% (w/w), were accurately weighed and dissolved in a 1% (w/v) lactic acid solution. The tetracycline-chitosan dispersion (200 mL) was added drop wise via a 2 mm diameter nozzle into gently agitated solutions (400 mL) of sodium tripolyphosphate (TPP) (3%, w/v) by employing a peristaltic pump (Aeromatic AG, Switzerland) at a flow rate of 3.5 mL/min. The droplets instantaneously gelled into discrete tetracycline-chitosan microspheres upon contact with the surface of the TPP solution.

The tetracycline microspheres were left overnight in a dark area to cure in the TPP solution. After this period, the TPP solutions were decanted and the microspheres were washed several times with distilled deionised water. The batches were spread on aluminium trays and oven dried (Series 2000, Scientific, SA) at 40 °C until constant weight.

2.2.2. Experimental design

A Box-Behnken experimental design was employed to statistically optimise the formulation parameters of a tetracycline microsphere preparation for maximum bioadhesivity and controlled drug release. Response surface methodologies, such as the Box-Behnken and Central Composite Designs, model possible curvature in the response function. The Box-Behnken design was specifically selected since it requires fewer treatment combinations than a Central Composite Design in cases involving three or four factors. The Box-Behnken design is also rotable and contains statistical "missing corners" which may be useful when the experimenter is trying to avoid combined factor extremes. This property prevents a potential loss of data in those cases.

Generation and evaluation of the statistical experimental design were performed with the Microsoft Excel 2002 Add-In, Essential Regression and Experimental Design Software Version 2.2 (USA). The studied factors were tetracycline hydrochloride concentration (TC), tripolyphosphate concentration (TPP) and chitosan concentration (CC), as these factors were shown in preliminary studies to have a significant effect on both bioadhesivity and drug release. The response variables were the maximum detachment force (MDF) and the mean dissolution time (MDT) measured from the microspheres. In order to produce formulations displaying an optimal MDF with a controlled release profile, formulations were optimised for bioadhesivity and then assessed for controlled drug release. In the same way, these formulations were optimised for MDT and then assessed for bioadhesion.

A design matrix comprising of 16 experimental runs was constructed. An interactive second order polynomial model was utilised to evaluate both the response variables:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1X_1 + b_5X_2X_2 + b_6X_3X_3 + b_7X_1X_2 + b_8X_1X_3 + b_9X_2X_3 \quad (1)$$

Table 1
Independent variables: factors and their levels for the Box-Behnken design

Factors	Level		
	−1	0	1
X_1: Tetracycline concentration (TC)	10	17.5	25
X_2: Tripolyphosphate concentration (TPP)	3	6	9
X_3: Chitosan concentration (CC)	3	5	7

where b_0–b_9 are the regression coefficients, X_1–X_3 the factors studied and Y is the measured response associated with each factor level combination.

Preliminary studies provided a setting of the levels for each formulation variable. Table 1 summarises the factors and their levels and Table 2 summarises the design matrix with the experimental runs, factor levels and combinations and the measured responses, i.e. MDF and MDT.

2.2.3. Evaluation of the tetracycline loaded microspheres

2.2.3.1. Uniformity of drug content and size analyses. Oven dried microspheres (500 mg) were crushed in a mortar and pestle and transferred into 100 mL volumetric flasks containing a small amount of phosphate buffer pH 6.8. The flasks were kept in an ultrasonic bath for 30 min and thereafter made up to volume. Solutions were filtered (0.45 μm Cameo® filters) and the amount of drug was measured by ultraviolet spectroscopy at a λ_{max} of 275 nm (Shimadzu UV–vis 1650 PC spectrophotometer, Japan). Assays were performed in triplicate and standard deviations were within approximately 2%.

Determination of microsphere size was undertaken using sieve analyses using a Retsch sieve agitator. Microspheres in the range (1400–1700 μm) were employed for the purposes of this study as the highest percentage yield of microspheres (85.02%) was obtained in this size range.

2.2.3.2. Bioadhesivity. The in vitro bioadhesion of the microspheres was evaluated with a digital force gauge (Lutron FG5000A, Korea) attached to a crosshead pulley. An excess of microspheres was thinly sprinkled onto an aluminium disc 13 mm in diameter, pre-coated with a thin film of cyanoacrylate adhesive in order to retain the microspheres. The microspheres were hydrated with phosphate buffer pH 6.8 (30 μL) for a period of 3 min. Thereafter, the disc containing the attached microspheres was attached to the force gauge using a thin non-elastic connector. The force gauge was then lowered by a crosshead pulley in such a way that the surface of the microspheres was in contact with a mucin suspension (30%, w/w) contained in a glass petri

Table 2
Experimental runs, factor combinations and mean maximum detachment forces for the Box-Behnken design

Experiment	Factor combinations at different levels			MDF (mN)	$MDT_{13\%}$ (h)
	TC (%, w/w)	CC (%, w/w)	TPP (%, w/v)		
1[a]	17.5	5	6	240	20.87
2	25	5	9	180	17.02
3[a]	17.5	5	6	250	25.07
4	17.5	3	3	150	24.79
5	10	7	6	150	32.57
6	10	5	3	220	42.51
7	25	5	3	130	14.37
8	10	3	6	430	29.89
9	25	7	6	120	12.91
10	25	3	6	410	13.71
11	17.5	3	9	300	31.77
12[a]	17.5	5	6	240	20.81
13	10	5	9	470	19.85
14[a]	17.5	5	6	260	20.47
15	17.5	7	9	300	16.99
16	17.5	7	3	210	20.93

[a] Indicates the center points of the design.

dish placed on a thermostatically controlled water bath (37 ± 0.5 °C). On contact with the mucin, a constant force of 0.2 N using a mass piece was maintained for 5 min. Thereafter, the crosshead pulley was raised at a speed of 15 mm/s until a peak detachment force was obtained. The mean ± S.D. of 10 individual replicates were expressed as the force required to separate the microspheres from the mucin (maximum detachment force). As a control, the bioadhesion experiments were conducted on drug-free non-pareils (sugar beads without a bioadhesive component).

