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Biodegradable Microspheres For Controlled Delivery Of Metronidazole In The Treatment Of Periodontal Diseases: Formulation consideration

GAURAV TIWARI

Department of Pharmaceutics, Pranveer Singh Institute of Technology, Kalpi road, Bhauti, Kanpur-208020 (Uttar Pradesh), India

Corresponding Author tiwari_mpharm@rediffmail.com

ABSTRACT

This study reports on the development of novel biodegradable microspheres prepared by oil-in-water–oil (O/W/O) double emulsion technique using the blends of poly (D, L-lactide-co-glycolide) (PLGA) and polycaprolactone (PCL) in different ratios for the controlled delivery of metronidazole (MTZ). Metronidazole encapsulation of up to 40% was achieved within the polymeric microspheres. Blend placebo microspheres, drug-loaded microspheres were analyzed by Fourier transform infrared spectroscopy (FT-IR), which indicated no interaction between drug and polymers. Differential scanning calorimetry (DSC) on drug-loaded microspheres confirmed the polymorphism of MTZ and indicated a molecular level dispersion of MTZ in the microspheres. Scanning electron microscopy (SEM) confirmed the spherical nature and smooth surfaces of the microspheres produced. Mean particle size of the microspheres as measured by dynamic laser light scattering method ranged between 100 and 200 µm. *In vitro* release studies performed in 7.4 pH media indicated the release of MTZ from 7 to 11 days, depending upon the blend ratio of the matrix. Up to 11 days, MTZ concentrations in the gingival crevicular fluid were higher than the minimum inhibitory concentration of MTZ against most of the periodontal pathogens. Statistical analyses of the release data were performed using the analysis of variance (ANOVA) method.

KEYWORDS

Blends; Microspheres; PLGA; PCL; Periodontal pocket; Metronidazole

INTRODUCTION

Gingivitis and periodontitis are pathological states affecting the gingival, subgingival, periodontal and adjacent tissues[1]. Together with conventional therapy, based on scaling and surgery, the use of antibiotics or antimicrobials (e.g. tetracycline, minocycline, clindamycin, metronidazole and chlorhexidine) has been proposed[2]. In particular, the tetracycline family of antibiotics was found effective against the microorganisms associated with periodontitis in the gingival crevice[3]. The antibiotic therapy of periodontal diseases is mainly based on two different approaches: extensive oral rinses with solutions and systemic administration. On the other hand, both approaches can be unsuccessful and/or produce adverse problems. In fact, the first one could result in a failure of antibiotics to reach the deeper subgingival tissues, while the second one could present disadvantages such as (a) bacterial resistance to the administered antibiotic and (b) unpleasant or toxic

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side effects as a consequence of the systemic regimen[4]. Because of these considerations, a variety of specialized local delivery systems (i.e. intrapocket devices) were designed to maintain the antibiotic in the gingival crevicular fluid at a concentration higher than that achieved by systemic administration[5]. Fibres, films and microparticles made of biodegradable or non-degradable polymers have been recently proposed as effective methods to administer antibacterial agent for periodontal therapy[6]. Together with these solid devices, recently, semi-solid formulations such as Elyzol ® 25% dental gel, consisting of metronidazole crystals suspended in a lipid matrix have been proposed[7]. In fact, in spite of relatively faster release of the incorporated drug (with respect to fibres or microparticles), gels can be more easily prepared and administered. Moreover, they possess a higher biocompatibility and bioadhesivity, allowing adhesion to the mucosa in the dental pocket and, finally[8], they can be rapidly eliminated through normal catabolic pathways, decreasing the risk of irritative or allergic host reactions at the application site. Aliphatic polyesters such as polycaprolactone (PCL), poly (3 hydroxybutyrate) (PHB), poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and its copolymers with glycolic acid i.e., poly (D,L-lactide-coglycolide) (PLGA) [8-15] have been widely used to formulate the controlled release devices. Among these, PLGA provides a wide range of degradation rates, from months to years, depending upon its composition and molecular weight[9-15]. Polycaprolactone (PCL) is an important member of aliphatic polyester family[16], which has been used in combination with polymers like cellulose propionate, cellulose acetate butyrate, PLA and PLGA to manipulate the drug release rates from microsphere[17-21]. In the present study, we report the preparation of a controlled release system prepared from blend microspheres of PLGA and PCL loaded with an antibiotic (MTZ), which is effective for treatment of periodontal disease. Various microsphere formulations were prepared by (O/W/O) double emulsion solvent evaporation method by varying the polymer blend composition (PLGA: PCL, 100/0, 80/20, 60/40, 40/60 and 0/100) and % drug loading (30 and 60% of dry polymer weight). The formulation and process variables affecting the preparation of microspheres and *in vitro* drug release characteristics have been investigated. *In vitro* release was performed on both the microsphere and gel formulations in pH 7.4 phosphate buffer solution (PBS) at 37 °C. Scanning electron microscopy was employed to investigate the morphology of the microspheres. The size of the microspheres was studied by dynamic laser light scattering technique.

