Effect of Additives on Release Profile of Leuprolide Acetate in an *In Situ* Forming Controlled-Release System: *In Vitro* Study

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ABSTRACT: *In situ* forming drug delivery system is prepared by phase inversion technique using poly (D,L-lactic-*co*-glycolide) and leuprolide acetate dissolved in *N*-methyl-2-pyrrolidone. The effects of ethyl heptanoate and glycerol additives are important determinant as rate modifying agents on the drug release kinetics in biodegradable *in situ* forming porous systems of poly(D,L-lactide-*co*-glycolide) (PLGA) in *N*-methyl-2-pyrrolidone (NMP). The release performance and porous structure morphology are investigated by scanning electron microscopy and UV–visible spectroscopy techniques to study the effect of additives. The experimental results exhibit the crucial role of ethyl heptanoate and glycerol at different loadings (1, 3, and 5% w/w) on release profile of leuprolide acetate loaded on poly(D,L-lactide-*co*-glycolide)hydroxylated (PLGA-H). Both additives at different concentrations reduce the burst effect, while increasing duration of drug release. Ethyl heptanoate, however, shows stronger effect than glycerol. The results of morphological studies show that ethyl heptanoate reduces the porosity of the polymer surface and interconnected tear-like structures of the bulk disappear while the sponge-like structures are observed. In this system glycerol reduces the surface porosity intensively, while the interconnected tears change into channel-like structures. Therefore, morphological results confirm the effect of additives on leuprolide release profile. J Appl Polym Sci 107: 3781–3787, 2008

Key words: drug delivery systems; phase separation; additives; morphology

INTRODUCTION

The development of new injectable drug delivery systems have received considerable attention over the past few years. The advantages of these delivery systems may include ease of application, localized delivery for a site-specific action, prolonged delivery periods, lesser body drug dosage with concurrent reduction in possible undesirable side effects common to most forms of systemic delivery, and improved patient compliance and comfort.[1,2] *In situ* forming systems, semisolid drug injection depots are a member of novel drug delivery systems family.

The sol–gel system is prepared by dissolving a water-insoluble and biodegradable polymer in a biocompatible organic solvent such as *N*-methyl-2-pyrrolidone. When the polymer solution is injected into the body, the organic solvent dissipates into the surrounding tissue as the water permeates into the implant. This process leads to phase separation and subsequent coagulation of the polymer to form an implant *in situ* (sol–gel system).[3] Phase inversion is a common technique for preparation of polymeric porous media with symmetric and asymmetric structures. In this process, usually a polymer is dissolved in an appropriate solvent, by introducing the polymer solution into the nonsolvent, after casting on a suitable support or being injected in a biological media.[4,5]

A sizable part of the porous media is prepared by controlled phase separation of polymer solution.

Evidently, the porous morphology, polymer and additive properties, solution concentrations, and preparation conditions of drug release device have great influence on membrane performance drug delivery process. The phase separation can be induced in several ways such as evaporation of volatile solvent or cooling the polymer solution temperature. The wet phase separation is the usual method to membrane or porous media structure formation. This method is carried out by immersing the shaped polymer solution into the nonsolvent bath where solvent and nonsolvent exchange takes place. A combination of these processes may also extend to membrane preparation.[6–9] For drug delivery application, the active drugs

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are added to the polymer solution to produce a ready-to-use homogeneous solution or a dispersion which depend upon the solubility of the drug.[3]

In situ forming formulation has considerable capacity for bursting effect, especially in the first few hours after injection into the body. Since these injection implant systems are administered as a liquid, there is a lag between injection and coagulation of the solid implant. During this lag time the initial burst of drug may exceed the plasma concentration achieved using conventional implant systems.[2]

The polymer concentration increases at interface immediately after immersion precipitation. This initial burst of drug has been linked to tissue irritation and sometimes to systemic toxicity. Because of this unwanted phenomenon, the use of this system has been limited only to drugs with a vast therapeutic index. However, the attainment of uniform release kinetics across the fixed and desired delivery time length is another important sensation of *in situ* forming systems.[4] It is noted that the drug release profile from sol–gel matrix consists of four stages: (i) the initial stage involves the sudden burst of drug, and hence all drugs near the surface are dissolved in surrounding aqueous media and are released quickly from the interconnected pores and channels; (ii) in the second stage, the nonentailment drugs in matrix are released by diffusion mechanism and are passed through channels with constant rate; (iii) in the third stage, the sudden increase of drug release happens which may be attributed to degradation of polymer matrix and it causes a second burst in release profile; and (iv) finally, although the process is continued, but due to low concentration of drug, the release rate is diminished.[10] To control the initial burst effect, four parameters, examined by several researchers, can be taken into account: concentration of polymer solution,[11] molecular weight of polymer, type of solvents,[12,13] and the addition of a surfactant or rate modifying agent.[14] It is important to mention that these parameters influence the rate of precipitation of the polymer.[2]