2.2.3.3. In vitro drug release kinetics. For the purpose of this study, a shaking water bath apparatus was used (Memmert, Germany). The dissolution medium comprised of phosphate buffer saline (PBS) (pH 6.8) (100 mL) (37 ± 0.5 °C). At the start of the dissolution test (0 min), a specific quantity of microspheres was emptied into the dissolution bottles and the apparatus agitated (100 strokes/min). At the end of the predetermined time intervals (e.g. 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7 and 8 h), aliquots (2 mL) were removed from each bottle and filtered through 0.45 μm Cameo® filters. An equal volume of drug-free medium (2 mL) to that of the aliquot removed was replaced into each dissolution vessel, to maintain a constant volume of medium during the dissolution test. The samples (suitably diluted as required) were analysed using UV spectroscopy at a λ_{max} of 275 nm (Shimadzu UV–vis 1650 PC spectrophotometer, Japan) to determine the percentage drug released. All experiments were performed in triplicate. For determination of drug release mechanisms from the optimal formulation, the dissolution data was modelled using using WinNonlin®, Version 3.1 (Pharsight, CA). The Power Law expression together with its geometry-independent form (Peppas, 1985) as well as the Hopfenberg model (Hopfenberg, 1976), a geometry dependent equation, were applied to the drug release data.

2.2.3.4. Antimicrobial efficacy. For the purpose of this study, the antimicrobial activity of drug-loaded, drug-free microspheres and tetracycline HCl alone was evaluated. Each experiment was carried out in triplicate. For the microspheres, samples collected from the in vitro release study of drug-loaded and drug-empty microspheres at different time intervals (0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7 and 8 h) were tested against *Staphylococcus aureus* ATCC 29213, one of the isolates found in periodontitis patients (Parthasarathy et al., 2002; Saini et al., 2003). Tetracycline HCl (pure drug) at different concentrations in phosphate buffered saline (pH 6.8) (0–128 μg/mL) was also tested against the same strains.

Molten agar media was transferred to sterilised petri dishes and allowed to solidify. The plates were swabbed with the culture of the microorganism. Wells, equidistant from one another were made in the solidified medium using a sterilised well borer. The solutions (100 μL) collected (in vitro drug release aliquots and pure drug solutions) were filtered through sterilised Millipore membrane filters (0.45 μm) and carefully filled into the wells. Samples were allowed to diffuse for 2 h at room temperature. The plates were then incubated for 48 h at 37 ± 0.5 °C. The diameter (mm) of zone of growth inhibition surrounding each agar well was measured using a calliper.

2.2.3.5. Hydration dynamics. To determine the hydration dynamics of the tetracycline microspheres, a Textural Profile Analyser (XT2*i*, Stable Micro Systems, UK) fitted with a 2 mm cylindrical steel probe and 25 kg load cell was used. Textural profile analysis (TPA) was performed by placing microspheres in the buffer medium pH 6.8 (37 ± 0.5 °C) and hydrating them over 8 h. At appropriate time intervals, the microspheres were removed and subjected to textural analysis. At each sample point, a quantity of 10 microspheres was analysed. During a typical test, the probe was advanced at a predetermined velocity into the sample in accordance with the following parameters: pre-test and post-test speeds, 2 mm/s; test speed, 1 mm/s; maximum compression force, 40 N; trigger force, 0.1 N. Data acquisition was performed at 200 points/s via the Texture Expert for Windows Software, Version 1.20. The data are presented as peripheral hydration zones (mm) versus hydration time (h) and matrix deformation energy (J) versus hydration time (h).

2.2.3.6. Erosion and swelling. Erosion and swelling of the microspheres were determined under conditions identical to those described for the dissolution testing. Water uptake and mass loss were determined gravimetrically according to the following equations (Durig and Fassihi, 2002; Seo et al., 2002):

$$\text{Degree of swelling (water uptake)} = \frac{\text{wet weight} - \text{original dry weight}}{\text{original dry weight}} \quad (2)$$

$$\text{Erosion (\% mass loss)} = \frac{\text{original weight} - \text{remaining dry weight}}{\text{original weight}} \times 100 \quad (3)$$

At predetermined times, the hydrated microspheres were carefully removed from the dissolution bottles and tapped gently with tissue paper to remove excess surface water. After determining the wet weight, the microspheres were dried at 40 °C until constant weight, before reweighing to determine the remaining dry weight. Experiments were performed in triplicate.

2.2.3.7. Thermal properties. DSC experiments were performed on excipients, drug-loaded microspheres and drug-empty microspheres. Thermograms were captured using a Mettler-Toledo TC15, TA Controller System (Switzerland) with a differential scanning calorimeter equipped with a computerised data station (Mettler DSC 20, Mettler-Toledo AG, Switzerland). An indium calibration was performed for the analyses. The heating rate (30–450 °C) was 10 °C per min. Measurements were performed in triplicate.

2.2.3.8. Morphology. The morphology of the microspheres was determined by scanning electron microscopy. No chemical fixation or freeze drying methods were used in the preparation of samples for SEM. In this study, samples were mounted on round brass stubs and sputter coated under an argon atmosphere with gold–palladium using a Polaron SEM Coating Unit E5000 (Japan). Samples were examined under the Philips SEM 500 scanning electron microscope.

2.2.3.9. Surface pH. As acidic or alkaline pH may cause irritation to the oral mucosa (Bottenberg et al., 1991), it was necessary to determine whether the surface pH of the microspheres could be maintained close to neutral pH during the retention and drug release period. A specific quantity of microspheres was allowed to swell in contact with 1 mL of phosphate buffer (pH 6.8) in glass tubes. The surface pH was noted by bringing a glass micro-electrode (Mettler Instrumente, Germany) near the surface of microspheres and allowing it to equilibrate for 1 min. Measurements were taken after hydrating the microsphere surfaces for 3 min and thereafter at specific time intervals (i.e. 2, 4, 6 and 8 h). Experiments were performed in triplicate.

3. Results and discussion

3.1. Formulation optimisation

3.1.1. Fitting of bioadhesion data to the model

Based on the experimental design, the factor combinations yielded different mean maximum detachment forces. Table 2 summarises the experimental runs, their factor combinations and the levels of experimental units used in the study as well as the bioadhesive forces obtained for each factor combination. In order to determine the levels of factors which yielded optimal bioadhesivity, mathematical relationships were generated between the dependent and independent variables. Using the software described earlier, the model was fitted to the data. Repeated backward stepwise regression was used to eliminate the insignificant effects and to generate the equation for the response parameter (MDF). The regression equation together with the statistically significant coefficients and the regression significance generated for the response variable from the above procedure is presented in Table 3.