MATERIALS AND METHODS

Materials

Metronidazole HCL was kindly received as a gift sample by Siemens laboratories, gurgaon, India. Poly (D, L-lactide-coglycolide) (PLGA) 50:50 Resomer® RG 504 (Mw=60,000) was purchased from Boehringer Ingelheim (Ingelheim, Germany). PCL (Mw=32 kDa) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Analytical reagent grade samples of poly (vinyl alcohol) (Mw=125,000 and 98% hydrolyzed), dichloromethane (DCM), sodium chloride were all purchased from S.D. Fine Chemicals (Mumbai, India). Dialysis membrane-110 was purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Double-distilled water was used throughout. All chemicals were used without further purification.

Methods

Preparation of microspheres and drug loading. An O/W/O double emulsion solvent evaporation technique with some minor modifications was

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adopted to formulate the MTZ-loaded PLGA/PCL blend microspheres. In this method, MTZ equivalent to 30 or 60% (w/w) dry weight of the polymer was dissolved in 2 mL of DCM to form MTZ solution; 0.3 g of the polymer dissolved in 10 mL of distilled water used as an aqueous phase; 2 mL of above-prepared MTZ oil phase was added to 10 mL of aqueous solution and emulsified using a probe ultrasonicator (UP 400s, Dr. Hielscher, Germany) for 2 min to form a stable O/W emulsion. Then, this stable O/W emulsion was slowly added into 100 mL of DCM containing 0.5% of PVA, 4% NaCl and emulsified using Eurostar (IKA Labortechnik, Germany) mechanical stirrer at 400 rpm to form the O/W/O emulsion at the ambient temperature. Further, solvent removal and hardening of the microspheres was achieved by continued stirring for up to 2 h[22-25]. Later, the microspheres were isolated by filtration and washed with distilled water several times to remove PVA. The microspheres thus produced were dried at ambient temperature for 24 h and then followed by vacuum drying at 25 °C for 12 h to remove the residual solvent. Placebo microspheres were prepared in a similar way; these were used as control formulations in characterization studies. Compositions of various formulations along with formulation codes are summarized in Table 1.

Table1.

Results of % encapsulation efficiency and mean particle size of various formulations.

Formulation code	Ratio of PLGA:PCL	% MTZ loaded	% Encapsulation efficiency	Mean particle size (mm)
F1	100:0	60	28.01±0.53	201±1.52
F2	80:20	30	16.2±0.26	145±1.73
F3	80:20	60	18.20±0.36	165±1.52
F4	60:40	30	11.32±0.20	132±1.15
F5	60:40	60	13.36±0.11	162±1.15
F6	40:60	30	14.35±0.17	101±1.52
F7	40:60	60	14.2±0.26	117±1.73
F8	0:100	60	9.00±0.20	95±0.57

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Table2.
First order rate constant, half life and shelf life of formulation F3 at elevated temperatures

Temperature	First order rate constant ($days^{-1}$)	Half life (days)	Shelf life (days)
40^0C	3.26×10^{-4}	2125.76	321.11
50^0C	6.28×10^{-4}	1103.50	167.73
60^0C	7.64×10^{-4}	907.06	137.8
25^0C	1.5×10^{-4}	4620	702.24

Metronidazole content

To determine the MTZ content, microspheres were dissolved in DCM and MTZ was extracted with pH 7.4 phosphate buffer solution (PBS)[26] followed by UV spectrophotometric analysis. Briefly, 10 mg of each batch of MTZ-loaded microspheres was dissolved in dichloromethane and then 10 mL of PBS was added into this solution to extract MTZ several times. The above suspension was vigorously mixed by vortexing, allowed to get the clear solution, separated and filtered through 0.45 mm filter (Sartorius, Germany) to remove the polymeric debris. The clear solution was analyzed for MTZ content by UV spectrophotometer at λmax value of 319 nm.