The occurrence of ''secondary burst'' in the drug release is due to increase in the rate of polymer degradation and subsequently the surface area. In other words the main reason for poly(D,L-lactide-*co*-glycolide) (PLGA) degradation is due to hydrolysis in presence of water. This is explained theoretically as there are two different trends of approach on hydrolytic degradation of aliphatic polyesters. One being macroscopically regarded as a homogeneous process attributed to bulk degradation and the other regard the surface erosion as a heterogeneous process responsible for ester bond cleavage. The arguments in favor of autocatalytic bulk degradation are based on the fact that the onset of mass loss lags behind molar mass decreases in which its distribution displays unimodal pattern.

The heterogeneous degradation is assigned to diffusion-reaction phenomena. In this approach it has been demonstrated that polymer matrix is initially homogeneous in the sense that the average molar mass is the same throughout the matrix. Once it is in contact with aqueous medium, water penetrates into the polymer matrix resulting in the cleavage of ester bonds. Each ester bond cleavage forms a new carboxyl end group that according to autocatalysis, accelerates the hydrolytic reaction of the remaining ester bonds.

Following the initial bulk degradation the situation becomes totally different when soluble oligomeric compounds are generated in the matrix. The soluble oligomers that are close to the surface can escape from the matrix before total degradation, whereas those located inside the matrix can hardly diffuse out of the matrix. When the aqueous medium is phosphate buffered at neutral, as is the case *in vivo* the neutralization of carboxyl end groups present at the surface can also contribute to decrease in surface acidity. Therefore, autocatalysis is larger in the bulk than at the surface, thus leading to a surface-interior differentiation. Similar features are observed for PLGA of different percent compositions. From then on, pores start to be formed in the polymer until it is fragmented which causes the increase in the surface area of the copolymer and the entrapped drug is released leading to a secondary burst.[15]

It has been discovered that water-soluble or insoluble additives as rate-modifying agents provide significantly improved control on the sustained release character of the polymer system. The choice of rate modifying agent employed depends on the types and ratios of polymer/solvent of the systems.[16] Preferred rate modifying agents are dimethyl citrate, triethyl citrate, ethyl heptanoate,[17–19] glycerin, and hexanediol.[20] These substances are biocompatible and approved by FDA.

In this study, our aim was to prepare an *in situ* forming PLGA system loaded with leuprolide acetate of 7.5 mg to be released in one month. Leuprolide acetate is a potent lutenizing hormone-releasing hormone (LH-RH) agonist that is useful in the treatment of hormonal related prostate cancer, endometriosis, and precocious puberty. Sustained leuprolide levels cause desensitization and down-regulation of pituitary-gonadal axis, leading to suppressed levels of leutenizing and sex hormones.[3] Initial GnRH analog dosage forms required daily administration by injection (1 mg/mL, s.c.),[20] but in its *in situ* forming formulations release occurs within 1–6 months, such as Eligard ™, an example of long-acting injectable *in situ* forming of leuprolide acetate in drug marketing formulated by QLT.

We have focused our study on the deeper understanding of controlled-release mechanism and specifically on reduction of burst effect with the help of

polar and nonpolar additives such as glycerol and ethyl heptanoate to obtain the optimum results for an *in situ* formulation. This comparison is made for first time and the results have demonstrated that the effect of a nonpolar additive on producing the best formulation has paved the way for taking a new direction in designing an *in situ* formulation carrying a specific drug such as leuprolide acetate. The study of morphological changes of the polymer is another important direction to comprehend the basis of controlled release mechanism. Our findings have clearly demonstrated that the structural surface and bulk changes during the course of drug release are direct evidence for the trend observed in drug release profile. In this study also it has been confirmed that the phase inversion phenomenon of polymer solution plays vital role in structural changes of controlled release systems.

