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Preparation of poly(lactic acid)/chitosan nanoparticles for anti-HIV drug delivery applications

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ABSTRACT

Poly(lactic acid) (PLA)/chitosan (CS) nanoparticles were prepared by emulsion method for anti-HIV drug delivery applications. The prepared PLA/CS nanoparticles were characterized using DLS, SEM, and FTIR. The hydrophilic antiretroviral drug Lamivudine was loaded into PLA/CS nanoparticles. The encapsulation efficiency and *in-vitro* drug release behaviour of drug loaded PLA/CS nanoparticles were studied using UV spectrophotometer. In addition, the cytotoxicity of the PLA/CS nanoparticles using MTT assay was also studied. The *in-vitro* drug release studies showed that drug release rate was lower in the acidic pH when compared to alkaline pH. This may due to repulsion between H^+ ions and cationic groups present in the polymeric nanoparticles. Drug release rate was found to be higher in the 6% drug loaded formulation when compared to 3% drug loaded formulation. These results indicated that the PLA/CS nanoparticles are a promising carrier system for controlled delivery of anti-HIV drugs.

1. Introduction

Biodegradable polymers have found much application in the field of controlled drug delivery. After drug depletion, the carrier degraded in the body to form products that are easily resorbed or eliminated. PLA is a biodegradable polymeric material with low toxicity, excellent biocompatibility and bio-absorbability *in vivo*. However the low hydrophilicity and high crystallinity of PLA reduce the degradation rate, which results in poorer soft tissue compatibility (Suh, Hwang, Lee, Han, & Park, 2001). As a biomedical material, PLA can be processed into solid products such as rods (Bergma, De Bruijin, Rozema, & Bos, 1995) bone screws (Park & Kim, 2004) suture lines, drug carriers (O'Donnell & Mc Ginity, 1997) and microspheres (Ma, Gao, Gong, & Shen, 2003) as well as porous structure such as scaffolds (Kim, Yu, Hsiao, & Chu, 2003), non-woven fabrics (Lathia, Leodore, & Wheatley, 2004) and hollow microspheres (Gonzalez, Ruseckalite, & Cuadrado, 1999).

CS is obtained by alkaline deacetylation of chitin, which is the principal component of the protective cuticles of crustaceans (such as crabs, shrimps, and lobsters) and of the cell walls of some fungi (such as *Aspergillus* and *Mucor*) (Muzzarelli, 1977; Roberts, 1992). It is able to open the tight junctions and in this way allows paracellular transport across the epithelium. Both nasal and oral drug delivery research has demonstrated that significantly higher amounts of macromolecular drugs can be transported after co-administration with chitosan (Inez et al., 2003). CS is soluble in aqueous solutions of various acids, but has no amphiphilic property and cannot form micelles in water. Apart from its biodegradable character in physiological conditions, CS has reactive amine and hydroxyl groups, which offer possibilities of modifications through graft reaction and ionic interactions. CS, being a natural polymer, has been degraded *in vivo* by enzymes such as lysozyme and chitosanase, into oligomers and further to *N*-glucosamine, which is endogenous to the human body (Khor, 2001). CS is also highly biocompatible and low toxicity material and is a good candidate for drug delivery systems (Agnihotri & Aminabhavi, 2004; Jayakumar, Nwe, Tokura, & Tamura, 2007; Jayakumar, Prabaharan, Nair, & Tamura, 2010b; Jayakumar, Prabaharan, Reis, & Mano, 2005; Jayakumar, Reis, & Mano, 2007; Jayakumar et al., 2010a; Prabaharan & Mano, 2005).