Particle size measurements

Particle size was measured by dynamic laser light scattering (Mastersizer 2000, Malvern, UK). Sizes of the completely dried microspheres of different formulations were measured by dry sample technique using a dry sample adapter[27]. The completely dried particles were placed on the sample tray with an inbuilt vacuum under a compressed air system, which was used to suspend the particles. The laser obscuration range was maintained between 1 and 2%.

Scanning electron microscopic (SEM) studies

The volume-mean diameter (Vd) was recorded. After the measurement of particle size of each sample, the dry sample adopter was cleaned thoroughly to avoid cross contamination. Each batch was analyzed in triplicate, but average values were considered in data analysis.

Fourier transform infrared (FTIR) spectral studies

FTIR spectra were taken on a Nicolet (Model Thermo 5700, Milwaukee, WI, USA) instrument to investigate the possible chemical interactions between the drug and the blend matrix. Samples were crushed with KBr to get the pellets by applying a pressure of 300 kg/cm2. FTIR spectra of placebo microspheres, pristine and MTZ-loaded microspheres were scanned in the range between 4000 and 500 cm-1.

Differential scanning calorimetric (DSC) studies

The nature of drug present in the formulations was assessed by performing DSC on PLGA, PCL, placebo microspheres and MTZ-loaded microspheres. DSC measurements were done on a Rheometric Scientific (DSC-SP, Surrey, UK) by heating the samples at the heating rate of 10 °C/min in a nitrogen atmosphere (flow rate, 10 mL/min).

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SEM images were taken on placebo microspheres, microspheres prepared with 60% MTZ loading and microspheres after the drug release study. Microspheres were sputtered with gold to make them conductive and placed on a copper stub. Scanning was done using JEOL model JSM-840A, Japan. The thickness of the gold layer accomplished by gold sputtering was about 15 nm.

In vitro drug release studies

In vitro drug release from different formulations was investigated in PBS (without enzymes). Microspheres (25 mg) were suspended in 1 mL PBS of pH 7.4 and placed within a dialysis bag[28]. The sample within the dialysis bag was kept in a conical flask containing 50 mL of PBS as the dissolution medium and the flask was shaken at 50 rpm. The whole assembly was maintained at 37 °C using a thermostatic water bath (Grant, model GR 150, GP 200, UK). The amount of drug released was determined by withdrawing each time 3 mL aliquots at the selected specific time intervals. The volume withdrawn was replenished with an equal volume of fresh and prewarmed PBS at 37 °C. Samples were analyzed by UV spectrophotometer (Secomam, Anthelie, France) at the λ max value of 319 nm using PBS as the blank. In the case of commercial MTZ gel formulation, in vitro drug release was performed in pH 7.4 PBS using Keshary–Chien diffusion cell. The dialysis membrane containing MTZ gel was mounted between the donor and the receptor compartments of the diffusion cell, which were held securely by the springs. The donor compartment was empty and open to air, while the receptor compartment was filled with PBS and stirred at 100 rpm using a magnetic stirrer (Jenway, Mode 1103, UK). The whole assembly was maintained at 37 °C using a thermostatic water bath (Grant, model GR 150, GP 200, UK). The amount of drug released was determined by withdrawing1 mL aliquots at the selected specific time intervals. The volume withdrawn was replaced with an equal volume of fresh and prewarmed PBS at 37 °C. Samples were analyzed by UV spectrophotometer (Secomam, Anthelie, France) at the λmax value of 319 nm using PBS as the blank.

