J Mater Sci (2011) 46:2510–2517
DOI 10.1007/s10853-010-5101-4

Fabrication and characterization of porous poly(lactic-*co*-glycolic acid) (PLGA) microspheres for use as a drug delivery system

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Received: 13 September 2010 / Accepted: 19 November 2010 / Published online: 3 December 2010
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Abstract In this work, Simvastatin (SIM) loaded porous poly(lactic-*co*-glycolic acid) (PLGA) microspheres were fabricated using the W/O/W1/W2 double emulsion and solvent evaporation method. The optimal conditions for fabricating porous PLGA microspheres were determined to be 20% distilled water (v/v), 10% PLGA (m/v), and a 4:1 ratio of internal polyvinyl alcohol (PVA) to dichloromethane (DCM). The pores size distribution of porous PLGA microspheres was varied from 0.01 to 40 µm, while their particle displayed a bimodal size distribution that had two diameter peaks at around 100 µm and 500 µm. The SIM encapsulation efficacy was found to be very high with a yield near 80% and the porous PLGA microspheres showed the excellent biocompatibility. In addition, the drug release profile was found to be significantly different from a temporal basis. Base on the combined results of this study, SIM loaded PLGA microspheres holds great promise for use in biomedical applications, especially in drug delivery system or tissue regeneration.

Introduction

Biodegradable polymer particulates such as microparticles and microspheres have received a significant amount of attention as drug delivery systems (DDS) due to their potential use in locally targeted pharmaceutical delivery [1], medical applications [2], and regeneration of injured tissues [3]. For microspheres to aid in healing injured tissues and for the delivery of drugs, they must be easy to fabricate, biocompatible, and biodegradable [4, 5]. In regards to tissue regeneration, the major role of the microspheres not only aids in repairing injured tissue but also contains hollow shells to provide a large surface area for adherent cells to attach and proliferate [6]. Among the variety of candidates, poly(lactic-*co*-glycolic acid) (PLGA) microspheres have been widely used for DDS and regenerative medical applications, due mainly to its biocompatible and degradation by metabolic products that are eliminated from the body [7, 8].

Several techniques have been used to fabricate biodegradable microspheres, including the emulsion solvent evaporation method [9], rapid solvent removal by introducing a temperature gradient [10], and the solution induced phase separation [11]. Among these approaches, solvent evaporation method is the most common method used to fabricate biodegradable microspheres aimed at delivering drugs and proteins [12–15]. The procedure of preparing microspheres using this technique is relatively simple; however, many different parameters can effect on the morphology and structure of the microspheres, which has been extensively documented [7, 9]. The sphere size distribution, drug encapsulation, and formation of hollow shells are often poorly controllable when using emulsion fabrication routes. Recently, fabrication of microspheres with precisely controlled and monodisperse size distributions were achieved by spraying polymer containing solutions through nozzles with acoustic excitation [16]. Otherwise, the Simvastatin (SIM) drug, which has reported to stimulate bone formation, was incorporated into

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microspheres to evaluate the stability of microspheres after formation [17, 18]. Because porous biodegradable microspheres are desirable for DDS applications [19]. Therefore, we developed a SIM loaded porous PLGA microspheres using the W/O/W1/W2 method with the aim of using for DDS. In this method, distilled water was used to create water droplets (internal aqueous phase) inside the PLGA phase by an ultrasonic probe machine, which was key to form porous structures of the PLGA microspheres.

In this study, the fabrication, characterization, and in vitro study of the porous PLGA microspheres were investigated. FT-IR was used to verify the incorporation of some components of the microspheres whereas the crystalline structure of those components was also investigated by XRD and DSC diffraction. Osteoblast cells were seeded on the porous microspheres and the cell seeded aggregation on the surface of microspheres was examined in 1, 3, and 7 days. The drug release profile confirmed a burst release in the first 2 weeks of the experiment, which was then released in a sustained manner in the following 2 weeks. When the SIM drug was loaded into the interior, the microspheres were still able to promote cellular aggregation and displayed excellent biocompatibility and biodegradability. These results suggest that SIM loaded porous PLGA microspheres hold promise for use as a DDS in bone tissue regeneration.