New drug delivery technologies are revolutionizing the drug discovery; development and creating R&D focussed pharmaceutical industries to increase the momentum of global advancements. In this regard, novel drug delivery systems (NDDS) have many benefits, which includes improved therapy by increasing the efficiency and duration of drug activity, increased patient compliance through decreased dosing frequency and convenient routes of administration and improved site specific delivery to reduce the unwanted adverse effects (Bajaj & Desai, 2006). The present work

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concerns the preparation and characterization of PLA/CS nanoparticles by emulsion solvent evaporation system for controlled drug delivery applications. In this study, Lamivudine was selected as a model drug. Lamivudine belongs to a class of drugs named nucleoside analogues. It is a potent and selective inhibitor of type 1 and type 2 human immunodeficiency virus (HIV) (Anderson, 2002; Clercq, 2002; Yzdanian, 1999). Lamivudine is hydrophilic, has a pK_a of 4.3 and exists primarily in un-ionized form when dissolved in distilled water. It is considered as class 1 in the biopharmaceutics classification system, which means that it has high permeability and high solubility (Jozwiakowski, Nguyen, Sisco, & Spancake, 1996). Dosage and duration of Lamivudine therapy should be individualized according to the requirement and response of the patient. The daily-recommended dose is 150 mg b.i.d. (Kao, Wu, Chen, Lai, & Chen, 2000). The oral administration of Lamivudine exhibits side effects in the gastro-intestinal treat (GIT) as well as in the central nervous system (CNS). Thrombocytopenia, paraesthesias, anorexia, nausea, abdominal cramps, depressive disorders cough, skin rashes, etc. have also been reported as possible adverse reactions (Caroline & Faulds, 1997).

Controlled release preparation helps to achieve a maximum therapeutic effect with simultaneous minimization of adverse effects. Micro- and nano-particulate drug delivery posses many advantages such as high bioavailability, rapid kinetic of absorption as well as avoidance of hepatic first pass effect and improvement of patient compliance (Chen, 1992). In this paper, the preparation, characterization, cytotoxicity and *in-vitro* dug release behaviour of Lamivudine drug loaded PLA/CS nanoparticles is described in detail.

2. Materials and methods

2.1. Materials

Chitosan (CS), poly(lactic acid) (PLA), dichloromethane, poly(ethylene oxide) (PEO), phosphate buffer saline (PBS) and acetic acid were obtained from Sigma–Aldrich, USA. Lamivudine drug was purchased from Sigma, USA. All other chemicals were of analytical grade.

2.2. Preparation of PLA/CS nanoparticles

About 100 mg of PLA was dissolved in 10 ml of dichloromethane to form a fine dispersion. This solution was rapidly poured into 10 ml of 1% acetic acid solution containing 40 mg of CS and 200 mg of PEO. The mixture was sonicated for 15 min to form an emulsion and vigorously stirred until the organic solvent evaporated. Finally, the nanoparticles were precipitated by adding water and then lyophilized.

2.3. Preparation of Lamivudine loaded PLA/CS nanoparticles

To encapsulate hydrophilic drug w/o/w emulsion technique was applied. Aqueous drug solution (2 ml) was first poured into a polymer solution (100 mg of PLA dissolved in 10 ml dichloromethane) to form a w/o emulsion. The w/o emulsion was the rapidly poured into 10 ml of 1% acetic acid solution containing 40 mg of chitosan and 200 mg of PEO. The mixture was sonicated for 15 min to form an emulsion, and then vigorously stirred. Stirring was continued until the organic solvent had evaporated. The nanoparticles were precipitated by adding water and then lyophilized. Fig. 1 shows the preparation method of Lamivudine loaded PLA/CS nanoparticles.

2.4. Cell culture and cytotoxicity studies

Mouse fibroblast cell line (L929) was cultured in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), and 100 U/ml penicillin/streptomycin. Cytotoxicity of the PLA/CS nanoparticles was evaluated using standard MTT assay by an indirect extract method (ISO 10993-5). Briefly, 1 g ethanol sterilized PLA/CS nanoparticles was incubated with 5 ml extraction media (α MEM with 10% FBS) with agitation for five days for extraction. The extract was collected and was used for the cytotoxicity assay. Positive control materials and negative control materials were similarly tested alongside to validate the test results. The presence of cytotoxic leachates is indicated by loss of cell viability. Cells were seeded onto 96-well plates with a density of 10^4 cells/well and kept in CO_2 incubator under standard culturing conditions. After 24 h, 25%, 50%, 75% and 100% of the extract from the nanoparticles replaced the culture media. The presence of cytotoxic leachates in the extract was verified by MTT assay after incubating the cells with the extract for 24 and 48 h. In the assay, fresh media containing 10% of MTT replaced the medium and the plate was incubated at 37 °C in CO_2 incubator for 4 h. Then the medium was removed, 100 μl of solubilization buffer (Triton X-100, 0.1 N HCl and isopropanol) was added to each well to dissolve purple formazan crystals. The absorbance of the solution was measured in a microplate reader (Power Wave XS, BioTek) at a wavelength of 570 nm.

