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Preparation and Characterization of Simvastatin Loaded PLGA Microparticles for Tissue Engineering Applications

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ABSTRACT

Simvastatin has been reported to promote osteoblastic activity and inhibit osteoclastic activity. The successful use of simvastatin to promote *in vivo* bone formation depends on the local concentration, and there have been continuous efforts to find an appropriate delivery system for local delivery. Controlled drug delivery approaches based on microparticles could be a promising approach for sustained-localized delivery of simvastatin. In this study, simvastatin-loaded PLGA microparticles were prepared by using a modified single emulsion-solvent evaporation method. Uniform, spherical simvastatin loaded PLGA microparticles of size below 10µm were produced by adopting three different drug polymer ratios such as 1:40, 1:20 and 1:10 with encapsulation efficiency above 85%w/w irrespective to the drug polymer ratio and maximum simvastatin loading within PLGA microparticles was observed at drug polymer ratio of 1:10. Two stage release of simvastatin from microparticles was observed for 45 days, illustrating a controlled release. Simvastatin loaded PLGA microparticles are compatible with hFOB cells and induced *in vitro* bio-mineralization during 11 days treatment. These studies illustrate the feasibility of achieving local delivery of simvastatin to induce *in* vivo bone formation activity by suitably engrafting simvastatin loaded microparticles within porous scaffolds.

Keywords: bone tissue engineering, localized delivery, PLGA microparticles, simvastatin, osteoblastic activity, bio-mineralization.

1. INTRODUCTION

Controlled release technology enables the delivery of a biologically active agent at specific time intervals with defined concentration by encapsulating it within a biodegradable polymer in the form of a microspheres. Microencapsulation of bioactive substances continues to be an important formulation strategy since its inception about 72 years ago. Starting first with the aim to protect vitamins from oxidation[1] it took some decades until polylactic acid (PLA) and later its co-polymer, e.g., poly(lactic-co-glycolic acid) (PLGA), were evaluated as biodegradable and biocompatible polymers for drug delivery[2-7].

PLGA is one of the most extensively investigated polymers for drug delivery and tissue engineering[8-10]. Many patents and research papers had been reported PLGA as a promising excipient with high commercial interest. PLGA offers unique properties for drug delivery purposes such as: world-wide approval for medical use, biodegradability, biocompatibility, ease of encapsulating wide range of pharmaceutical agents (both hydrophilic and hydrophobic) and eventually their controlled release. Moreover, its release properties can be easily modified by varying composition (lactide/glycolide ratio), molecular weight and chemical structure. In this way, a wide range of *in vivo* lifetimes of PLGA can be obtained: from 3 weeks to over a year[11-14].

In PLGA co-polymers, lactic acid is more hydrophobic than glycolic acid and hence lactide-rich PLGA copolymers are less hydrophilic, absorb less water and subsequently degrade more slowly. In this study we used PLGA 50:50 since it is known to be degrade in physiological

environment for a period of 4-6 weeks. Furthermore, the type of the polymer end group will impact the water-uptake and degradation rate of particles. In this study, we used Resomer (RG 504 H) which is having free carboxyl groups as end groups, which may lead to much more swelling of the matrix in compared to Resomer (RG 504) which is a capped polymer bearing an aliphatic group at the very end group of the polymer chain. The Tg (glass transition temperature) of the PLGA copolymers are above the physiological temperature of 37º C and hence they are glassy in nature. Thus they have a fairly rigid chain structure at room temperature, which gives them significant mechanical strength to be formulated as drug delivery devices[15].

The PLGA co-polymer undergoes degradation in an aqueous environment through hydrolytic cleavage of its backbone ester linkages. Hydrolysis of the polymer backbone is accompanied by gradual erosion of the device, leads to the complete degradation of PLGA in physiological environment. The microencapsulation of hydrophobic pharmaceutical compounds within PLGA by the single emulsion-solvent evaporation method has been investigated extensively during the past few decades. The basic mechanism involved in formation of particles by this method depends on emulsification of a polymeric solution (oil or organic phase) with an aqueous continuous phase resulting in the formation of o/w emulsion. A schematic diagram of this method is shown in Fig.1[16-17].

In this method, the hydrophobic drug substance is dissolved the polymer/ solvent system to form dispersed phase (oil phase) of the emulsion. This dispersed phase is homogenized in continuous phase (aqueous phase) which contains a surfactant. Homogenozation process is continued until the solvent partitions into the aqueous phase and is removed by evaporation. This process results in hardened microspheres which contain the active drug. Homogenization process had been widely employed to disperse the oil phase in aquoues phase with the help of a homogenizer, which is equipped with a rotor and stator type blade. The rotar blade is attached to a variable high speed motor, with which it can rotate and produce high shear forces between stator and rotar. This high shear forces result in formation of small particles. The O/W emulsion solvent evaporation system has been successfully employed to encapsulate poorly water soluble drugs including chlorpromazine, prednisolone, and hydrocortisone[18-20]. Sansdrap and Moes investigated the influence of several process parameters, including agitation rate, surfactant concentrations, organic phase volume and drug loading on microspheres of nifedipine, a poorly- water soluble calcium-channel blocking agent[21].

Simvastatin is a well-known member of the statin family. Statins are potent pro-drugs of hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors that block conversion of HMG-CoA to mevalonic acid, which is needed for cholesterol biosynthesis[22]. Simvastatin has a very low aqueous solubility, approximately 1.4 µg/mL, because it has a rigid and hydrophobic section that is covalently linked to the HMG-like moiety. It has an inactive lactone ring in HMG-like portion.