The initial model was refined to include in the model only those terms for which the level of significance was below or equal to $P \leq 0.05$. Statistical testing (ANOVA) indicated that the regression model obtained was statistically significant ($P = 0.011$).

Table 3
Regression equation, significant coefficients, terms and regression significance of the MDF model

Coefficients	Term	Value	*P*-value
b_0		278.12	0.00851
b_1	TPP	45.40	0.00534
b_2	CC	−31.87	0.03600
b_3	TC × TPP	−1.309	0.03926
Regression significance			0.011

Response = $b_0 + b_1 \times \text{TPP} + b_2 \times \text{CC} + b_3 \times \text{TC} \times \text{TPP}$.

3.1.2. Examining the coefficients for maximum bioadhesivity and response surface plots

The resultant equation for maximum detachment force is given below:

$$\text{MDF} = 278.12 + 45.40 \times \text{TPP} - 31.87 \times \text{CC} - 1.309 \times \text{TC} \times \text{TPP} \quad (4)$$

The equation represents the quantitative effect of the formulation parameters upon the response. Following the resultant polynomial equation (Eq. (4)) and Fig. 1a and b, it can be seen that tripolyphosphate concentration had a positive effect on bioadhesion. It has been documented that flexibility of the polymer chains is required for interpenetration and entanglement with mucin. Highly crosslinked polymers decrease the mobility of the individual polymer chains and can therefore lead to decreased bioadhesive strength (Ahuja et al., 1997; Vasir et al., 2003). Therefore, these findings were contrary to what was expected; namely a decrease in the bioadhesive force with an increase in TPP due to an increased crosslinking and hence decrease in the effective length of the polymeric chain for penetration into the mucus layer and hence bioadhesion. Rather, the increased bioadhesion with an increase in crosslinking could be attributed to the fact that an increased interaction of chitosan with the negatively charged TPP instead of mucin may have led to more sites on the negatively charged sialic acid residues of mucin being additionally available for interaction with the positively charged drug.

Chitosan concentration showed a negative effect on bioadhesion. At a higher chitosan concentration, coiling of the polymer molecules may have occurred reducing the flexibility of the polymeric chain thereby reducing the bioadhesive strength. At lower chitosan concentrations, the polymer structure of the microspheres may have been looser and the polymer chains therefore had more space to extend within the mucin.

While the effect of drug concentration itself was a statistically insignificant main effect, a negative

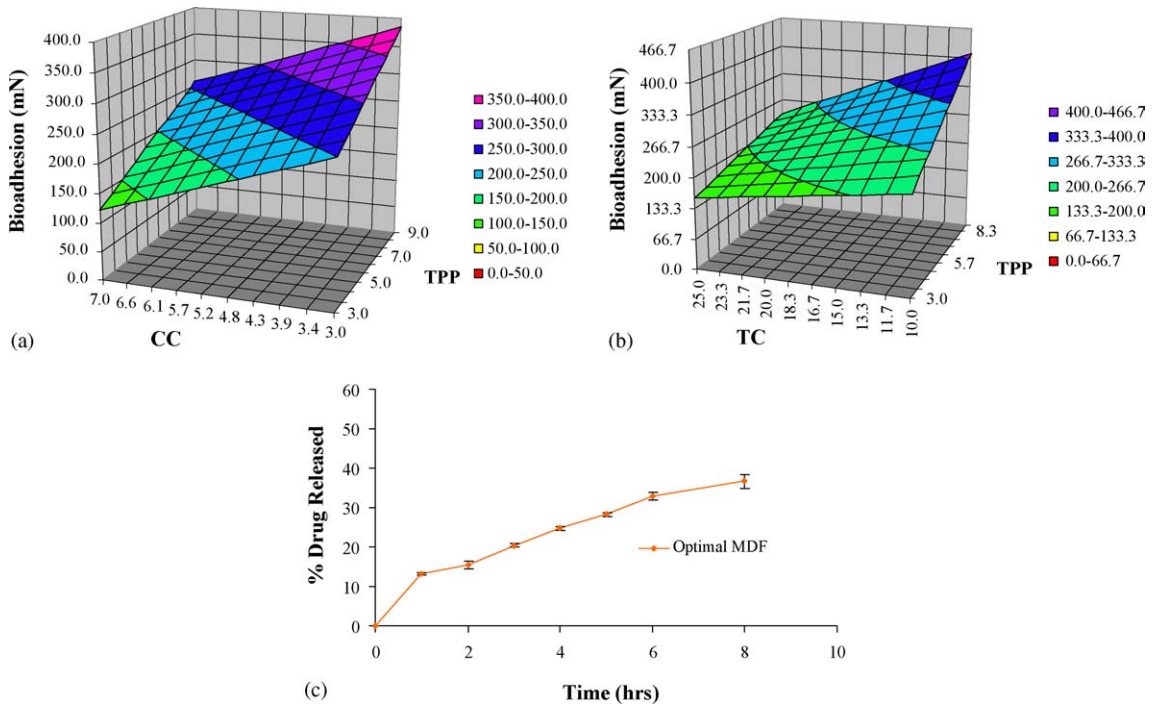

Fig. 1. (a) Response surface plot illustrating the influence of tripolyphosphate (TPP) and chitosan concentration (CC) on bioadhesion. (b) Response surface plot illustrating the influence of tripolyphosphate (TPP) and tetracycline HCL loading on bioadhesion. (c) Drug release profile of optimal formulation identified from the MDF model.

interaction effect between drug concentration and tripolyphosphate concentration was identified. Thus far, studies on the quantitative and combined effects of formulation parameters on the bioadhesion of microspheres have been lacking.

3.1.3. Prediction of the optimal bioadhesive formulation

Using the model generated with the MDF as the response variable (Eq. (4)), the optimisation tool in the experimental design software was used to identify a formulation with maximum bioadhesivity. It predicted a MDF of 473 mN with a formulation comprising of 10% (w/w) tetracycline HCl concentration, 9% (w/v) TPP concentration and 3% (w/w) chitosan concentration. Using this formulation, a batch of microspheres was prepared and its bioadhesivity was measured. The actual experimental value obtained was 469 ± 20.25 mN ($n = 10$). Since the predicted value (473 mN) and experimental value (469 mN) obtained were statistically similar (Wilcox test, $P = 0.548$) ($P > 0.05$), the model generated in this study was validated.