2.5. Drug encapsulation efficiency

The obtained nanoparticles was frozen and lyophilized by a freeze dryer system to obtain a dried nanoparticle product. The

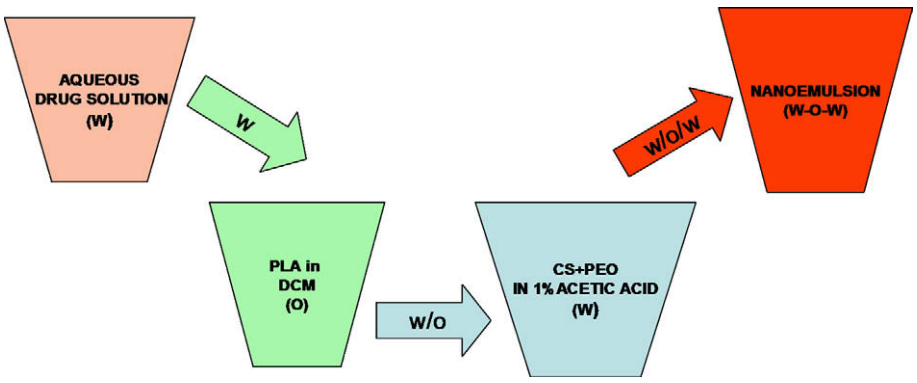

Fig. 1. Schematic representation of the synthesis of Lamivudine loaded PLA/CS nanoparticles by emulsion solvent evaporation technique.

weighed product of nanoparticles was washed with distilled water and then centrifuged and the supernatant was collected. The solution was measured by UV spectrophotometer (UV-1700 Pharma Spec, Shimadzu) at the wavelength 270 nm (Fernandes, 2006) and the weight of drug was calculated by use of a calibration curve.

$$\text{Encapsulation efficiency} = \frac{\text{Weight of Lamivudine drug in nanoparticles}}{\text{Weight of Lamivudine drug initially}} \times 100\%$$

2.6. In-vitro drug release studies

The *in-vitro* drug release tests were carried out on all formulations (3% and 6% drug loaded samples). Fifty milligrams of each sample was suspended in 100 ml of PBS buffer at various pH at 37 °C and placed in a incubated shaker at 120 rpm. At predetermined time intervals, 3 ml of aliquots was withdrawn and the concentration of drug released was monitored by UV spectrophotometer (UV-1700 Pharma Spec, Shimadzu) at 270 nm. The dissolution medium was replaced with fresh buffer to maintain the total volume. The drug release percent can be determined by the following equation:

$$\text{Drug release } [\%] = C(t)/C(0) \times 100$$

where $C(0)$ and $C(t)$ represents the amount of drug loaded and amount of drug released at a time t, respectively. All studies were done in triplicate.

2.7. Characterization techniques

2.7.1. Size determination by dynamic light scattering

The particle size of the prepared nanoparticles was characterized using Nicomp, 380 ZLS particle size analyzer at 25 °C. The samples were diluted until they were transparent so as to ensure free diffusion and unhindered Brownian motion of the particles and to prevent the formation of artefacts.

2.7.2. Scanning electron microscopy (SEM)

The surface morphology of the prepared nanoparticles was analyzed by scanning electron microscopy Jeol JSM-649OLA. SEM samples were prepared using platinum sputter coating with a high resolution Coater. Measurements were made using the Sigma Scan Pro software (Jandel Scientific). SEM images of the nanoparticle were taken after degradation for 48 h at various pH.

2.7.3. Infrared (FTIR) spectrophotometry

IR spectra were recorded on Perkin-Elmer, Spectrum RX1 Fourier transform infrared spectrophotometer. PLA/CS nanoparticles loaded with Lamivudine drug or PLA/CS nanoparticles alone were mixed with KBR and pressed to plate for measurement.