Materials and methods

Materials

Polyvinyl alcohol (PVA) was purchased from Sigma-Aldrich, Inc, (St. Louis, MO, USA). PLGA was obtained from Aldrich (St. Louis, MO, USA). SIM was purchased from Sigma-Aldrich Chemie GmbH. DCM (99.5%) was obtained from Samchun pure chemical Co., Ltd, (10B-5L, 440-3, Mogok-dong, Pyeongteak city, Gyeonggi-do, Korea). Cell culture studies were carried out with the ATCC Cell Line (CCL-1^{TM}, NCTC clone 929 [L cell, L-929, derivative of Strain L], Korea). All other chemicals and solvents were of analytical reagent grade.

Fabrication of porous PLGA microspheres

Simvastatin (SIM) loaded porous PLGA microspheres were fabricated using the W/O/W1/W2 method. First, PLGA was dissolved in DCM with or without SIM to create a homogenous solution. Then distilled water was added and emulsified using a homogenizer (Sonifier-Brason 450) to form the first emulsion droplet phase (W/O). The second emulsion droplet phase (W/O/W1) was prepared by pouring the first emulsion droplet phase into a 1% internal PVA solution (W1) and stirring at a fast rate. Then the second emulsion droplet phase was poured into 200 mL of 0.1% external PVA solution (W2) to form W/O/W1/W2 phase and stirred for 45 min to allow for solvent evaporation and stabilization of the microspheres. The microspheres were isolated and washed three times with distilled water before lyophilized using a free-dry machine.

Characterization of porous PLGA microspheres

The morphology of porous PLGA microspheres was examined using a scanning electron microscope (SEM, JSM-635F, Jeol). The pore size and particle size distribution of the porous PLGA microspheres were determined using a particle size analyzer (Quantachrome instrument poremaster). PLGA, PVA, SIM, their physical mixture, and SIM loaded porous PLGA microspheres were characterized by attenuated reflectance Fourier transform spectroscopy (FT-IR) (Spectrum GX, PerkinElmer, USA). The X-ray diffraction (XRD) (CuKα, D/MAX-250, Rigaku, Japan) technique was used to characterize SIM, PLGA, and SIM loaded porous PLGA microspheres. DSC measurements (METTLER TOLEDO KOREA–DSC822e) were used to characterize SIM, physical mixture of SIM, PLGA, and PVA, then SIM loaded PLGA microspheres were also characterized.

Drug encapsulation and in vitro study

Drug encapsulation efficacy experiments were performed to determine the actual amount of SIM that was entrapped into the porous PLGA microspheres. A known amount of SIM loaded porous PLGA microspheres sample was dissolved in acetonitrile to form the sample solution. Then sample solutions were taken and the SIM peak was detected using a UV-spectrophotometer (UV-1240, Shimadzu, Japan) at 240 nm.

In vitro degradation and SIM drug released from the 0.2 g of porous microspheres were determined in phosphate buffer saline (PBS, 0.15 M, pH 7.4) at 37 °C. SIM released was determined using a UV-spectrophotometer at a wavelength of 240 nm. A standard calibration curve of known amounts of SIM was used to quantify the amount SIM loaded and released.

The cytotoxicity of the porous PLGA microspheres was estimated in vitro using the MTT assay. Extraction media was prepared by immersing the specimens in Rosewell Park Memorial Institute (RPMI 1640 medium: HyClone, Logan, UT, USA) over the course of 1 day. Osteoblast cells were seeded at a density of 1×10^5 cells/well and then incubated at 37 °C for 24 h. After 1 day, the cell culture media was removed with an aspirator, and the extraction media from the porous PLGA microsphere was

added to the 96 wells at different extraction media concentrations (12.5, 25, 50, and 100%). After 72-h incubation, 20 μL of the MTT solution was added to each well and incubated for 4 h. Then after careful removal of the media, 200 μL of dimethylsulfoxide (DMSO; Samchun, D0458, Korea) was added to each well to extract the formazan crystals while shaking. The absorbance intensities were measured at 595 nm using an ELISA plate reader (EL 312e, Bio-Tek).

The aggregation of osteoblast cell on porous PLGA microspheres was investigated after adding 0.1 g of porous microspheres to the culture plate (24 wells). Following this, 1 mL of media was added to create the microsphere suspension in media. 100 μL of the microsphere suspension was transferred to the culture plate (96 wells). The suspension was incubated for 30 min, and then the media was discarded before the cells were seeded. Then osteoblast cells were seeded at a density of 10^4 cells/cm^2 in DMEM and cultured for 1, 3, and 7 days at 37 °C in a humidified air atmosphere with 5% CO_2. After 1, 3, and 7 days of culture, cellular constructs were harvested and subsequently cells were stained with propidium iodide for 30 min and washed twice with PBS. Finally, samples were observed by confocal laser scanning microscopy (Nikkon eclip 56 E 600, Japan).