Maeda, et al., and Skoglund, et al, reported that, oral dosing of rats with simvastatin increased cancellous bone volume and formation transverse area of fracture callus as well as mechanical properties compared to un-treated control counter parts[23-24]. Furthermore, simvastatin has been reported to support bone morphogenetic protein (BMP)-induced osteoblast differentiation through antagonizing TNF-α-to-Ras/Rho/ mitogen activated protein kinase and augmenting BMP-Smad signalling[25]. However, some researchers reported skeptical result of simvastatin on bone formation by investigating oral dosing and systemic effects of simvastatin[26-27]. However, the main difference between systemic and local delivery of simvastatin is effective concentration at the desired site. Former is absorbed by gastro-intestine system and mainly works in the liver and the latter works directly on the desired site. With 60 mg dosage forms, serum concentrations of simvastatin and simvastatin acid are 18.7 +/- 4.7 ng/ml and 3.5+/-0.5 ng/ml, respectively. Further, the biological half-life of simvastatin acid is 5.9 +/- 0.3 hr[28]. In i*n vivo* conditions, the inactive lactone ring of simvastatin is enzymatically hydrolyzed to its active hydroxyacid form[29-30]. Hence, localized delivery of simvastatin at desired site of bone regeneration is required in order to obtain desired therapeutic concentrations of simvastain.

For local delivery of simvastatin, controlled release system based on PLGA microparticles could be a promising approach. This paper is focused on preparation and characterization of simvastatin loaded PLGA microparticles by single emulsion-solvent diffusion technique and characterized in terms of surface morphology, encapsulation efficiency and PLGA particles loaded with 10% w/w simvastatin were further characterized in terms of drug release studies, cell viability test and pNPP assay.

2. MATERIALS AND METHODS

Preparation of simvastatin loaded PLGA microparticles

PLGA microparticles were prepared by the modified single emulsion-solvent diffusion method as described by Jain, et al., [31]. Briefly, 4 ml of organic phase constituting 200 mg of PLGA dissolved in ethylacetate along with various amounts of simvastatin such as 5 mg, 10 mg and 20 mg in order to obtain drug-polymer ratio of 1:40, 1:20 and 1:10 respectively. The resulting organic phase was added slowly to 25 ml of aqueous phase containing 1% (w/v) PVA solution and homogenized at 13,500 rpm at room temperature for 3 minutes. The resulting emulsion was then transferred into 25 ml of water at 30°C and stirred at 100 rpm with hot plate-magnetic stirrer until the solvent was evaporated (approximately 3-4 hrs). The resulted PLGA microparticles were recovered by centrifugation at 10,000rpm for 15 min at 4 °C, washed three times with water to remove residual PVA, re-

suspended in 1.5ml d dH_2O and then lyophilized (Freezone 6, Labconco, MO: −57 °C, 0.03 mbar, 24 h). Blank PLGA microparticles were prepared in a similar fashion except for the lack of drug added in the organic phase.

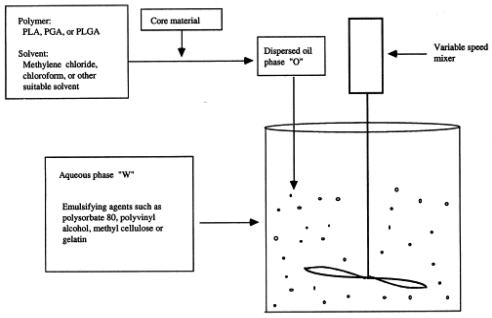

Figure 1. A schematic diagram of O/W Emulsion- solvent evaporation technique (Adopted from O'Donnell, 1997)

Microparticles characterization

Microparticle yield

One of the primary requirements for most of the methods of microencapsulation is to obtain a high yield of microspheres[31]. In this work, we determined the yield of microspheres produced using the single emulsion technique. The microsphere yield, expressed as a percentage, was calculated as follows

$$\%\,Yield = \frac{\text{Weight of microparticles obtained}}{\text{Weight of the polymer + Weight of Drug}} \times 100$$

.....(1)

The weight of the microparticles, expressed in mg, was determined post lyophilization. The weights of the polymer and drug, expressed in mg, were the initial amounts of the polymer and drug added in the emulsion process.

Encapsulation efficiency and drug loading study

Drug content in quantitavely weighed 1 mg of PLGA microparticles was determined by using a suitably modified HPLC method as described by Gupta et al.,[32]. Briefly, the chromatographic procedure is as follows: HPLC analysis was performed with an Agilent Series 1120 HPLC (Agilent Technologies, Wilmington, DE). The chromatographic separations were performed using Phenomenex® 100 C18, 5 µm, 250 mm X 4.6 mm. column, at 45ºC, eluted with mobile phase at the flow rate of 1.0 mL/min. The mobile phase consisted of acetonitrile and 50mM potassium di-hydrogen phosphate buffer (65:35, v/v), apparent pH adjusted to 4.5±0.1 with phosphoric acid solution, filtered through 0.45 µm nylon filter and degassed in ultrasonic bath prior to use. Column was equilibrated for 5 min. by using mobile phase after each run. Each sample was passed through a 0.22 mm poly tetra fluoro ethylene (PTFE) membrane filter before HPLC analysis and each sample analyzed in triplicate and results are indicated as an average of three.

The procedure for analyzing drug loading and encapsulation is as follows:1 mg of quantitavely weighed microparticles were dissolved in 1 ml of ethyl acetate and vortexed for 15 minutes. After agitation, the suspension was centrifuged at 10,000 rpm for 15min. The supernantant was collected and subjected for HPLC analysis for determining the simvastatin content as described earlier. Simvastatin loading and encapsulation efficiency were determined by Eqs. (2) and (3), respectively.

$$\%\,Drug\ loading = \frac{\text{amount of drug in microparticles}}{\text{Amount of microparticles}} X100$$

……..(2)

$$encapsulation\ efficiency = \frac{\text{Actual drug loading in microparticles}}{\text{Theoretical drug loading}} X100$$

……..(3)

Particle size

Microparticle size was determined by laser diffraction using a Malvern Master Sizer Sirocco 2000 (Malvern Instrument Ltd., Worcestershire, UK). Lyophilized microparticles (<5mg) were resuspended in 1 ml of dH_2O and particles size recorded in triplicate of each batch of microparticles.