In addition, we used Ultrasonicator-Misonix (Model 2510E DTH/100 W–42 kHz) and Probe sonicator-Sonics (Model CV18/130 W–20 kHz) to prepare monodispersed nanoparticles.

3. Results

PLA/CS nanoparticles (around 300 nm) were synthesised by emulsion and solvent evaporation technique (Dutta, Tripathi, Chattopadhyaya, & Dutta, 2005). The polymer PEO was used as a stabilizer for the preparation and it is easily washed out after synthesis. This route is very advantageous; since it avoids the use of any surfactants, which remain to be a contaminant after synthesis of nanoparticles.

Lamivudine drug was loaded into the PLA/CS nanoparticles system at the time of synthesis itself. The amount of drug added was maintained at 3% and 6% weight of the total base polymers to get two different formulations. Dichloromethane, which was used as a solvent for PLA, was completely evaporated with no residuals in the nanoparticles. PEO, which was used as a stabilizer for the emulsion, was successively washed away. Then the drug loaded PLA/CS nanoparticles were purified without any contaminants and then lyophilized.

Fig. 2 shows the particle size distribution of the prepared nanoparticles was in the range of 300–350 nm as measured by DLS technique. It was also evident from the figure that particles were monodispersed.

Fig. 3 shows the surface morphology of the prepared nanoparticles. The SEM images showed that the PLA/CS nanoparticles had spherical morphology (Fig. 3A and B). Fig. 3C and D shows the SEM images of Lamivudine drug loaded nanoparticles. The surface morphology of the drug loaded nanoparticles and the size of nanoparticles were the same as when prepared without drug.

A prior assessment in the FTIR spectra (Fig. 4I) of PLA/CS nanoparticles showed the characteristic peaks of PLA and CS individually. Compared with the controlled spectrum of CS and PLA, the well-resolved peaks of PLA/CS nanoparticles were identified. The peaks at 1654 and 1322 cm^{-1} correspond to the amide group of CS. The multiple peaks at 898 and 1262 cm^{-1} are the result of polysaccharide structure of CS. The peak at 1322 cm^{-1} is the characteristic band of CH_3 symmetrical deformation mode. The peaks between 3200 and 3350 cm^{-1} are due to the hydrogen bonded —OH group. The peak around 1760 cm^{-1} is attributed to carboxylic groups on the PLA side chains. Four bands present in the Lamivudine drug spectrum at 3448, 2999, 1767 and 1458 cm^{-1}, were due to the formation of N—H, O—H, C=O, C=N linkage, respectively (Fig. 4II). In addition, the broad peak between 1000 and 1100 cm^{-1}, shows the interaction between polymer and drug (Fig. 4II-A). This

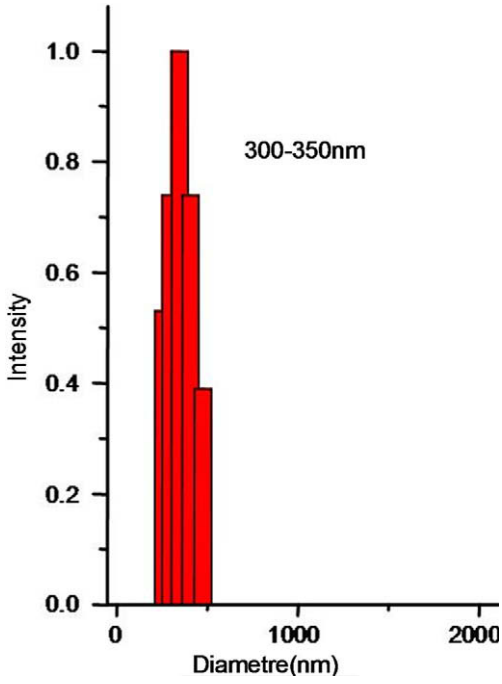

Fig. 2. DLS data of the prepared PLA/CS nanoparticles.