Stability Study

The solid state reactions are slow and it is customary to use stress conditions in the investigation of stability. This approach is not always straight forward and due care must be exerted in the interpretation of the data. High temperatures can drive moisture out of sample and render a material apparently stable that would otherwise be prone to hydrolysis. Therefore the stability study of microspheres was performed at elevated temperature and different humidity conditions**.** A stability study was performed to determine the effect of temperature and humidity. The formulation F3 was selected for the stability study[29].

Solid state stability at elevated temperatures

The formulation F3 (100 mg) was placed in glass vials. These glass vials were kept at 40^0C, 50^0C and 60^0C for 3 months. The samples so stored were examined for caking, liquefaction, discolouration and odour. The samples (5 mg) was withdrawn from vials after 15, 30, 60, and 90 days, dissolved in *n*-octanol, diluted suitably and drug remains to degraded was determined by UV spectrophotometer at 319 nm.

Rate of degradation (k) of microspheres in solid state were determined at 40^0C, 50^0C and 60^0C by equation 1.

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$$k = 2.303/t \log Co/C \quad \text{---------- (1)}$$

Where, k = first order degradation rate constant
t = time at which sample was withdrawn
Co = Concentration of drug at time (t=0)
C = Concentration of drug after time

The value of k at 25^0C was obtained by plotting log k versus 1000/T (absolute temperature), this k value (Fig. 5) was used to determine half life and shelf life ($t_{10\%}$) of microspheres at room temperature, by equation 2 and 3[30].

$$\text{Half life } (t_{1/2}) = 0.693/k \quad \text{---------- (2)}$$
$$\text{Shelf life } (t_{10\%}) = 0.152 \times t_{1/2} \quad \text{---------- (3)}$$

Statistical analyses

Statistical analyses were done using the SPSS statistical package. Analysis of variance followed by the least significant difference (LSD) procedure was used for comparison of drug release rates from different formulations and $p<0.05$ was considered to be significant.

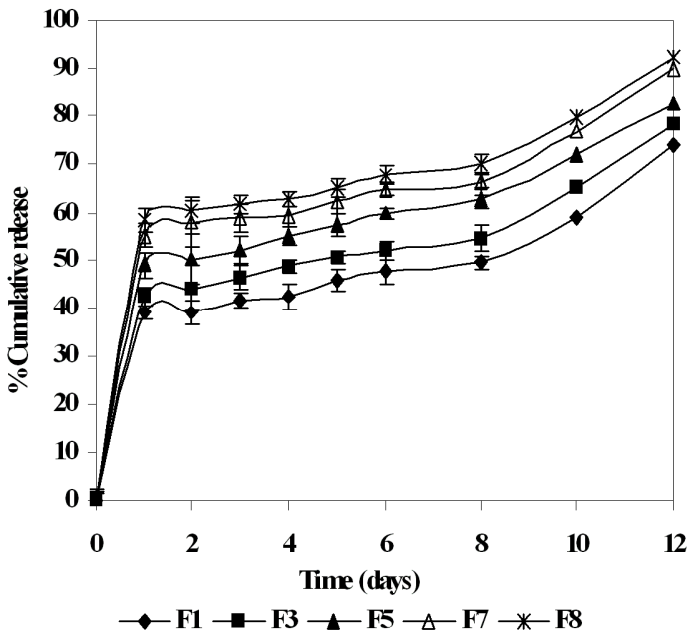

Figure 1 In vitro release profiles of formulations containing different blend compositions

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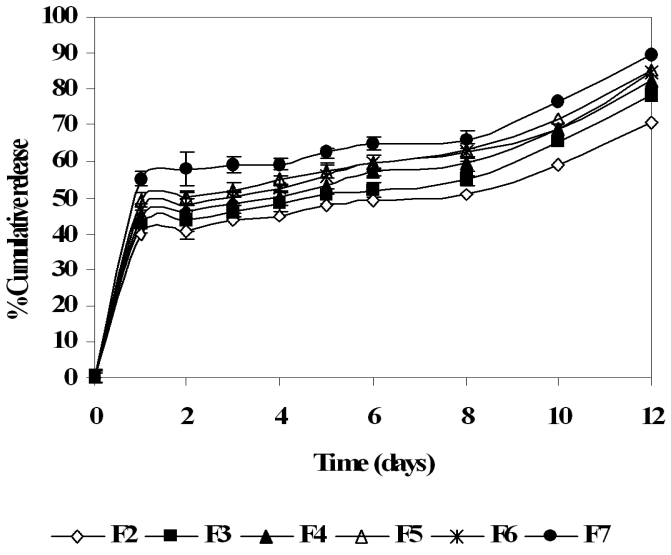