The in vitro drug release profile of the optimal formulation identified above was determined. A controlled release profile was observed (Fig. 1c), with only 36.65% drug release measured at the end of 8 h. Although tetracycline HCl is a freely soluble drug, its release behaviour is strongly influenced by the nature of crosslinking, microsphere structure and swelling/erosion dynamics. On the basis of the concept of swellable microspheres, drug release from the microspheres would be markedly hindered if optimum swelling were not attained (Pillay and Fassihi, 1999). Thus, at pH 6.8, the chitosan microspheres did not demonstrate significant swelling (Fig. 4; Section 3.2.2) or solubility hence retarding drug release. Other studies have also reported such retarded drug release for the local treatment of periodontitis (Schwach-Abdellaoui et al., 2000; Perugini et al., 2003).

3.1.4. Fitting of the dissolution data to the model

Since only 36.65% drug was released at the end of 8 h, the possibility of obtaining a formulation displaying a controlled release profile with a higher drug release at the end of 8 h was further investigated. The mean dissolution time ($MDT_{13\%}$) determination was applied to analyse dissolution profiles obtained for all formulations constructed from the experimental design. The MDT is defined as the sum of different release fraction periods (release areas) during dissolution studies divided by the initial loading dose as calculated by the following equation (Kim and Fassihi, 1997):

$$\mathrm{MDT} = \sum_{i=1}^{n} t_i \frac{M_t}{M_\infty} \tag{5}$$

where M_t is the fraction of dose released in time $t_i = (t_i + t_{i-1})/2$ and M_∞ is the total amount of drug released. Since, a possibility to increase the drug release was considered increasing the MDT was employed to generate a formulation with the highest amount of drug released at the end of the test period. Based on the experimental design, the factor combinations yielded different mean dissolution times. Table 2 summarises the experimental runs, factor combinations, levels of experimental units as well as the mean dissolution times obtained for each factor combination.

In order to determine the levels of factors which yielded optimal mean dissolution time, mathematical relationships were once again generated between the dependent and independent variables. Using the software described earlier, the model was fitted to the data. The regression equation together with the statistically significant coefficients and the regression significance generated for the response variable from the above procedure are presented in Table 4. The initial model represented by Eq. (1) was refined by including in the model only those terms for which the level of significance was below or equal to $P \leq 0.05$. Statistical testing (ANOVA) indicated that the regression model obtained was statistically significant.

Table 4
Regression equation, significant coefficients, terms and regression significance of the MDT model

Coefficients	Terms	Value	*P*-value
b_0		76.04	8.943×10^{-6}
b_1	TC	−2.801	0.000289
b_2	TPP	−4.600	0.01670
b_3	TC × TPP	0.281	0.00670
Regression significance			0.00027

Response = $76.04 + b_1 \times \mathrm{TC} + b_2 \times \mathrm{TPP} + b_3 \times \mathrm{TC} \times \mathrm{TPP}$.

3.1.5. Examining the coefficients for maximum drug release and response surface plots

The mathematical relationship generated for the response variable (MDT) is expressed as the polynomial equation:

$$\text{MDT} = 76.04 - 2.801 \times \text{TC} - 4.600 \times \text{TPP} + 0.281 \times \text{TC} \times \text{TPP} \quad (6)$$

Eq. (6) indicates that tetracycline HCl concentration and TPP concentration had a negative main effect on the MDT, i.e. at high drug and TPP concentrations, the rate of release of the drug from the chitosan microspheres decreased. In addition, an interaction effect between tetracycline HCl concentration and TPP concentration affected the MDT positively.

3.1.6. Prediction of the optimal drug release formulation

Using the model generated with MDT as the response variable (Eq. (6)), the optimisation tool in the experimental design software was used to identify a formulation with a maximum MDT (i.e. fastest drug release achievable). It predicted a maximum MDT of 40.82 with a formulation comprising of 10% (w/w) tetracycline HCl concentration, 3% (w/v) TPP concentration and 3% (w/w) chitosan concentration. Using this formulation, three batches of microspheres were prepared and an in vitro drug release study was performed. The actual experimental MDT obtained was 40.42 ± 3.37. The predicted and experimental values were found to be statistically similar (Wilcox test, $P = 0.548$) ($P > 0.05$) thus validating the model generated in this study.

3.1.7. Selection of an optimal formulation for bioadhesivity and drug release

Using the Box-Behnken experimental design, two formulations were obtained: one displaying a maximum bioadhesivity (optimal MDF) and the other maximum drug release (optimal MDT). In order to select a formulation with suitable bioadhesion and drug release over the 8 h period, the maximum detachment forces and dissolution profiles of both formulations were compared. The bioadhesion data and the drug release profiles of the optimal MDF formulation and optimal MDT formulation are presented in Fig. 2a and b.

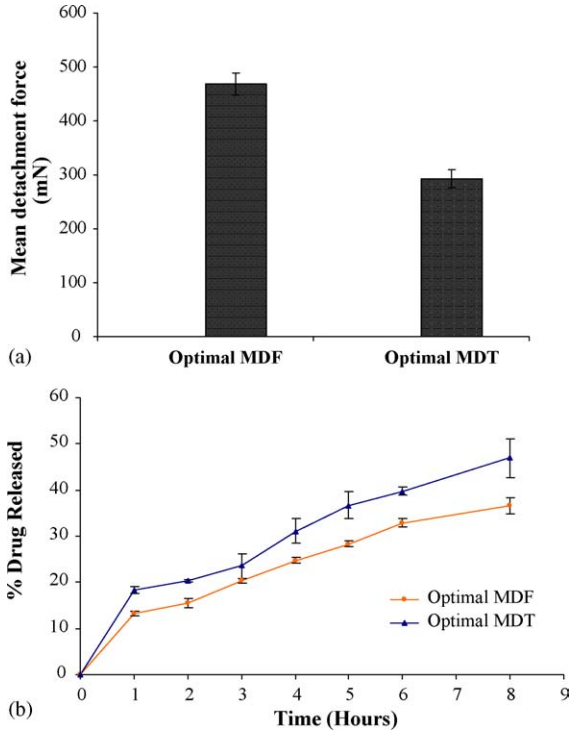

Fig. 2. (a) Comparison of maximum detachment forces of the optimal formulations from the two models. (b) Comparison of dissolution profiles from optimal formulations generated from the MDT and MDF models.