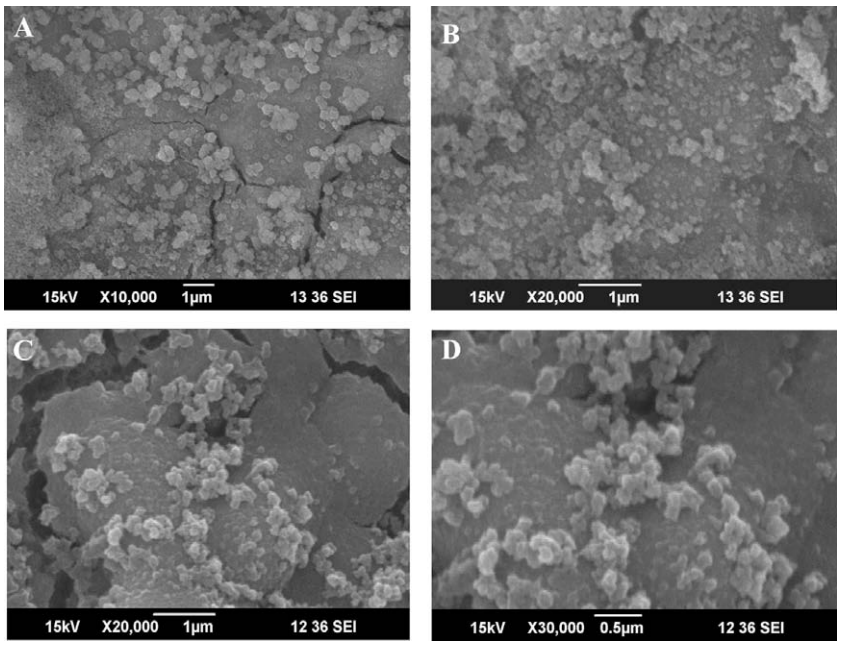

Fig. 3. SEM image of PLA/CS nanoparticles (A and B) and Lamivudine drug loaded PLA/CS nanoparticles (C and D).

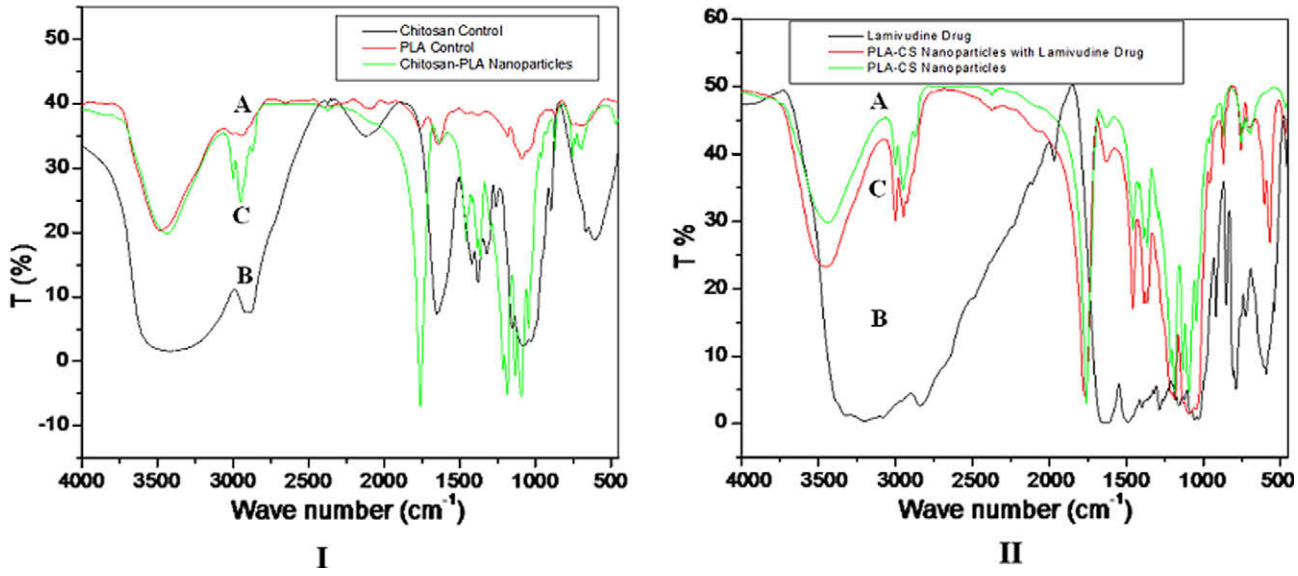

Fig. 4. (I) FTIR spectra of (A) PLA (control), (B) CS (control) and (C) PLA/CS nanoparticles and (II) FTIR spectra of (A) PLA/CS nanoparticles, (B) Lamivudine drug and (C) Lamivudine drug loaded PLA/CS nanoparticles.