Results and discussion

Figure 1 shows the morphology of the porous PLGA microspheres observed by SEM. The microspheres were mostly spherical in shape and almost all were separated from each other. However, a few microspheres had aggregated, as highlighted by the arrows in Fig. 1a. In the first emulsion phase, distilled water droplets were dispersed into PLGA droplets to form distilled-water–PLGA microdroplets. In the second emulsion phase, distilled water droplets were effused internally into the internal PVA solution when stirring and the external PVA solution was left behind allowing for the formation of pores, and simultaneously this prevented the coalescence of microdroplets [7, 20, 21]. In fabrication process, many parameters were varied to determine the optimal condition fabrication, as shown in Table 1. The optimal condition created the homogenous porous structure of PLGA microsphere, as shown in Fig. 1b. Similarly with previous report, distilled water as internal aqueous phase was a key to create porous structure of PLGA microspheres [4, 9]. While porous microspheres have been fabricated using a variety of methods, to our knowledge, no previous methods have used distilled water for easy formation of porous structure on PLGA microspheres. Thus, the W/O/W1/W2 is a new method for stably fabricating porous PLGA microspheres.

The SEM surface image of the porous PLGA microsphere is shown in Fig. 2a. From this, diameter of pores on the surface of microsphere was determined to range from 2 to 8 μm. Figure 2b shows a cross-sectional SEM image of

Table 1 Distilled water volume, PLGA concentration, and PVA/DCM volume ratios of PVA to DCM fabricate porous PLGA microspheres

No	Distilled water (V/V) (%)	PLGA (M/V) (%)	PVA: DCM (V/V)
1	0	10	4:1
2	10	10	4:1
3	20	10	4:1
4	20	5	4:1
5	20	8	4:1
6	20	10	4:1
7	20	10	1:3
8	20	10	3:2
9	20	10	2:1
10	20	10	3:1
11	20	10	4:1

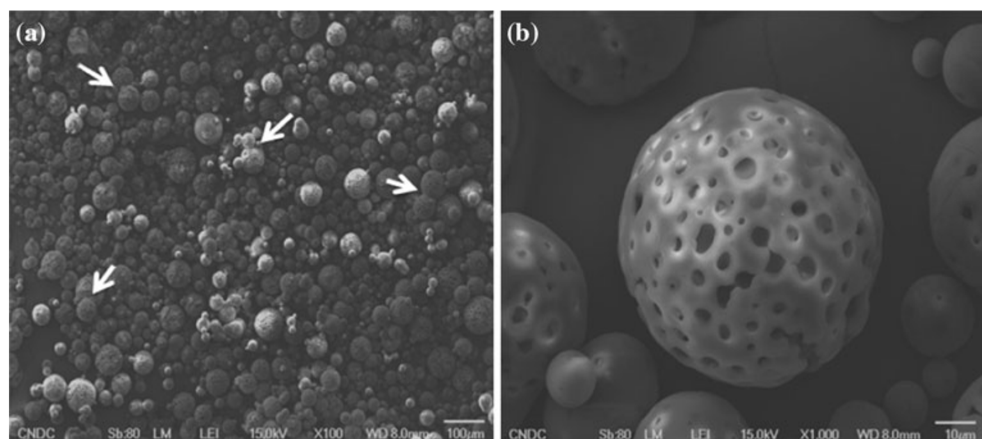

Fig. 1 SEM morphology of: **a** porous PLGA microspheres, **b** high magnification of **a**