Table No. 1. Effect of initial amounts of SIM on average particle size of microparticles (mean ± SD, n=3)

Formulation	Average particle size (in µm)
BLMP	6.4±1.8
MSIM-01	6.2±1.2
MSIM-02	6.8±1.6
MSIM-03	7.2±1.9

Table No. 2 Effect of initial amounts of SIM on % Yeild, Drug loading and entrapment efficiency (mean ± SD, n=3)

Formulation name	Wt of SIM used (in mg)	Wt of PLGA used (in mg)	% Yield of microparticles	% SIM loading	Encapsulation Efficiency(%
BLMP	0	200	92.5±2.5	-	-
MSIM-01	5	200	90±0.75	2.31±0.1	85.31±3.8
MISIM-02	10	200	87±1.7	4.68±0.14	85.27±2.5
MSIM-03	20	200	85±1.4	9.94±0.22	92.2±2.1

Surface morphology by scanning electron microscopy (sem)

Microparticles surface morphology was assessed by scanning electron microscopy (SEM) Freeze-dried PLGA microparticles were fixed onto metallic studs with double-sided conductive tape (diameter 12 mm, Oxon, Oxford instruments, UK) and coated with gold for 4 minutes under nitrogen atmosphere in a blazers of a sputter coating unit (Agar Sputter coater, Agar Scientific Ltd., Essex, UK). A LEO 1450 VP (Leo Electron microscopy Ltd., Cambridge, UK) scanning electron microscope was used with an acceleration voltage of 1.00 kV and a secondary detector. The magnification was adjusted till a clear image of the surface of the microparticles appeared and the picture was then recorded.

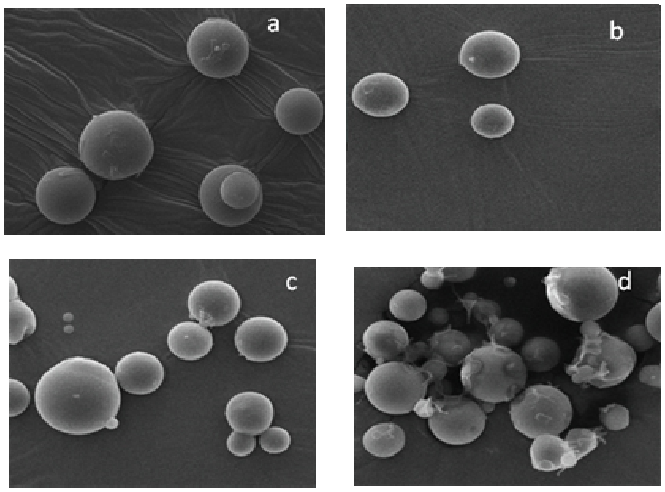

Figure 2. SEM images of freeze dried, a)Blank microparticle; b)MSIM-01;c)MSIM-02; d)MSIM-03.

In vitro simvastatin release studies

In vitro simvastatin release from optimized PLGA microparticles was determined by quantitavely weighing lyophilized MSIM microparticles (10 mg) and dispersing in 1 ml of phosphate buffer (100 mM, pH 7.4) (PBS) containing 0.02% w/v sodium azide as a preservative and incubated at 37°C under gentle shaking at 100 rpm. At appropriate intervals, samples were centrifuged at 10,000 rpm for 15 min. The supernatant was assayed for simvastatin release as it described earlier in of this article, and pellet was re-suspended in fresh 1 ml PBS containing 0.02% w/v sodium azide. Each microparticle batch was analyzed in triplicate and results were expressed as mean ±SD.

hFOB cell culturing

hFOB (ATCC, MA) pre-osteoblastic cells were cultured under standard conditions (5% CO_2, 37°C). Cells were routinely grown to 80% confluence in T175 culture flasks (Sarstedt, Ireland) containing culture media; a 1:1 ratio of Hams F12 and Dulbecco's modified Eagle's medium (without phenol red), 10% foetal bovine serum, 1% penicillin/streptomycin 10mg/ml (Sigma-Aldrich). Expanded hFOB cells of passage 5-15 were harvested with trypsin–EDTA treatment, centrifuged and re-suspended in the culture medium.

Cell viability studies

Simvastatin incorparated microparticles and blank microparticles formulated by adopting the optimized protocol for maximum simvastatin encapsulation efficiency were subjected to cell viability study with hFOB cells by alamar blue assay. hFOB cells were seeded into two 48 well tissue culture plates (one for 24 hrs and another for 48hrs) at a cell density of 50,000 cells per well (250 µl of 200,000 cells/ml stock). The plates were incubated at 37 C in humidified 5% CO_2 overnight to allow the cells to attach to the bottom of the wells. The medium from each well was replaced with 250 µl of fresh medium and to each wells 50 µl of microparticle suspension containing various amounts of simvastatin incorporated PLGA microparticles such as 0, 0.1mg, 1mg and 10 mg was added. The plates were placed on an orbital shaker (Biosan, Riga, Latvia) in the incubator at 37ºC and 5% CO_2, and the assay was carried out at the end of 24h and 48 h, using Alamar blue assay kit (Invitrogen, Biosciences, Dublin). After 24 h of incubation, the medium from each well from plate 1 was replaced with 400 µl of fresh medium containing 10% v/v of Alamar blue reagent and incubated for 3 h. After 3 h of incubation, 100 µl of the supernatant from each well was plated in triplicate into a 96-well plate and absorbance of the samples was read on a spectrometer (Varioskan Flash, version 4.00.53, using SkanIt Software 2.4.3 RE for Varioskan Flash soft ware, Thermo scientific, Waltham, MA, USA) at 590nm and 610nm. Percentage reduction of the Alamar blue solution was determined according to the manufacture's specifications. The same procedure was repeated for 2nd plate after 48 hrs of incubation.