The bioadhesion results (Fig. 2a) showed that the optimal MDF formulation exhibited a higher maximum detachment force (469 ± 20.25 mN) than the optimal MDT formulation (293 ± 17.03 mN). Based on the bioadhesion results it can be seen that the optimal MDF formulation was far more likely to prolong the retention time on the mucosa than the optimal MDT formulation.

From the drug release results (Fig. 2b) it can be seen that the release of tetracycline HCl from the optimal MDT formulation was higher than that of the optimal MDF formulation, i.e. at 1 h the optimal MDT formulation showed 18.19% drug released compared to 13.23% from the optimal MDF formulation. At the end of the 8 h test period the optimal MDT formulation showed 46.93% drug released while the optimal MDF formulation showed 36.65% drug released. However, on closer examination of the dissolution profiles, it was observed that the release profiles of both the optimal formulations appeared to be similar. In order to confirm the similarity of these dissolution profiles the *similar-*

ity factor were used. The similarity factor denoted as f_2 (Moore and Flanner, 1996) directly compares the similarity between percentage drug dissolved per unit time for a test and reference product, in this case optimal MDF and optimal MDT. The similarity factor (f_2) is a logarithmic transformation of the sum-squared error of differences between the test T_j and reference product R_j over all time points:

$$f_2 = 50 \log \left\{ \left[1 + \left(\frac{1}{n} \right) \sum_{j=1}^{n} |R_j - T_j|^2 \right]^{-0.5} \right\} \times 100 \quad (7)$$

In general, f_2 values higher than 50 (50–100) show similarity of the dissolution profiles. The calculated f_2 value obtained in this study was 61.03, indicating that the dissolution profiles of both optimal formulations were indeed similar and drug release with the formulation from the MDT model not significantly faster.

Although the optimal MDT formulation showed a slightly faster drug release than the optimal MDF formulation, the release of the drug from both formulations was similar. Furthermore, the bioadhesivity of the optimal MDT formulation was significantly lower than that of the optimal MDF formulation. Therefore, the slightly faster release provided by the optimal MDT formulation did not warrant its choice as the optimal formulation for the local treatment of periodontal diseases. Hence, based on the results obtained, the optimal MDF formulation comprising of 3% (w/w) chitosan, 10% (w/w) tetracycline HCl and 9% (w/v) TPP was selected as the optimal formulation for localised periodontal therapy.

3.2. Characterisation of the optimised formulation

The optimal formulation identified via the Box-Behnken design was characterised in terms of hydration properties, drug release mechanisms, antimicrobial activity, thermal behaviour, morphology and surface pH and the results are presented hereunder.

3.2.1. Hydration dynamics

To investigate the swelling behaviour of the optimised bioadhesive controlled release formulation, the chitosan microspheres were subjected to textural profile analysis. The data were expressed as peripheral hydration zones (mm) versus hydration time (h) and matrix deformation energy (J) versus hydration time (h). The peripheral hydration zone is calculated as the distance travelled by the probe prior to any significant deflection in force. Matrix deformation energy is the work performed in the penetration of the hydrated or unhydrated matrices. This value is calculated from the AUC of a force applied (N) versus distance plot.

Textural measurements revealed that minimal hydration of the microspheres occurred (Fig. 3a and b). This may be related to the reduced solubility of chitosan at pH 6.8 and hence its inability to hydrate in this media. In addition, the profiles suggest that the chitosan microspheres had a highly crosslinked internal matrix while the peripheral area was porous. This porous peripheral zone had the ability to attract the influx of buffer medium. However, the dense internal structure may have resisted the diffusion of the buffer medium. This is apparent from the rapid initial hydration of the microspheres which occurred within 0.5 h, followed by a plateau indicating the absence of any further hydration. These results correlated with the results obtained in the swelling study where rapid swelling occurred within the first hour and thereafter remained constant (Fig. 4).

The degree of hydration showed an excellent correlation with the energy required for matrix rupture (deformation), i.e. due to the absence of significant

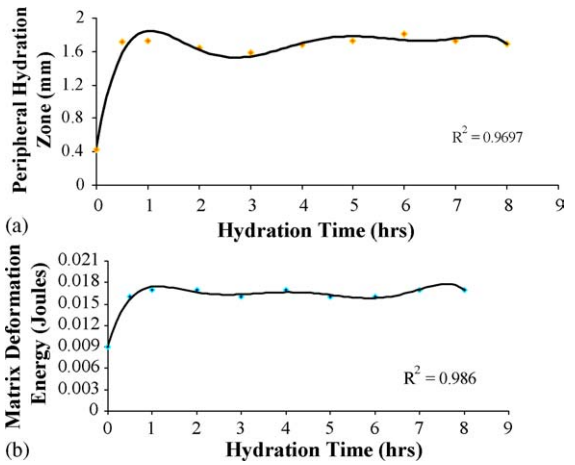

Fig. 3. (a) Hydration dynamics of the optimised chitosan microspheres. (b) Matrix deformation energy of the optimised chitosan microspheres.

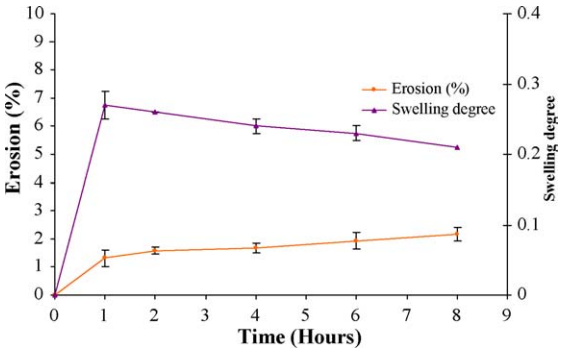

Fig. 4. Erosion and swelling behaviour of the optimised chitosan microspheres.

hydration, the microspheres were highly resistant to rupture but tended to fracture instead. Hence, the deformation energy was not lowered with increasing exposure of the microspheres to the hydration medium (Fig. 3b). The sixth order polynomial regression coefficients of the peripheral hydration zone and matrix deformation energy were 0.9697 and 0.986, respectively, which essentially indicates that both profiles were similar in shape, indicating their close correlation.