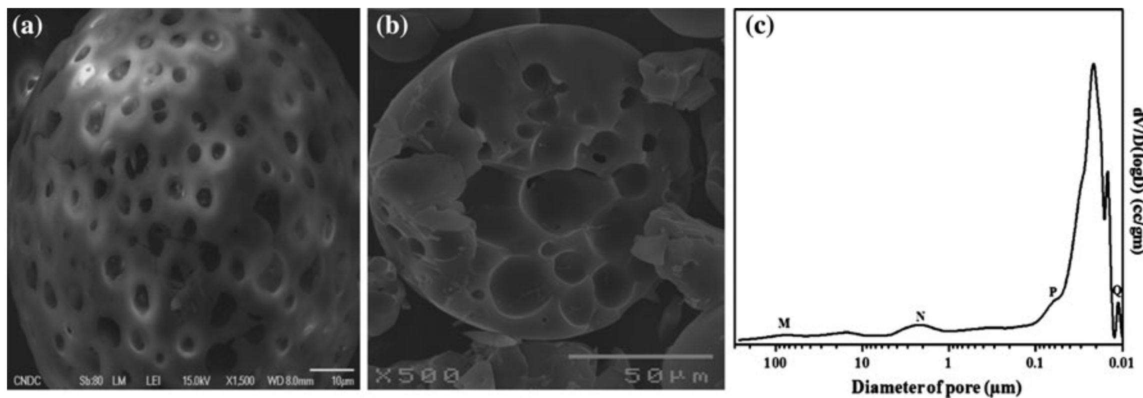

Fig. 2 SEM morphology of outer surface of porous PLGA microsphere (**a**), its cross section (**b**) and its pore size distribution (**c**)

the porous PLGA microsphere. In this image, open pores inside the microsphere were observed. These pores had diameters ranging from 10 to 40 µm. There was discernible difference in morphology between the surface and the internal region of PLGA microspheres. This may be due to the difference between the osmotic pressure of the internal aqueous solution and external aqueous solution through procedure fabrication [22, 23]. The pore size distribution measured using a particle size analyzer is shown in Fig. 2c. These peaks (marked from P to Q) were observed as the main peaks, with a few minor peaks that were marked from M to N. The porous PLGA microsphere had some open pores that resided on the surface and interior of microsphere. Their diameters varied from 0.3 to 40 µm, which corresponded to the peaks from M to N. These others varied from 0.01 to 0.05 µm, which corresponded to the peaks from P to Q [24] although these pores were not observed by SEM. Conclusively, the porous PLGA microspheres that possessed so many hollow shells can provide a large surface area, allow free exchange of oxygen and nutrients for adherent cells attachment and proliferation.

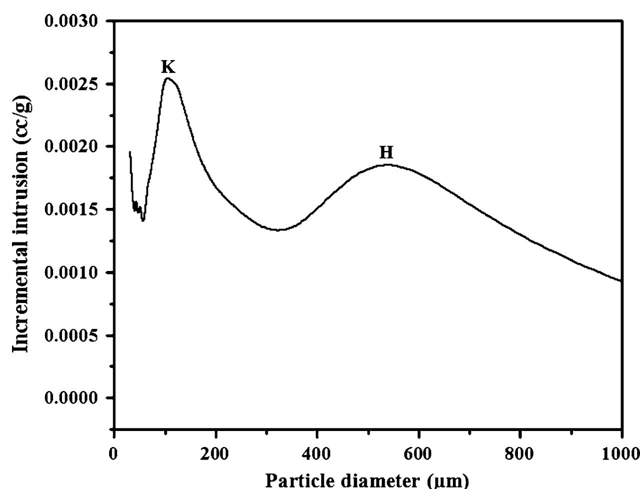

Fig. 3 The particle size distribution of porous PLGA microspheres

Figure 3 shows the particle size distribution measured using a particle size analyzer. Two peaks (marked K and H) were observed as the main peaks, with a few minor peaks [24]. Thus, when porous PLGA microspheres were fabricated, the coalescence of emulsion droplets was the main cause of inherent thermodynamic instability in the interface between the water-rich and oil-rich phases, which tends to reduce the interface area between the two immiscible phase [25]. Because the porous PLGA microspheres were stably fabricated, almost meta-stable emulsion droplets had collided prior to coalescence so that almost porous PLGA microspheres had been separated, as shown in Fig. 1. As result, the diameter of microspheres was a little smaller than 100 µm, which corresponded to the peak labeled K. However, the peak labeled H was due to the aggregation of a few microspheres, which most likely occurred when a few meta-stable emulsion PLGA droplets coalescence in the external PVA solution.