Figure 2 In vitro release profiles of formulations containing different blend compositions

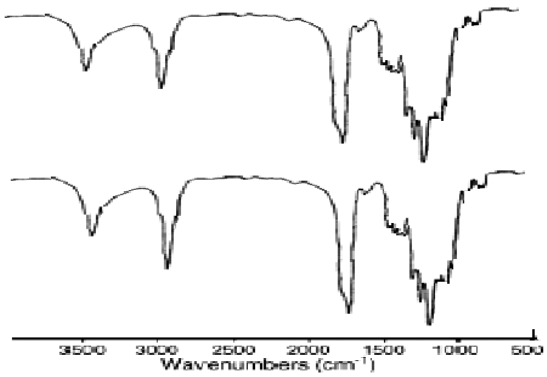

Figure. 3. FTIR spectra of (a) placebo microspheres (b) MTZ loaded microspheres.

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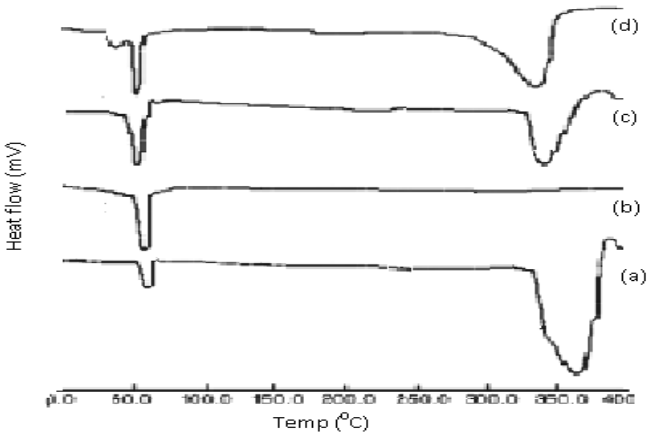

Figure 4. DSC curves of (a) plain PLGA, (b) plain PCL, (c) placebo microspheres and (d) MTZ-loaded microspheres.

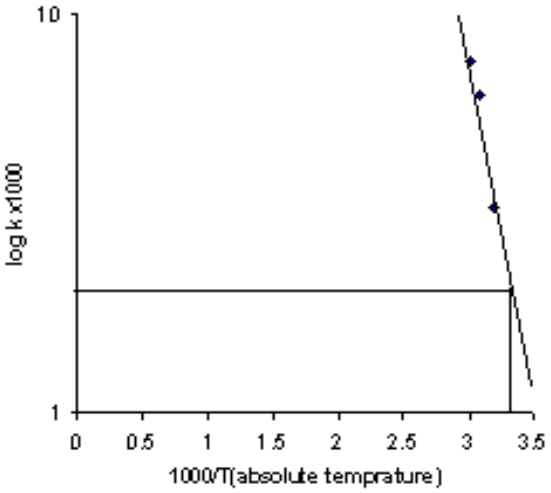

Figure 5. Graph between log k x10^3 and 1000/T (absolute temperature)