3.2.2. Erosion and swelling

The swelling and erosion behaviour of the optimised chitosan microspheres in phosphate buffered saline pH 6.8 is shown in Fig. 4. It can be seen that the chitosan microspheres displayed a limited amount of swelling with a maximum degree of swelling achieved after 1 h of exposure to the dissolution medium. Thereafter, minimal changes in the swelling degree took place. This minimal swelling is attributed to the reduced solubility of chitosan at pH 6.8 (Singla and Chawla, 2001) and consequently the inability to absorb water and swell. In addition, the limited amount of swelling may have been related to the degree of crosslinking. In addition, it can be seen that minimal erosion of the microspheres occurred over the 8 h period.

3.2.3. Drug release kinetics

Dissolution data derived for the chitosan microsphere formulation were subjected to model analysis to determine the mechanism of drug release. Table 5 outlines the model used and illustrates the data generated by use of the kinetic models.

The Akaike Information Criterion (AIC) is a measure of the goodness of fit of a particular model based on the maximum likelihood. When comparing several models for a given set of data, the model associated with the smallest value of AIC is regarded as giving the best fit out of that set of models and is calculated as follows:

$$\mathrm{AIC} = N_\mathrm{d} \ln \mathrm{SSR} + 2P \tag{8}$$

where N_d represents the number of data points, SSR the sum of squares and P denotes the number of parameters used in the model. In addition, other parameters, the condition number (CN) and Schwartz criteria (SC) were used as confirmatory indicators of the soundness of statistical and experimental interpretation (Costa and Lobo, 2001).

From model fitting, it became evident that the choice of the most suitable model was based on the AIC and SC values. CN values were not conclusive and therefore placed last in order of priority (Table 5). Using the statistical fit factors, the lower AIC and SC values for Model 1 and Model 2 suggested better model suitability. Using Model 1, an n value of 0.57 was

Table 5
Drug release kinetic data derived from various mathematical models

Model	k_1	k_2	n	Akaike Information Criterion (AIC)	Schwartz criteria (SC)	Condition number (CN)
$\frac{M_t}{M_\infty} = k_1 t^n$ (Model 1 = Power Law)	0.114	–	0.567	−52.659	−54.579	19.02
$\frac{M_t}{M_\infty} = k_1 t^n + k_2 t^{2n}$ (Model 2 = derivative of Power Law)	0.083	0.034	0.404	−51.504	−54.384	136.5
$\frac{M_t}{M_\infty} = 1 - (1 - k_1 t)^n$ (Model 3 = Hopfenberg model)	0.054	–	1	−31.328	−32.289	1
	0.030	–	2	−33.751	−34.711	1
	0.021	–	3	−34.583	−35.543	1

$n = 1$ for a slab, $n = 2$ for a cylinder and $n = 3$ for a sphere.

obtained, indicating that drug release from chitosan microspheres was regulated through Fickian diffusion. Similarly, using Model 2, it was observed that the dominant mechanism in the release process was diffusion. Statistically, the marginally smaller relaxational constant k_2 (0.034) in comparison to the Fickian constant k_1 (0.083) confirmed that diffusion was the primary operating release mechanism for this formulation.

The contributions of surface erosion on drug release are considered to be less significant due to the small k_1 value obtained using Model 3 (Table 5). These conclusions are supported by experimental data generated from erosion studies (Fig. 4) where minimal polymer erosion was observed.

In conclusion, it was clear that drug release from chitosan microspheres followed Fickian diffusion. This type of diffusional release occurs by the usual molecular diffusion of the drug due to a chemical potential gradient.

3.2.4. Antimicrobial studies

The in vitro antimicrobial activities of the drug-loaded microspheres, drug-free microspheres and tetracycline HCl (pure drug) were evaluated in PBS pH 6.8 against *S. aureus*, an isolate found in the periodontal pockets of patients with periodontitis. The method used for this study was similar to that used in other oro-dental studies (Ali et al., 1994).

From the results shown in Table 6, it can be seen that the minimum concentration of drug required for demonstrating zones of microbial growth inhibition in PBS pH 6.8 was ≈1 μg/mL. The in vitro release study therefore revealed that the concentration of the drug released at each time point throughout the 8 h test period was above 1 μg/mL (Fig. 5), i.e. concentration of drug released was in the range 6.03–51.42 μg/mL. In a similar study design, Uzunoglu et al. (2000) also showed that the amount of drug released from natamycin chitosan beads over 2 days was above the minimum concentration (2 μg/mL) required for inhibiting growth of *C. albicans* for periodontal therapy.

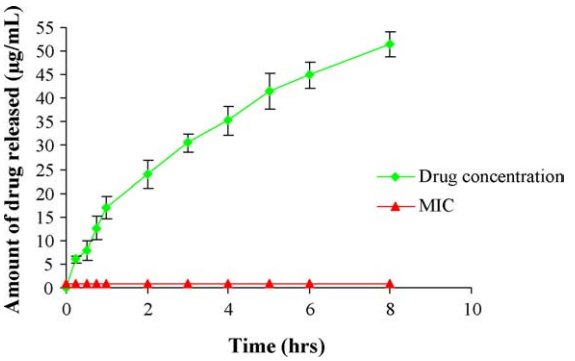

Fig. 5. In vitro drug release from the optimised microspheres against the minimum concentration of tetracycline HCl for microbial growth inhibition.

In vitro dissolution samples showed inhibition of growth of *S. aureas* throughout the test period (Table 7), confirming the antimicrobial activity of the microspheres. The in vitro release samples obtained form the drug-free microspheres (Table 7) showed no inhibition of growth of the microorganism. This is contrary to

Table 6
Inhibition of growth of tetracycline HCl (pure drug) solutions against *S. aureus*

Concentration (μg/mL)	Mean zone diameter (mm ± S.D.)
0	0
0.25	0
0.5	0
1	1.68 ± 0.09
2	2.03 ± 0.09
4	2.15 ± 0.20
8	2.54 ± 0.23
16	2.84 ± 0.07
32	3.03 ± 0.14
64	3.14 ± 0.13
128	4.09 ± 0.12

0: No zone of inhibition.