Figure 4 shows the FT-IR transmittance spectra of PLGA, PVA, SIM, their physical mixture, and the SIM loaded porous PLGA microspheres. From the PLGA spectra (Fig. 4a), the peaks of –OH stretching vibrations (3,200–3,500 cm^{-1}), –CH, –CH_2, –CH_3 stretching (2,850–3,000 cm^{-1}), carbonyl-C=O stretching vibrations (1,700–1,800 cm^{-1}), C–O stretching (1,050–1,250 cm^{-1}) were observed, which is in the agreement with previously publish data [26]. The FT-IR spectrum of PVA showed many characteristic peaks of PVA including a broad band at 3100–3400 cm^{-1} due to O–H stretching vibration and another band at 2,930 cm^{-1} corresponding to the C–H stretching vibration, as shown in Fig. 4b [27]. In Fig. 4c, the FT-IR spectra of the SIM showed prominent absorption at 3,553 cm^{-1} (Free O–H stretching vibration), 3,011, 2,959, and 2,872 cm^{-1} (C–H stretching vibrations), 1,714 cm^{-1} (stretching vibration of ester and lactone carbonyl functional group) [28]. Among these SIM peaks, those peaks at 3,533 cm^{-1} and 1,714 cm^{-1}, which were not found in PLGA (Fig. 4d), were clearly distinct in the

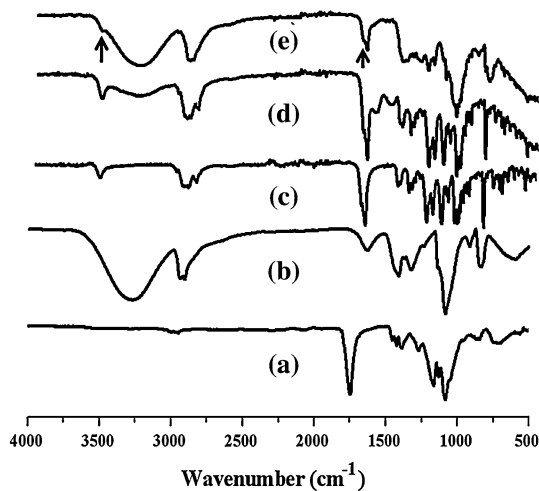

Fig. 4 FT-IR spectra of PLGA (*a*), PVA (*b*), SIM drug (*c*) physical mixture of SIM, PVA and PLGA (*d*) and SIM loaded porous PLGA microspheres (*e*) (Note the peaks present in SIM loaded porous PLGA microspheres were at 3,553 and 1,714 cm^{-1})

loaded porous PLGA microspheres (Fig. 4e) confirming successful SIM incorporation.

Figure 5 depicts the XRD spectrum of SIM, PLGA, and SIM loaded porous PLGA microspheres. The presence of several distinct peaks in the XRD spectrum of SIM at a diffraction angle of 2θ 7.85°, 9.35°, 10.92°, 12.85°, 15.62°, 16.48°, 17.24°, 18.81°, 22.58°, 25.95°, 28.38°, and 31.99° revealed that SIM drug was present in the crystalline form, as shown in Fig. 5a [28]. However, these peaks were not observed in the XRD spectra of PLGA and SIM loaded porous PLGA microspheres (Fig. 5b, c). Therefore, the spectrum of the SIM loaded porous PLGA microspheres indicated that the drug would be either molecularly dispersed in the polymers or distributed in an amorphous state.

Figure 6 shows the DSC thermogram of the SIM drug, the physical mixture of SIM–PLGA–PVA and SIM loaded porous PLGA microspheres. Figure 6a showed an endothermic peak of melting at 139.5 °C for the SIM drug, which is in agreement with the study by Seoung et al. [28]. This SIM melting peak was observed in the SIM–PLGA–PVA physical mixture (Fig. 6b). However, the complete disappearance of the endothermic peak corresponding to the SIM drug (Fig. 6c) was observed in the SIM loaded porous PLGA microspheres, indicating the formation of an amorphous inclusion complex and the molecular encapsulation of the drug inside porous PLGA microspheres [29–31].

Porous PLGA microspheres were immersed in PBS to investigate the degradation and SIM drug release. Porous PLGA microspheres were used as the control sample, as shown in Fig. 7a. The morphological surface changes in the porous PLGA microspheres after 2 and 4 weeks of degradation. The morphology of the porous PLGA microspheres changed after the first 2 weeks, as shown in Fig. 7b, which was due to the swelling of the PLGA material. The rapid deformation observed in the following 2 weeks was due to the porous structure of the microsphere, which allowed PBS to easily penetrate into the microsphere matrix and hydrolysis the polymer [32]. Therefore after 4 weeks, the porous PLGA microspheres collapsed and their spherical structure was lost, shown in Fig. 7c. This phenomenon could also be obvious in terms of the mass loss, as shown in Fig. 7d. At the end of the degradation period, the porous PLGA microspheres were approximately 40% of their original weight.