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RESULTS AND DISCUSSION

In this study, O/W/O method was used to entrap the oil soluble drug (MTZ) in the microparticles[30-33]. MTZ was first dissolved in dichloromethane and emulsified in a solution of blend polymers to form the primary emulsion; this stable O/W emulsion was slowly added into 100 ml of DCM containing 1% of PVA, 6% NaCl and emulsified using mechanical stirrer at 400 rpm to form the O/W/O emulsion at the ambient temperature. Further, solvent removal and hardening of the microspheres was achieved by continued stirring for up to 2 h. After solvent evaporation, the polymer precipitates and microparticles were solidified, but the encapsulation efficiency obtained was quite small. This solid–oil–water technique (S/O/W) was recommended before for producing microspheres with high drug loadings[34]. Even by following this method, encapsulation efficiency was still small. Finally, slight modification was made by incorporating NaCl in an external dichloromethane solution. This way, the solubility of drug in the external phase was decreased. During the preparation step, NaCl concentration and stirring time were optimized at 4% and 2 h, respectively. The results of mean particle size and size distributions of microspheres as recorded by laser light diffraction technique on a population basis were found to be unimodal with a narrow size distribution. Calculated values of volume-mean diameter, % encapsulation efficiency and % drug loading of different formulations presented in Table 1 display the systematic dependence on the amount of drug incorporated as well as the ratio of PLGA to PCL. Particles are all spherical in shape with sizes ranging from 90 to 200 µm. Particle size of plain PLGA is greater than blend microspheres containing different amounts of PCL. Results of mean particle size for formulations F1, F5 and F8, respectively, for plain PLGA, blend of PLGA/PCL and plain PCL depicted in Fig. 1 show an increase in size from 100 to 147 µm and 116 to 200 µm for 30% and 60% drug containing microspheres, respectively (F1 to F8). This would cause an increase in viscosity of the dispersed phase (polymer solution), resulting in a poorer dispersability of PLGA solution into the aqueous phase. However, the high viscous resistance against the shear forces during emulsification is possible such that coarse emulsions were obtained at higher ratios of PLGA, resulting in bigger particles during the diffusion step. This can be explained as due to greater probability of the desolvated drug (or small aggregates formed from these molecules) to coalesce in a more concentrated solution, thereby forming larger size particles. However, by increasing the PCL content of the blend, the particle size was smaller because PCL chains have higher flexibility than PLGA chains; however, crystallinity of PCL is less affected by solvent evaporation rate or by addition of MTZ. During solvent evaporation, high diffusion rate of MTZ in the PCL might have lead to low entrapment efficiency of MTZ in PCL microspheres, thereby reducing the particle size. Two different loadings of MTZ viz., 30 and 60 wt % were employed for encapsulation. Results of % encapsulation efficiency, also included in Table 1, exhibit an increase with increasing drug loading as well as increasing the amount of PLGA in the blend microspheres. Thus, microspheres containing different ratios of PLGA and 30% MTZ (i.e., formulations F2, F4 and F6) exhibited encapsulation efficiencies 15.3, 10.3 and 8.9%, respectively. For formulations containing different ratios of PLGA and 60% MTZ (i.e., formulations F1, F3, F5, F7 and F8), encapsulation efficiencies are, respectively, 24.7, 17.6, 13.1, 12.3and 9.0%. Thus, it is evident that %

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encapsulation efficiency of the microspheres decreases with increasing amount of PCL in the blend.

FTIR STUDY

FTIR spectral data[35] could confirm the chemical stability of MTZ in the blend microspheres. FTIR spectra of (a) placebo microspheres, (b) MTZ-loaded microspheres are displayed in (Fig. 3). A band appearing at 3388 cm-1 is due to O–H stretching vibrations, while those observed at 2924 and 2855 cm-1 are due to the C–H stretching vibrations. Bands at1458 and 1328 cm-1 are due to $–CH_2$ bending and C–H bending vibrations, respectively. The bands at 1219 and 1171 cm-1 belong to C–N stretching vibrations. The spectra of MTZ loaded microspheres are not characteristically different from the spectra of the placebo microspheres. After drug loading into blend microspheres, in addition to characteristic bands of the blend polymers, some additional bands have also appeared due to MTZ remaining in the blend matrix. Some bands of MTZ are not prominent in drug-loaded microspheres since these are identical to those of placebo microspheres and appear at almost the same wavenumbers. Notice that peaks appearing at 3412, 2946, 2865, 1725, 1615, 1577, 1461 and 1294 cm-1 for MTZ are also appearing in MTZ-loaded microspheres, indicating the chemical stability of MTZ even in the blend matrix.