Table 7
Inhibition of growth of in vitro dissolution samples against *S. aureus*

Time (h)	Mean zone diameter (mm ± S.D.)	
	Drug-loaded chitosan microspheres	Drug-free chitosan microspheres
0.25	1.03 ± 0.05	0
0.5	1.14 ± 0.07	0
0.75	1.23 ± 0.05	0
1	1.45 ± 0.09	0
2	1.56 ± 0.11	0
3	1.84 ± 0.07	0
4	1.99 ± 0.12	0
5	2.10 ± 0.09	0
6	2.20 ± 0.08	0
7	2.28 ± 0.10	0
8	2.35 ± 0.12	0

0: No zone of inhibition.

other studies that have shown that chitosan alone possesses antimicrobial activity (Ikinci et al., 2002; Jumaa et al., 2002). In this preparation, chitosan therefore does not afford any additional antimicrobial activity. The difference in results may be attributed to the different mediums in which chitosan was dispersed or dissolved. For instance, in the study of Jumaa et al. (2002) a chitosan emulsion was prepared by dispersing chitosan into a solution of sorbitol and lactic acid. However, in this study the pH of the dissolution medium was 6.8 and since chitosan is relatively unionised at this pH, its reduced solubility may have resulted in the absence of any antimicrobial activity.

3.2.5. Thermal properties

DSC analysis of the polymer, drug, drug-loaded and drug-free microspheres was performed in order to determine the thermal changes of chitosan and tetracycline before and after microsphere preparation. Fig. 6 shows the DSC thermograms obtained during the heating stage for tetracycline HCl (A), chitosan (B), chitosan:tetracycline HCl (1:1) mixture (C), drug-free (D) and drug-loaded chitosan microspheres (E). The DSC thermogram (A) of tetracycline HCl showed an exothermic peak with an onset from 228 °C to reach a maximum peak at 234 °C. This was in close agreement with the thermal behaviour of tetracycline HCl reported by Deasy et al. (1989). In this study, a typically broad endothermic peak at about 100 and 290 °C was observed for the chitosan polymer thermogram This has also been reported elsewhere (Sreenivasan, 1996; Tirkistani, 1998). The small endothermic peak at around 350 °C is found in thermograms A, C and E. Drug is present in all of these samples and it may be due to the decomposition of the drug.

The DSC curve for the 1:1 physical mixture of chitosan and tetracycline HCl (C) exhibited one peak at 233 °C. Also, the gradient of the decomposition curve for the physical mixture (C) is different from the gradient of the pure polymer (B) and drug (A), thereby indicating that an interaction occurred between chitosan and tetracycline. The DSC curve for the drug-free microspheres (D) showed two endothermic peaks at 225 and 340 °C. This curve differed from the DSC curve for tetracycline HCl alone (A) and the chitosan powder alone (B). The difference may have been due to an interaction between the amino groups of chitosan and the phosphate groups of tripolyphosphate

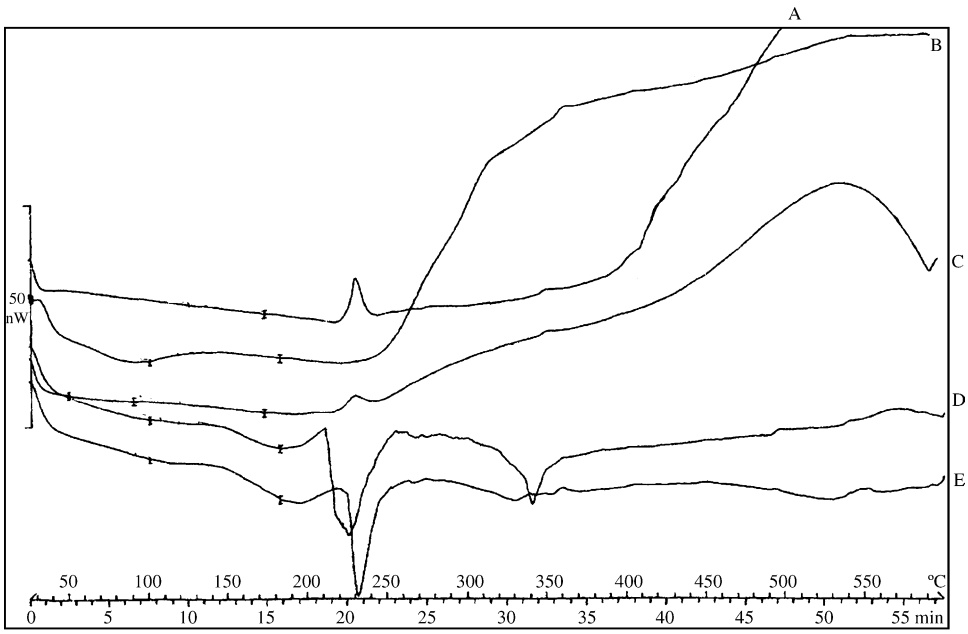

Fig. 6. Thermal profiles ($n = 3$) of tetracycline HCl (A), chitosan (B), chitosan:tetracycline (1:1) (C), drug-free microspheres (D) and drug-loaded microspheres (E).

during microsphere preparation. In the case of the drug-loaded microspheres (E), only one endothermic peak at 232 °C was present. The difference in the DSC curves between the drug-free microspheres and the drug-loaded microspheres may indicate that some interaction between chitosan and tetracycline HCl occurred and that the product was not a mere physical mixture.

3.2.6. Morphology

SEM was performed on the optimised chitosan microsphere formulation to assess their surface and cross-sectional morphological characteristics. In addition, the surface morphology of the microspheres before and after dissolution and bioadhesion were also assessed.