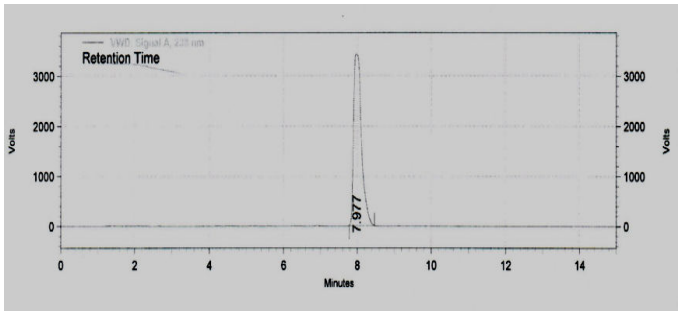

Figure 3. HPLC chromatogram of simvastatin extracted from microparticles.

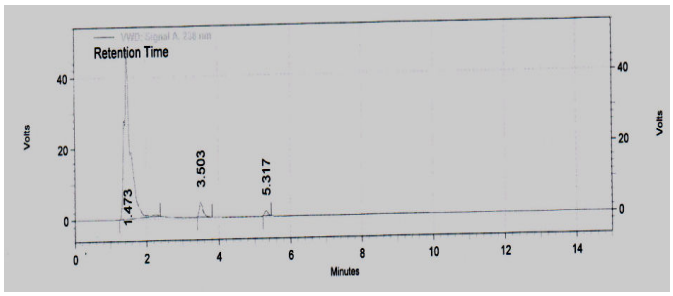

Figure 4. HPLC chromatogram of simvastatin released from microparticles. (Day 2.)

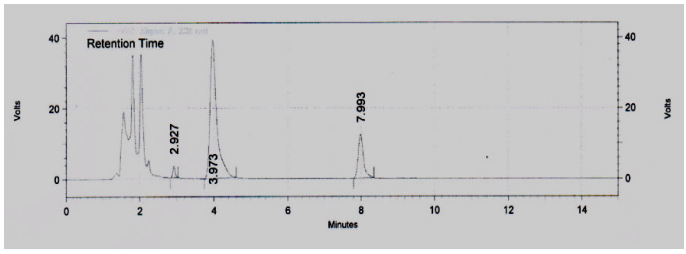

Figure 5. HPLC chromatogram of simvastatin released from microparticles (Day 10.) mixed with pure simvastatin in ethylacetate just before the analysis.

In vitro bio-mineralization test

In vitro bio-mineralization induced by various amounts of MSIM-03 on hFOB cells was assessed by using alkaline phosphatase activity (ALP) assay by conveniently modifying the procedure described by Gentile et al.,[33]. Briefly, hFOB cells were seeded into two 48 well tissue culture plates (one each for 2 d and for 11d) at a cell density of 50,000 cells per well (250 µl of 200,000 cells/ml stock). The plates were incubated at 37°C in humidified 5% CO_2 overnight to allow the cells to attach to the bottom of the wells.

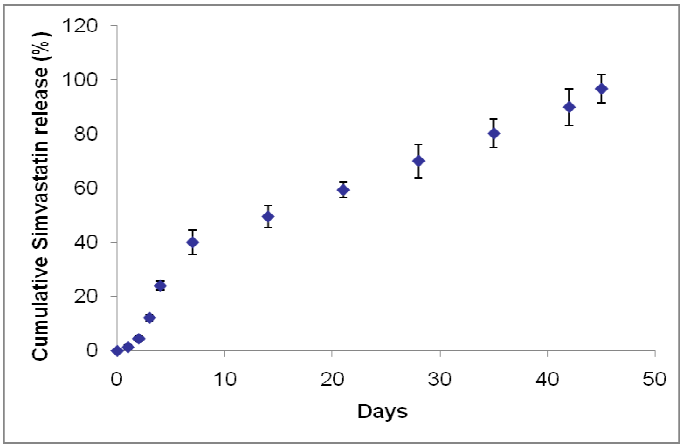

Figure 6 Cumulative % release of simvastatin from microparticles in PBS at 37 ºC (mean±SD, n=3)

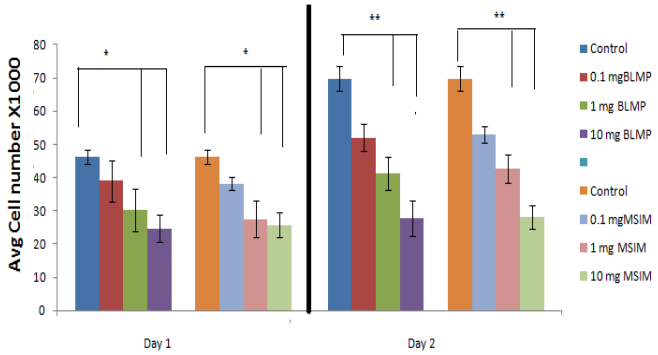

Figure 7. Effect of blank and simvastatin loaded microparticle treatment on cell viability of hFOB cells. Bars indicate the average cell number (mean ± SD; n=3; *is $p<0.05$, ** is $p<0.0001$)

The medium from each well was replaced with 250 µl of fresh medium and to each wells 50 µl of microparticle suspension containing various amounts of simvastatin incorporated PLGA microparticles such as 0, 0.1mg, 1mg and 10 mg was added. The plate was placed on an orbital shaker (Biosan, Riga, Latvia) in an incubator at 37ºC and 5% CO_2, and the assay was carried out at the end of 2 d and 11 d, using the p-Nitrophenyl Phosphate disodium salt (pNPP reagent) (Sigma, Ireland). After 2 d of incubation, the medium from each well from plate 1 was removed and each well and cell layer was washed two-three times with 300 µl of PBS, and replaced with 400 µl of PBS containing 20 % v/v of pNPP and incubated for 15 min in an incubator at 37ºC and 5% CO_2. After 15 min of incubation, 100 µl of the supernatant from each well was plated in triplicate into a 96-well plate and absorbance of the samples was read on a spectrometer (Varioskan Flash, version 4.00.53, using SkanIt Software 2.4.3 RE for Varioskan Flash soft ware, Thermo scientific, Waltham, MA, USA) at 405 nm. For plate 2, media was replenished every 2-3 days for 11 days by simply aspiring 250µl from each well and replacing with fresh equivalent medium. At the end of 11 days, pNPP activity was assessed as described earlier. Enzyme activity was expressed as a function of

OD at 405nm. The mean and the standard deviation were obtained from three different experiments[34].