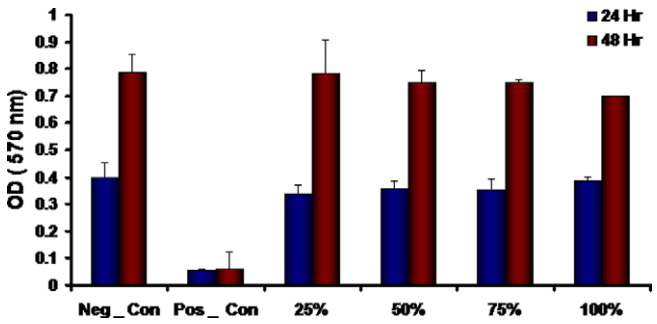

Fig. 5. Cytotoxicity studies of PLA/CS nanoparticles after MTT assay.

also confirms the drug was incorporated into the polymeric nanoparticles.

There was no toxicity in any of the concentrations of the extract, which contain all the possible leachable, and degradation products. Cells proliferated normally at 48 h compared to 24 h as evident by the MTT assay (Fig. 5). This result suggested that the synthesised PLA/CS nanoparticles did not have any toxic leachable or degradation products and it can be used for drug encapsulation applications.

Drug encapsulation efficiency was found by the above-described formula. Drug encapsulation efficiency was higher for 6% drug loaded formulation compared to the 3% drug loaded formulation. The 6% drug loaded sample gave an efficiency of 75.4% and 3% drug loaded sample gave an efficiency of 67.9%. Fig. 6 shows the drug release rate of the 6% drug loaded formulation and the 3% drug loaded formulation simultaneously. It is evident from the fig-

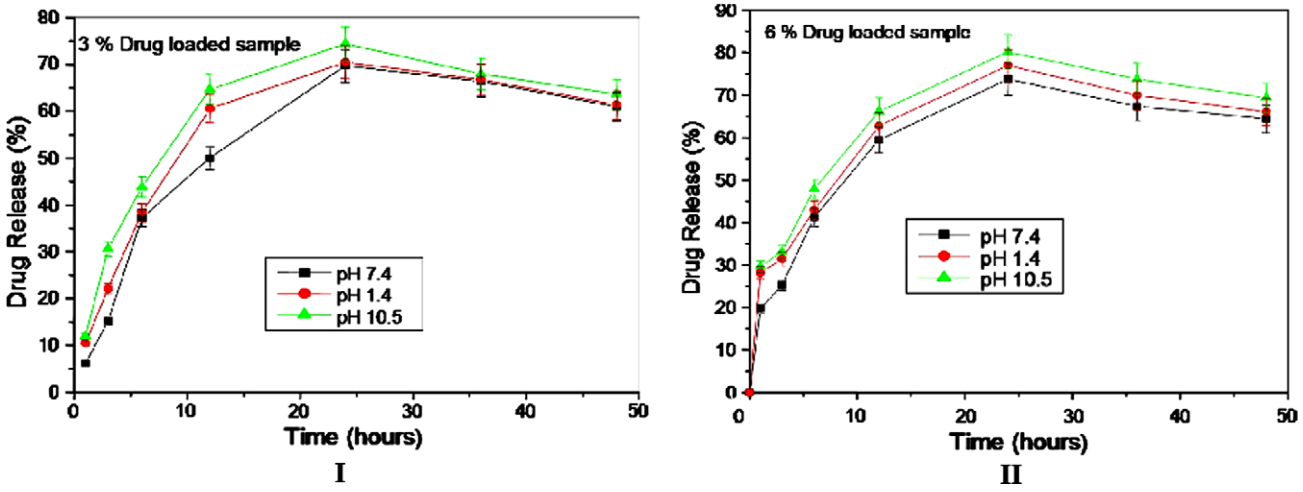

Fig. 6. Drug release rate of 3% (I) and 6% (II) drug loaded nanoparticles.