The SIM drug release curve from porous PLGA microspheres shows two main stages: the initial burst, mainly during the first 2 weeks where more than 40% of the drug was released, and a sustained release of almost at 68% of the SIM following 2 weeks. The high initial release rate can be explained by the highly porous structure of the PLGA microspheres and the high drug encapsulation efficacy (80%). The slower release rate was consistent with the fact that the porous structure of the microspheres collapsed with time [21]. However, in the release profile, a rapid

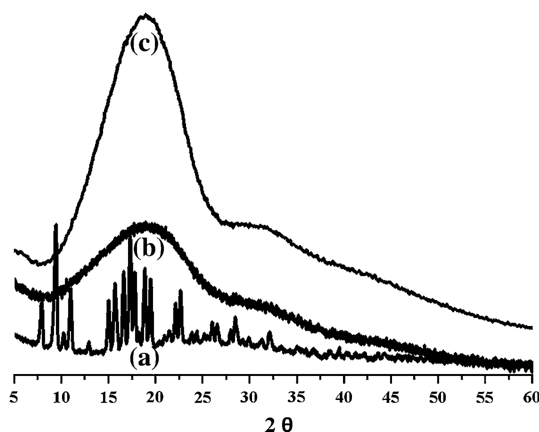

Fig. 5 X-ray diffraction spectra of SIM drug (*a*), PLGA raw material (*b*) and SIM loaded porous PLGA microspheres (*c*)

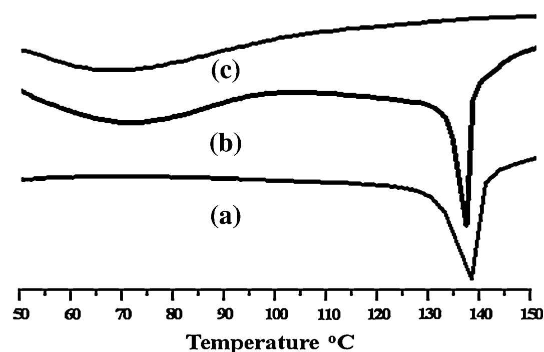

Fig. 6 DSC thermorgram of SIM (*a*), SIM–PLGA–PVA physical mixture (*b*) and SIM loaded porous PLGA microspheres (*c*)