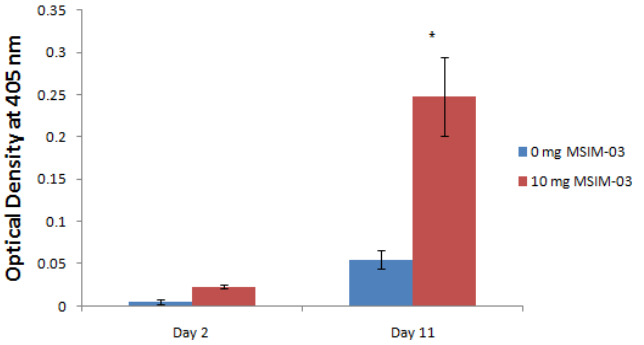

Figure 8.The ALP activity of hFOB cells in response to the treatment of MSIM . Bars indicate the average OD values (mean ± SD; n=3; *is p<0.05, ** is p<0.0001)

Statistical analysis

Experiments were run in triplicate for each sample. All data were expressed as mean ± SD for n=3. Statistical analysis was determined by using Analyse-it v2.22 software. The statistical differences between groups were calculated using Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA). Statistical significance was declared as significant(*) at $p<0.05$, and very significant(**) at $p<0.0001$.

3. RESULTS AND DISCUSSION

Particle size and Surface morphology

For encapsulating hydrophobic or poorly water-soluble drugs with PLGA microparticles, the oil-in-water (o/w) method is frequently used as this method is the simplest and easy to prepare microparticles[35]. The particle size is one of the important characteristics of the drug loaded microparticles as it could affect drug release properties of microparticles. Particle size determination studies reveal that the microparticles produced were uniform with a size range of 5-8 µm. and loading of simvastatin has not altered the particle size as represented in Table.1. Furthermore, it is evident from SEM images that the microparticles produced were spherical in shape with smooth surfaces indicating.that the loading of the drug did not lead to any significant changes in surface of the microparticles.

In general the size of microparticle formed depends on the extent of separation of oil phase containing polymer in continuous phase into small individual droplets, which can be achieved by applying shear forces. In this study, we used to homogenization process to induce the shear forces. Furthermore, 1%w/v of PVA was adopted as a stabilizer for microparticle formation which can reduce the interfacial tension of organic and water phase. The droplet break-up during the homogenization process is due to the turbulent flow of organic phase within the aqueous phase. This turbulent flow is controlled by inertial forces which are dependent on the viscosity of the polymer phase[36]. Hence, in this study, we used an optimum concentration of 50 mg/ml of PLGA to make the microparticles. Poly vinyl alcohol (PVA) is the most commonly used emulsifier in PLGA microparticle formulation as it is associated with the formation of relatively small particles with uniform size distribution[37].

Furthermore, the concentration of PVA used in continuous phase during emulsification influences the particle size. Increasing the PVA concentration or its molecular weight had been reported to result in smaller microparticles[38]. However, higher PVA concentrations in continuous phase may hinder the complete separation of nascent droplets due to bridging effect at overly high PVA concentration and may result in formation of a larger number of aggregated microparticles during solidification[39]. Hence in this study, poly vinyl alcohol (Mw: 30-70 kD, >87-90% hydrolized) at a concentration of 1%w/v was considered to make PLGA microparticles. The scanning electron micrograph images of the microparticles revealed their regular spherical shape, smooth surface and no aggregation. No difference was observed in the morphological properties of microparticles due to presence of the simvastatin (Fig. 2).

Encapsulation efficiency and simvastatin loading

The RP-HPLC analysis for the simvastatin loaded microspheres dissolved in ethyl acetate for determining simvastatin loading and encapsulation efficiency displayed a characteristic peak of simvastatin at around 7.9 min. which is similar to that of pure simvastatin (Fig. 3).

All microparticle formulations had high yield above 85% w/w recovery with respect to the initial amounts of polymer and drug used in the microparticle formulations and no significant differences were observed with varying amount of simvastatin added to the formulations. The results disclosed a maximum encapsulation (above 85%) of simvastatin within PLGA microparticles (Table. 2) which could be attributed to the hydrophobicity of the simvastain and very low water solubility,(solubility of simvastatin in PBS (1.1µg/ml))[29,40].

Simvastatin loading within PLGA microparticles was increased with the increase in initial amount of the formulation. However, maximum encapsulation of simvastatin of above 92% was observed 1:10 drug polymer ratio used for making microparticles. Owing to the dosage requirements and detection limitations we optimized 10% w/w loading of simvastatin in PLGA microparticles i.e. MSIM-03 as a suitable formulation for further studies.