Differential Scanning Calorimetric Study

DSC studies were performed to understand the nature of the encapsulated drug in the matrix. The physical state of MTZ in the polymer matrix would also influence its release characteristics. To probe this effect, DSC analysis was performed on (a) pristine PLGA, (b) pristine PCL, (c) placebo blend microspheres, (d) MTZ-loaded microspheres (Fig. 4). In the DSC curves displayed in Fig. 4, two characteristic features can be noticed. First, the endotherm peaks of PLGA and PCL polymers were observed at ~44 °C and ~57 °C, respectively, due to the glass transition temperature, Tg of PLGA and melting temperature of PCL. The Tg of PLGA was displaced to a lower temperature (~39.2 °C) with the drug-loaded microspheres as compared to neat PLGA. The melting peak of PCL in the blend is quite similar to that in the non-blended polymer both in shape and position. The melting endotherm of MTZ (~197 °C) was not detected in the drug-loaded microspheres. The absence of detectable crystalline domains in the blend microspheres along with the presence of MTZ degradation exotherm clearly indicates that drug was molecularly dispersed in the PLGA/PCL blend matrix.

In vitro release studies

In vitro release profiles of MTZ from formulations containing different blend compositions (Fig. 1) with fixed drug loadings (Fig. 2) are shown. In the literature[36], different aspects of drug release through PLGA matrices have been studied. In the present study, to overcome the limitation of PLGA microspheres, we have prepared the blend microspheres consisting of (50:50) PLGA and PCL. We found that MTZ release rate decreased in the order: PLGA, PLGA:PCL and PCL (i.e., formulations F1 to F8). However, PCL microspheres have shown higher burst release effects during the first day than PLGA: PCL and PLGA microspheres; thereafter, MTZ release was continued at a reduced rate with almost the complete release occurring in 11 days. A comparison of drug release from[35, 36] with the burst effect. Since PCL is a semi-crystalline polymer, water can penetrate easily into the amorphous region of the polymer. Degradation of PCL was very slow in an aqueous medium due to its semi-crystalline and hydrophobic nature[37]. Thus, drug release may be due to diffusion, but not due to polymer degradation. Data points in Fig. 1 and 2 represent the averages of triplicate

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measurements obtained within 3% standard deviations. With O/W/O technique, the burst effect was followed by a much slower release of MTZ, leading to a total release of 90% after 11 days. When MTZ is molecularly dispersed in the blend matrix, it leads to the migration at the surface of microparticles in addition to its leakage in the dissolution medium. In addition, porous structure of the microspheres as seen from. Permeability and drug release rates from microspheres are thus controlled by both blending as well as by changing the blend composition[38]. In all the formulations prepared in this study, we observed linear drug release rates after the burst effect for a period of 11 days; this could be the ideal treatment period for periodontal disease. Metronidazole release profile from blend microspheres containing MTZ (60%, w/w) was compared with a commercial sample of MTZ gel, which is based on a biodegradable PLA polymer containing MTZ (10%, w/w). Microspheres containing MTZ (60, % w/w) released 90% of drugs in 11 days. On the contrary, the commercial gel released 90% of MTZ in 7 days. Drug release from formulation F3 was statistically evaluated by ANOVA. The F value was found to be 0.691 (df=21, p=0.416), which indicated that there is no significant difference in the drug release from formulation F3 and MTZ gel. To study the effect of drug loading on release rates, formulations (F2, F3, F4, F5, F6 and F7) were chosen; the release rates are compared in (Fig. 2). Release rates vary depending on the extent of drug in the matrices, i.e., release was slower for formulations containing lower amount of drug, but the release rate increased at higher amount of drug in the microspheres. A comparison of drug release from formulations containing different drug loadings was evaluated by the ANOVA. The F value was found to be 1.862 (df=65, p=0.114), which indicated no significant difference in drug release rates from formulations containing different drug loadings. However, the initial burst effects remained the same for all formulations; this could be due to high aqueous solubility of MTZ as well as the method of preparation (O/W/O) of microparticles.

CONCLUSIONS

Results of in vitro suggest that the novel blend microparticles prepared from PLGA and PCL are effective in controlled delivery of metronidazole to periodontal pockets. In preparing the microspheres, polymorphism of the drug was retained without any chemical interactions with the polymers as indicated by DSC and FTIR measurements. Microspheres produced by o/w/o technique were in the size range of 90 to 200 µm, which depend upon blend composition as well as drug entrapment efficiency. In vitro release indicated a burst effect initially, which was followed by controlled release up to 11 days. Statistical analyses of the release data indicated that MTZ release was significantly affected by the composition of the blend ratio.

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