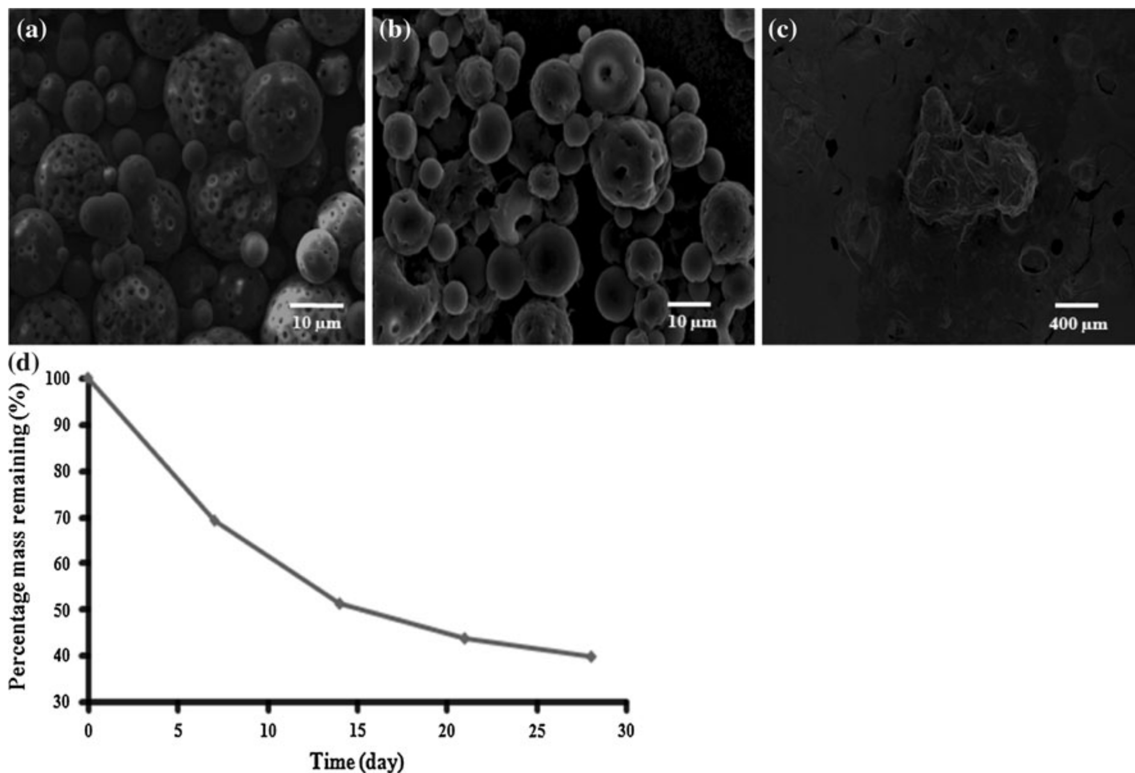

Fig. 7 SEM morphology of SIM loaded porous microspheres after fabrication (**a**), 2 weeks (**b**), 4 weeks (**c**); and percentage mass of sample remaining at defined time points (**d**)

burst release was observed between day 1 and day 2 (18%), then sustained release was observed between day 3 and day 4 (22%), as highlighted by the arrows in Fig. 8. This phenomenon may have occurred because of the amount conjugated drug on the surface of microspheres or the skin layer on microspheres surface [33].

The cytotoxicity of SIM loaded porous PLGA microspheres and porous PLGA microspheres were tested. The extracts containing the porous PLGA microspheres and SIM loaded porous PLGA microspheres did not inhibit cells metabolism relative to the control group. This indicated that cell metabolism in the presence of the SIM loaded porous PLGA microspheres was higher than in the presence of porous PLGA microspheres. Based on the quantitative scores, the extracts of porous PLGA microspheres did not display cytotoxicity (Fig. 9). Thus, SIM

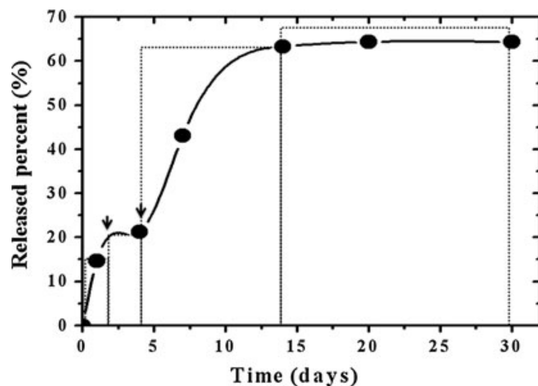

Fig. 8 Diagram illustrated the SIM drug release after 28 days in vitro study

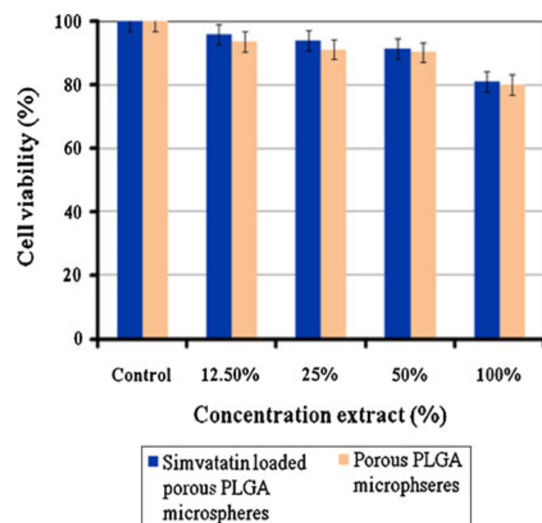

Fig. 9 Cytotoxicity of PLGA microspheres assessed using the MTT assay

loaded and unloaded porous PLGA microspheres displayed no cytotoxic effects and there was high biocompatibility in the presence of both SIM loaded porous PGLA microspheres and only porous PLGA microspheres.

Figure 10 shows that the round shape particles, which have a diameter around 100 µm, are the PLGA microspheres, while the objects that were about ten folds smaller in dimension were the osteoblast cells. From these images, it can be seen that the osteoblast cells could grow on the surface of PLGA microspheres and the cell distribution after 1, 3 and 7 days was different, as shown in Fig. 10b, c, d. Furthermore, the osteoblast cells tended to attach to the PLGA microspheres after 1 day (Fig. 10b) and grew 3 days after the cells were seeded (Fig. 10c). This occurred because of the high SIM encapsulation efficacy and the hollow shells, which formed a highly porous structure on the surface of PLGA microspheres. After 7 days of culture cell, cells not only attached and grew in the gaps between the porous PLGA microspheres but they also grew and proliferated across the entire surface of the SIM loaded porous PLGA microspheres, as shown in Fig. 10d [34].