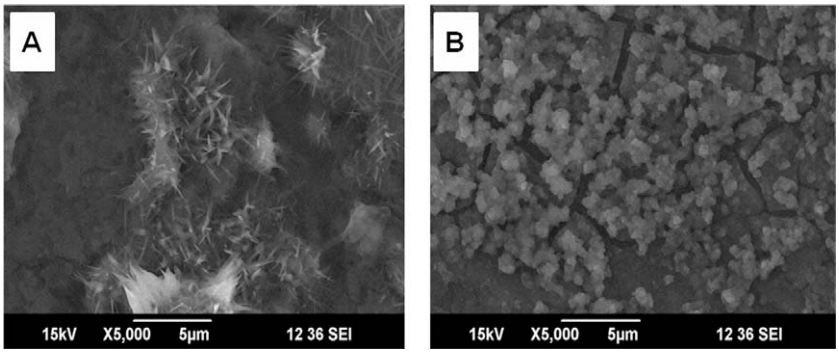

Fig. 7. SEM images of (A) basic and (B) acidic pH degraded drug loaded PLA/CS nanoparticles.

ures that both formulations gave almost similar controlled release. After 12 h, 3% drug loaded sample released 50.1%, 60.5% and 64.6% drug at pH 7.4, 1.4 and 10.5, respectively (Fig. 6I). After 24 h, Lamivudine drug concentration in buffer came around 69.7%, 70.4% and 74.3% at pH 7.4, 1.4 and 10.5, respectively (Fig. 6I). For 6% drug formulation, drug release percentage after 12 h was 59.5, 62.7 and 66.1 at pH 7.4, 1.4 and 10.5, respectively (Fig. 6II). After 24 h released concentration of drug became around 73.7%, 76.9% and 80.2% at pH7.4, 1.4 and 10.5, respectively (Fig. 6II).

The surface morphology of nanoparticle was analyzed using SEM (Fig. 7A and B) and found to be very different after each type of degradation.

4. Discussion

In the FTIR spectra of PLA/CS nanoparticles, the bands became sharper due to intramolecular hydrogen bonding. The characteristic peaks of drug Lamivudine remained the same in both the systems, indicating no existence of different association forms of Lamivudine with PLA/CS nanoparticles. Biodegradable polymers used in controlled drug delivery formulations must be chemically inert, non-toxic and free of leachable impurities. The degradation products that are tolerated with little or no adverse reactions within the biological environment are metabolized and removed from the body via normal metabolic pathways. Therefore we evaluated the toxicity of synthesised nanoparticles as described in the methods bonding.

The release of the drug from polymer nanoparticles is a rather complicated process. It can be affected by many factors such as polymer degradation, molecular weight, crystallinity, the binding affinity between the polymer and the drug and so on (Shin, Kim, Lee, Cho, & Sung, 1998). Drug loading is also a significant factor for influencing the drug release. Drug release rate was higher for 6% than 3% drug loaded formulation. Higher drug loading caused the drug to be released more quickly. In both the formulations, the drug release rate was higher for alkaline pH than acidic pH, and the drug release in the acidic pH was higher than in the neutral pH. The lower drug release rate in the acidic pH than the alkaline pH is attributed to the repulsion between H^+ ions and cations on the surface of CS, which slow down the hydrolysis (Proikalis, Tarantalli, & Andreopoulos, 2006; Zhao, Fu, Dennis, & Wu, 2004).

SEM images after basic degradation shows that the nanoparticles broken up and released the drug. SEM images after acidic degradation did not show this morphology and we believe that the release mechanism is one of diffusion (Prabaharan, Grailer, Pilla, Steeber, & Gong, 2009).

5. Conclusions

In conclusion, the PLA/CS nanoparticles with Lamivudine drug were prepared by emulsion technique and characterized for controlled drug delivery applications. The PLA/CS nanoparticles are found to be non-toxic in mouse fibroblast cells (L929). This confirms that this PLA/CS nanoparticles system can be used for

biomedical application including drug encapsulation studies. The degradation rate increases rapidly when pH increases in the range 8–13. Therefore such nanoparticles can entrap and protect the drugs at stomach environment (acidic pH) and then provide sustained release at neutral pH (intestine).

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