When examined at a magnification of 650× (Fig. 7a), the polymer surfaces of the microspheres appeared heterogeneous and porous. These findings are concordant with those of other researchers (Shu and Zhu, 2000; Ko et al., 2002) who also reported rough surface morphologies for chitosan microspheres prepared by the ionotropic gelation technique. However, Kumbar et al. (2002) reported smooth surface morphologies of chitosan microspheres when chitosan microspheres were produced in a w/o emulsion and then crosslinked with glutaraldehyde. The surface morphology observed may have important implications for bioadhesion, for the purposes of this study. It has been recently reported (Vasir et al., 2003) that microspheres with a coarser and more porous surface may offer enhanced bioadhesivity as compared to those with a

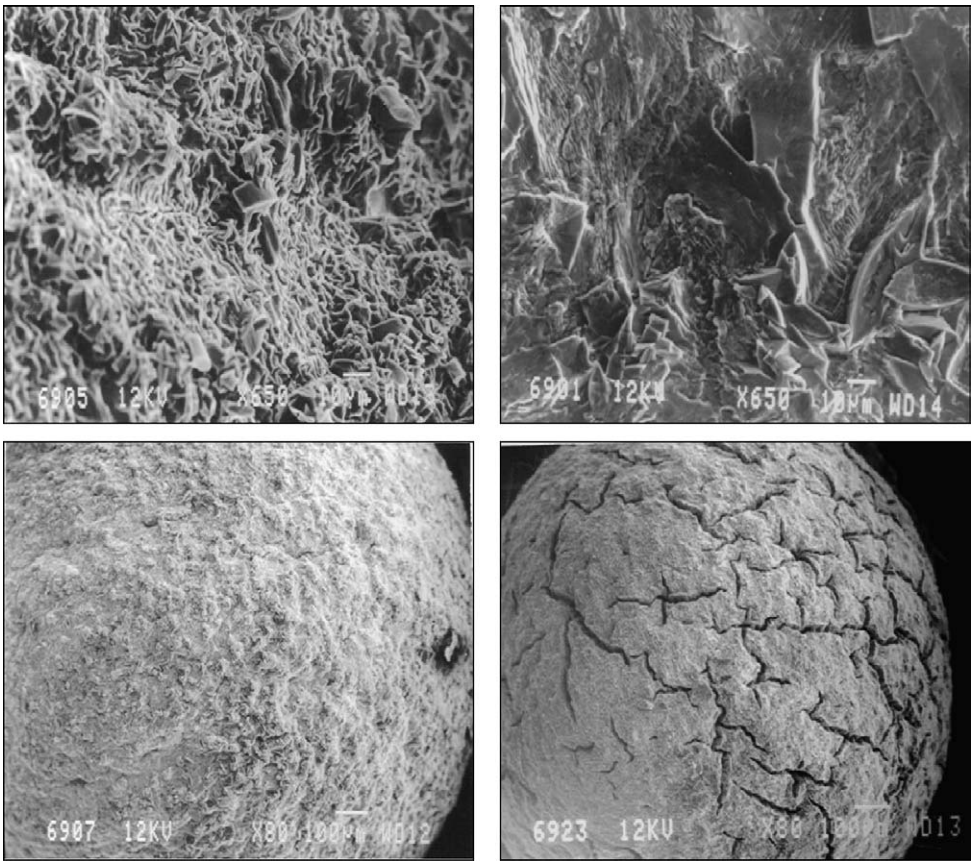

Fig. 7. (a) Surface morphology of a chitosan microsphere (650×). (b) Cross-section of a chitosan microsphere (650×). (c) Microsphere surface before dissolution (80×). (d) Microsphere surface 8 h post dissolution (80×).

Table 8
Surface pH changes of the optimised chitosan microspheres in PBS pH 6.8

Time (h)	Surface pH
0 (Initial reading after 3 min hydration)	6.32 ± 0.19
2	6.27 ± 0.07
4	6.30 ± 0.04
6	6.36 ± 0.06
8	6.46 ± 0.17

more smoother texture. Therefore, the rough, coarse structure observed in this study may have led to the observed bioadhesion.

Fig. 7b illustrates the cross-section of a drug-loaded chitosan microsphere. It is clear that internally the microspheres appear dense, and there are no distinct hollow cores. This was probably due to the uniformity afforded from complete crosslinking of the homogenous drug-chitosan dispersion. The surface morphologies of whole microspheres before and after exposure to the dissolution medium are shown in Fig. 7c and d. It can be seen that after exposure to the dissolution medium, there appeared to be the formation of 'cracks'/channels on the surface of the microspheres (Figs. 7c and d). This may be due to the penetration of the dissolution medium into the microspheres and the subsequent dissolution of the drug and hence its diffusion through the polymer matrix.

3.2.7. Surface pH

The surface pH of the microspheres was determined in order to confirm that the microspheres would not cause irritation in and around the periodontal pocket mucosa due to extremes in pH. Table 8 shows that the surface pH of the microspheres remained fairly constant at a pH of approximately 6.3–6.5 over the 8 h test period. Therefore, this study confirmed that the surface pH of the microspheres was within the neutral conditions of saliva and that no extremes in pH occurred throughout the test period.

4. Conclusions

The aim of this study was to identify optimal formulation parameters for a microsphere preparation with maximum bioadhesion and controlled drug release and to subsequently undertake a physicochemical/mechanical characterisation of the optimised formulation. A Box-Behnken experimental design was employed to identify optimal formulation parameters for maximum bioadhesivity and drug release. From the mathematical models generated in this study, an optimal formulation comprising of 3% (w/w) chitosan, 10% (w/w) tetracycline hydrochloride and 9% (w/v) tripolyphosphate was identified to provide maximum bioadhesion and a controlled drug release profile.

Microspheres were prepared according to the above formulation and subjected to characterisation. Texture profile analysis was employed to determine the hydration dynamics of the chitosan microspheres. The chitosan microspheres showed minimal hydration over the test period. It was postulated that the outer porous structure of the microspheres attracted the influx of water while the highly crosslinked internal structure resisted the diffusion of water, thus contributing to the decreased hydration. Drug release from the microspheres followed Fickian diffusion. Antimicrobial studies revealed that the release of the drug over an 8 h period was above the minimum concentration required for inhibition of microbial growth. Differential scanning calorimetric studies showed a possible interaction between tetracycline HCl and chitosan during microsphere preparation. Scanning electron microscopy proved useful in evaluating the integrity of the chitosan microspheres as well as elucidating the morphological changes due to drug release. The surface pH of the microspheres also remained constant at neutral pH throughout the study.

The bioadhesion, drug release and physicochemical characterisation data obtained in this study confirms the potential of this system for optimising localised periodontal therapy.

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