Release studies

Quantification of released simvastatin from PLGA microparticles was performed by RP-HPLC method. In general, HPLC system consists of a continuous phase and a mobile phase. The mobile phase acts as a carrier for the sample solution. The

columns are packed with the stationary phase, which was immobile and immiscible to the mobile phase forced through it. In Reverse Phase HPLC system, the retention of solute in column is as a result of the interactions between non polar components of the solutes and the non polar stationary phase. Therefore the more hydrophilic moieties elute faster than the hydrophobic ones. The RP-HPLC chromatogram for released simvastatin show a shift in retention time from 7.9 min. to 5.7 min. and a peak was observed at around 3.5.min., and at around 1.5 min. which kept increasing over time which could be due to simvastatin-acid, the active metabolite of simvastatin. This is the open hydroxy acid form of the closed lactone form of simvastatin and is more hydrophilic in nature (Fig. 4 & Fig. 5).

The lactone drug may have been hydrolyzed and consequently simvastatin acid, which is more hydrophilic in nature might have been detected before the characteristic peak of simvastatin. The AUC (area under curve) which was used to quantify the released simvastatin was the sum of all peaks which were observed during the chromatographic analysis[29,40].

Furthermore, for potential detection of simvastatin (which is not hydrolyzed) in release medium, 0.5 ml drug release supernatant was mixed with of 0.5 ml of ethyl acetate and subjected for HPLC analysis. Ethyl acetate was added with an idea to dissolve simvastatin if present in the sample. But, there was no peak observed at the retention time of 7.9 min. indicating that the released simvastatin is hydrolyzed in PBS. Blank PLGA microparticles were also subjected for release study similar to simvastatin loaded PLGA microparticles to check for peak if any, from polymer degradation. But these results confirmed the absence of simvastatin and simvastatin acid peaks. Moreover, HPLC analysis for simvastatin released samples on day 10 were mixed with of known amounts of simvastatin dissolved in ethyl acetate and mixed just before the analysis, was also performed and resulted a characteristic peak of simvastatin (at retention time around 7.9 min) and subsequent peaks of simvastatin-acid in a similar fashion(Fig.6).

Generally speaking, hydrophobic drug release from microparticle carrier systems occurs via diffusion down the concentration gradient. The homogenous distribution of hydrophobic drug within the polymeric matrix would result during the microparticles formulation, owing to its solubility in solvent, resulting in the formation of a monolithic solution system. Hence, as drug begins to release from the surface of the matrix, a concentration gradient is established to provide the driving force for drug diffusion from the interior of the matrix towards the surface for subsequent release[41].

The cumulative release versus time profiles of simvastatin from PLGA microparticles in PBS buffer (pH 7.4) was represented in Fig.7. reveals an initial slow release of simvastatin from microparticles which could be attributed to the hydrophobicity of simvastatin. Moreover, the interaction of hydrophobic drug molecules with the polymer matrix via hydrophobic binding forces or of amine-groups with the polymer carboxyl groups by ionic bonds might cause a trapping of the drug inside the particles and therefore, a slower release[42].

During first 24 hrs of release study, only 1%w/w of encapsulated simvastatin was released from micro-particles. Following to this, the release of simvastatin was increased to around 5 % w/w of encapsulated simvastatin (from day 1- day 2 of release studies), which is probably due to the diffusion of drug which is present at the surface of the microparticles. The presence of drug crystals at the surface of particles could be attributed to the solvent flux out of the oil phase during the solvent evaporation step of microparticle formation. The solvent flux might transport drug to the particle surface. Once the solvent partitioned into the water phase its dissolving power would have disappeared resulting in either precipitation of drug at the particle surface or in the suspension medium. Since simvastatin is hydrophobic in nature, it would have adsorbed at the surface of microparticles [43]. After this initial slow release, there was a rapid release of simvastatin from the microparticles (from day 2-day 7 of the release study) which could be attributed hydrolytic random chain scission process of the polymer resulting in the formation of pores within the microparticles. The pores which were formed would have resulted an increase in water uptake within the microparticles. During this release phase, the drug release is governed by diffusion-controlled mechanism through a network of water filled pores and channels. During this process of drug release, the molecular weight of the polymer decreases significantly, but no appreciable weight loss and no soluble monomer products were formed[44-46]. Around 40% w/w of encapsulated drug was released during this phase. Following to this rapid release phase, a more controlled release of drug from microparticles was observed (during day 7-day 45 of the release study) which could be attributed to the bulk erosion of the microspheres by hydrolysis of the polymer's ester bond linkages. During this release phase, there would be a rapid loss of polymeric mass due to the formation of soluble oligomeric and monomeric products of bulk polymer. Finally, soluble monomeric products formed were degraded into soluble oligomeric units resulting a complete release of encapsulated drug[47-49](around 96% w/w of encapsulated drug was released towards the end of 45 days release study).

Cell viability test

The effect of BLMP and MSIM-03 on hFOB cell viability was represented in Fig 8. It is evident from the histogram that there is a dose dependent reduction in cell number when hFOB cells are treated with blank PLGA microparticles and MSIM-03 particles after 1 d and 2 d of incubation. However, this treatment is not having any effect on cell proliferation which is evident from increased cell number after 2 d. Moreover, in case of cells treated with MSIM-03 particles, a slight increase in cell number observed in compare with that of blank microparticles, which could be

attributed to released simvastatin which would have promoted the cell proliferation rate and differentiation.

***In vitro* bio-mineralization**

The ALP activity of hFOB cells in response to the treatment of MSIM-03 particles. The histogram was plotted by taking number of days after treatment on X-axis and difference in OD at 405 nm between blank wells and treated wells. The results reveal that MSIM-03 induced in vitro bio-mineralization as the treatment time increased which could be attributed to the release of simvastatin from MSIM-03 microparticles. ALP is a good marker for the differentiation of osteoblastic cells. The increased expression of ALP levels after 11 days treatment of MSIM-03 on hFOB cells confirms release and the activity of encapsulated simvastatin.