Conclusion

SIM loaded porous PLGA microspheres were easily and stably fabricated using the novel W/O/W1/W2 double emulsion and solvent evaporation method. During fabrication some factors such as the distilled water content, PLGA concentration, or volume ratios of PVA/DCM affected the morphology of the resulting microspheres. Results from fabrication screens showed that highly porous PLGA microspheres were obtained at a distilled water concentration of 20%, PGLA concentration of 10%, and PVA/DCM volume ratio = 4:1. The SEM images and particle size analyzer revealed that the pore size distribution of the microspheres varied from 0.01 to 40 µm and the particulate size distribution was almost around 100 µm. Spectrum analysis by FT-IR, XRD, and DSC demonstrated that SIM was dispersed and distributed in the amorphous state within the polymer phase. In vitro studies showed that the sample was highly biocompatible and biodegradable. The high drug encapsulation efficacy into porous PLGA microspheres (80%) and porous structure promoted cell aggregation, proliferation. The drug release profile

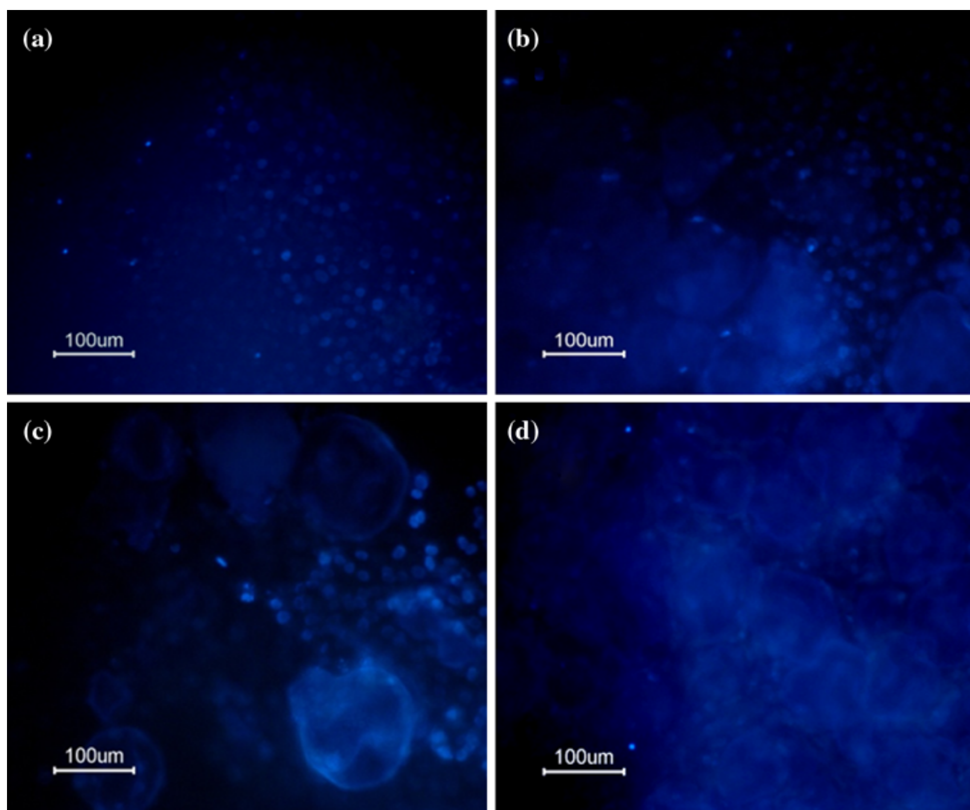

Fig. 10 Confocal microscopic images of cellular aggregates of SIM loaded porous PLGA microspheres in cell culture: control sample (**a**), after 1 day (**b**), 3 days (**c**), and 7 days (**d**) cultured cell

demonstrated that the drug was released in a burst during the first 2 weeks, which was then released in a sustained manner over the following 2 weeks. Thus, SIM loaded porous PLGA microspheres hold promise for use as a DDS in bone tissue regeneration.

Acknowledgement This work was supported by Mid-career Research Program through NRI grant funded by the MEST (2009-0092808) and Prof. Hun Mo Yang who was supported by Soonchunhyang University 2009 research year program.

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