4. CONCLUSION

In this study, we successfully incorporated simvastatin within biodegradable PLGA microparticles. The resulted microparticles displayed uniform spherical shape, size and controlled release of simvastatin for 45 days. Release of simvastatin from PLGA microparticles displayed two stages release: During first stage, initially there was a slow release mediated by diffusion of adsorbed simvastatin through the polymer network followed by a rapid release mediated by the diffusion of simvastatin through the fluid-filled within the particles as a result of pores formed within the particles. During second stage, a more controlled release was observed which could be attributed to the physical bulk erosion and/or hydrolysis of the polymer and finally complete degradation of polymer after 6 weeks of release study. Furthermore, simvastatin loaded PLGA microparticles treatment on hFOB cells induced in vitro bio-mineralization which indicates that the developed delivery system can be potentially applied for the purpose of bone regeneration by suitably incorporating within porous 3 D scaffolds for the purpose of spatio-temporal release of simvastatin.

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REFERENCES

1. Taylor HF. (1938). Vitamin preparations and methods of making same. US Patent 2,183,053 (12 December).
2. Kulkarni RK, Moore EG, Hegyelli AF, Leonard F. Biodegradable poly(lactic acid) polymers. J. Biomed. Mater. Res. 1971;5: 169–181.
3. Cutright DE, Beasley JD, Perez B. Histologic comparison of polylactic and polyglycolic acid sutures. Oral Surg. 1971;32: 165–173.
4. Brady JM, Cutright DE, Miller RA, Battistone GC. Resorption rate, route of elimination, and ultrastructure of implant site of polylactic acid in the abdominal wall of the rat. J. Biomed. Mater. Res. 1973;7: 155–166.
5. Laurencin, CT., Elgendy, H. (1994). The biocompatibility and toxicity of degradable polymeric materials: implications for drug delivery. In: A.J. Domb (Ed.), Polymeric Site-specific Pharmacotherapy (pp. 27–46). Chichester: Wiley.
6. Ignatius AA, Cleas LE. In vitro biocompatibility of bioresorbable polymers: poly(l, dl-lactide) and poly(l-lactide-co-glycolide). Biomaterials. 1996; 17: 831–839.
7. Beck LR, Cowsar DR, Lewis DH, Gibson JW, Flowers CE. New longacting injectable microcapsule contraceptive system. Am. J. Obstet. Gynecol. 1979; 135: 419–426.
8. Sanchez A, Tobio M, Gonzalez L, Fabra A, Alonso MJ. Biodegradable micro- and microparticles as long-term delivery vehicles for interferon-α. Eur J Pharm Sci.2003;18: 221–229.
9. Shakweh M, Besnard M, Nicolas V, Fattal E. Poly(lactide-co-glycolide) particles of different physicochemical properties and their uptake by Peyer's patches in mice, Eur J Pharm Biopharm. 2005;61(1-2): 1-13.
10. Song CX, Labhasetwar V, Murphy H, Qu X, Humphrey WR, Shebuski RJ, Levy RJ. Formulation and characterization of biodegradable microparticles for intravascular local drug delivery. J Control Release. 1997;43: 197–212.
11. Feng SS, Chien S. Chemotherapeutic engineering: application and further development of chemical engineering principles for chemotherapy of cancer and other diseases. Chemical Engineering Scienc.2003;58: 4087-4114.

12. Sandor M, Enscore D, Weston P, Mathiowitz E. Effect of protein molecular weight on release from micron-sized PLGA microspheres. J. Control. Release. 2001;76: 297–311.
13. Lam X, Duenas E, Daugherty A, Levin N, Cleland J. Sustained release of recombinant human insulin-like growth factor-I for treatment of diabetes. J. Cont. Release. 2000;67: 281–292.
14. Siepmann J, Gopferich A. Mathematical modeling of bioerodible, polymeric drug delivery systems. Adv Drug Deliv Rev. 2001;48: 229–247.
15. Tracy MA, Ward KL, Firouzabadian L,Wang Y, Dong N, Qian R, Zhang Y. Factors affecting the degradation rate of poly(lactide-co-glycolide) microspheres in vivo and in vitro. Biomaterials. 1999; 20: 1057–1063
16. Li M, Rouaud O, Poncelet D. Microencapsulation by solvent evaporation: State of the art for process engineering approaches. Int J Pharm. 2008;363(1-2): 26-39.
17. Passerini N, Craig D.Q. Characterization of ciclosporin A loaded poly (D,L lactide-co-glycolide) microspheres using modulated temperature differential scanning calorimetry. J. Pharm. Pharmacol. 2002;54: 913-919.
18. Suzuki K, Price JC. Microencapsulation and dissolution properties of a neuroleptic in a biodegradable polymer, poly(dl-lactide). J Pharm Sci. 1985;74(1): 21-24.
19. Smith A, Hunneyball IM. Evaluation of poly(lactic acid) as a biodegradable drug delivery system for parenteral administration. Int. J. Pharm. 1986; 30: 215–220.
20. Cavalier M, Benoit JP, Thies C. The formation and characterization of hydrocortisone-loaded poly((+/-.)-lactide) microspheres. J Pharm Pharmacol. 1986;38(4): 249-253.
21. Sansdrap P, Moes AJ. Influence of manufacturing parameters on the size characteristics and the release profiles of nifedipine from poly(DL-lactide-co-glycolide) microspheres. Int. J. Pharm. 1993;98(1-3): 157–164.
22. Sugiyama M, Kodama T, Konishi K, Abe K, Asami S, Oikawa S. Compactin and simvastatin, but not pravastatin, induce bone morphogenetic protein-2 in human osteosarcoma cells. Biochem Biophys Res Commun. 2000;271: 688-692.
23. Maeda T, Matsunuma A, Kawane T, Horiuchi N. Simvastatin promotes osteoblast differentiation and mineralization in MC3T3-E1 cells. Biochem Biophys Res Commun 2001;280: 874-877.
24. Skoglund B, Forslund C, Aspenberg P. Simvastatin improves fracture healing in mice. J Bone Miner Res. 2002;17: 2004-2008.
25. Yamashita M, Otsuka F, Mukai T, Otani H, Inagaki K, Miyoshi T, Goto J, Yamamura M, Makino H. Simvastatin antagonizes tumor necrosis factor-alpha inhibition of bone morphogenetic proteins-2-induced osteoblast differentiation by regulating Smad signaling and Ras/Rho-mitogen-activated protein kinase pathway. J Endocrinol. 2008;196: 601-613.
26. von Stechow D, Fish S, Yahalom D, Bab I, Chorev M, Muller R, Alexander JM. Does simvastatin stimulate bone formation in vivo? BMC musculoskeletal disorders. 2003;4:8.
27. Hsia J, Morse M, Levin V. Effect of simvastatin on bone markers in osteopenic women: a placebo-controlled, dose-ranging trial. BMC musculoskeletal disorders. 2002;3: 3-7.
28. Ucar M, Neuvonen M, Luurila H, Dahlqvist R, Neuvonen P, Mjorndal T. Carbamazepine markedly reduces serum concentrations of simvastatin and simvastatin acid. European journal of clinical pharmacology. 2004;59: 879-882.
29. Serajuddin AT, Ranadive SA, Mahoney EM. Relative lipophilicities, solubilities and structure- pharmacological considerations of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase inhibitors pravastatin, lovastatin, mevastatin, and simvastatin. J Pharm Sci 1991;80: 830-834.
30. Corsini A, Maggi FM, Catapano AL. Pharmacology of competitive inhibitors of HMG-CoA reductase. Pharmacol Res.1995;31: 9-27.
31. Jain RA. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomaterials. 2000;21: 2475-2490.
32. Gupta KR, Askarkar SS, Wadodkar SG. Stability indicating RP-HPLC method for simultaneous determination of Atorvastatin and Nicotinic Acid from

their combined dosage form. Eurasian J. Anal. Chem. 2009; 4(3): 294-303.

33. Gentile P, Chiono V, Boccafoschi F, Baino F, Vitale-Brovarone C, Vernè E, Barbani N, Ciardelli G. Composite films of gelatin and hydroxyapatite/bioactive glass for tissue engineering applications J. Biomat. Sci. Polymer edition. 2010;21: 1207-1226.
34. Nandagiri VK, Gentile P, Chiono V, Tonda-Turo C, Matsiko A, Ramtoola Z, Montevecchi FM, Ciardelli G. Incorporation of PLGA nanoparticles into porous chitosan–gelatin scaffolds: Influence on the physical properties and cell behaviour. J Mech Behav Biomed Mater. 2011;4(7):1318-27.
35. Albayrak, C. (2005). Induced phase transition method for the production of microparticles containing hydrophobic active agents. US Patent 6,899,898 (31 May).
36. Schuchmann HP, Danner T, Emulsification: more than just communication. Chem. Ing. Tech. 2004;76: 364–375.
37. Boury F, Ivanova T, Panaiotov I, Proust JE, Bois A, Richou J. Dynamic properties of poly(d,l-lactide) and polyvinyl alcohol monolayers at the air/water and dichloromethane/water interfaces. J. Colloids Interface Sci. 1995;169: 380–392.
38. Jobmann, M., Rafler, G. Controlled release systems of biodegradable polymers. 6th communication: control of particle size and size distribution of microparticles produced by the solvent evaporation technique. Pharm. Ind. 1998;60: 979–982.
39. Yang Q, Owusu-Ababio G. Biodegradable progesterone microsphere delivery system for osteoporosis therapy. Drug Dev. Ind. Pharm. 2000;26: 61–70.
40. Patel R, Buckton G, Gaisford S. The use of isothermal titration calorimetry to assess the solubility enhancement of simvastatin by a range of surfactants. Thermochimica Acta. 2007;2: 106-113
41. Huang X, Brazel CS. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. J Control Release. 2001;73(2–3):121–136.
42. Panyam J, Williams D, Dash A, Leslie-Pelecky D, Labhasetwar V. Solidstate solubility influences encapsulation efficiency and release of hydrophobic drugs from PLGA/PLA nanoparticles. J. Pharm. Sci. 2004;93: 1804–1814
43. Jalil R. Nixon JR. Microencapsulation using poly(l-lactic acid) I: microcapsule properties affected by the preparation technique. J. Microencapsul. 1989;6(4): 473–484.
44. Wu, XS. (1995). Preparation, characteriztion, and drug delivery applications of microspheres based on biodegradable lactic/glycolic acid polymers. In D. Wise (Ed.) Encyclopedic handbook of biomaterials and bioengineering (pp 1-41). New York: Marcel Dekker, Inc.
45. Lewis, DH. (1990). Controlled release of bioactive agents from lactide/glycolide polymers. In M. Chasin (Ed.) Biodegradable polymers as drug delivery systems (pp1-41). New York: Marcel Dekker, Inc.
46. Ramirez L, Pastoriza P, Herrero-Vanrell R. Biodegradable poly(DL-lacticco- glycolic acid) microspheres containing tetracaine hydrochloride. in-vitro release profile. J. Microencapsul.1999;16: 105–115.
47. Wada R, Hyon SH, Ikada Y. Lactic acid oligomer microspheres containing hydrophilic drugs. J Pharm Sci. 1990;79(10): 919-924.
48. Izumikawa S, Yoshioka S, Aso Y, Takeda YJ. Preparation of poly(l-lactide) microspheres of different crystalline morphology and effect of crystalline morphology on drug release rate. J. Control Release. 1991;15(2): 133-140.
49. Miller RA, Brady JM, Cutright DE. Degradation rates of oral resorbable implants (polylactates and polyglycolates): rate modification with changes in PLA/PGA copolymer ratios. J Biomed Mater Res. 1977;11(5): 711-719