EXPERIMENTAL

Materials

PLGA-H Resomer RG 504 H [poly (D,L-lactic-*co*-glycolide), 50 : 50, and intrinsic viscosity of 0.4 dL/g] was purchased from Boehringer Ingelheim, Germany. Leuprolide acetate (D-Leu-(des-Gly-NH_2)-LH-RH ethylamide) was obtained from Bachem, Switzerland. *N*-methyl-2-pyrrolidone (NMP), ethyl heptanoate, glycerol, sodium potassium tartrate tetrahydrate, sodium carbonate, $CuSO_4{\cdot}5H_2O$, Folin-ciocalteu's phenol reagent, NaOH, and sodium azide were obtained from Merck, Germany; potassium phosphate monobasic and Tween 80 were purchased from Aldrich, Germany. The phosphate buffer was prepared according to USP 30 with pH 7.2. In Figure 1, the chemical structures of glycerol and ethyl heptanoate and leuprolide acetate are shown.

Preparation of formulations

The formulations were prepared by dissolving the appropriate amount, 33% (w/w) PLGA-H polymer in NMP as polymer solvent. Seven formulations consisting of 0, 1, 3, and 5% (w/w) of ethyl heptanoate or glycerol and 3% (w/w) of leuprolide acetate were added to the polymer solution. The uniform polymer solutions were cast on a cylindrical vial at room temperature and were immediately immersed in the nonsolvent bath of phosphate buffer before any phase separation. Disks of 14 mm diameter and 2 mm thickness were prepared by pouring 0.2 g of each polymer-formulated solution to a cylindrical vial, adding buffer and allowed to become solidified to use in release study tests.

Drug release studies

The leuprolide acetate release studies were conducted in 15 mL polypropylene vials containing 10 mL of a 0.2*M* phosphate buffered saline (PBS) at pH 7.4 without stirring. Polypropylene vials were used to minimize protein absorption.[14] The vials were kept in a laboratory oven at 37°C. Samples of 2 mL volume were taken from release media (the release medium of samples was replaced by fresh buffer after each sampling) after 0, 1, 8 h and 1, 3, 7, 14, 21, 28 and 40 days and assayed for leuprolide presence.

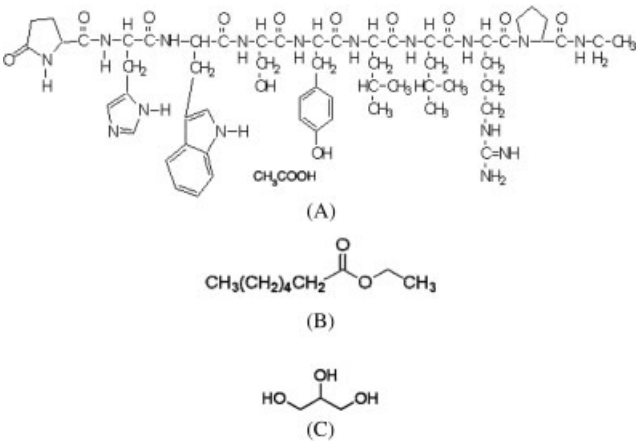

Figure 1 The chemical structures: (A) leuprolide acetate, (B) ethyl heptanoate, and (C) glycerol.

Determination of protein concentration

Protein concentration was determined by using a modified Hartree-Lowry assay method.[21–23] In our modification the required amount of sodium potassium tartrate tetrahydrate, $CuSO_4{\cdot}5H_2O$, and NaOH solutions were mixed thoroughly. The resulting solution was added into vials containing the required amount of buffer, folin reacting agent, and samples of release media and then mixed well. The final solutions were determined at 650 nm absorption.

Scanning electron microscopy

The cross section and surface morphology of polymer disks were studied by SEM using a Cambridge S360 scanning microscope. For this purpose, the disks were withdrawn after 24 h from release media, freeze dried (Zirbus, Germany) for 15 min and transferred into the microscope with a sample holder after sputtering with gold. The SEM analyses were carried out at room temperature and 10 kV with a magnification of 5000 for surfaces and 800 for cross sections.

Statistical analysis

To determine significant difference between the data obtained from drug burst release the One Way ANOVA statistical method was used. In this method

TABLE I
Precision and Accuracy of the Proposed Spectrophotometric Method

Analyte concentration (μg/mL)			Recovery	RSD	
Spiked	Mean determined	SD	(%)	(%)	*n*
48.5	51.2	2.4	105.6	4.7	7
120.7	128.0	4.4	106.0	3.4	7
168.5	179.2	1.7	106.3	0.9	7

two tests: least square difference and Scheffe were used. The formulations containing ethyl heptanoate with 1, 3, and 5% (w/w) and samples containing glycerol with 1, 3, and 5% (w/w) were compared by two and two. The significant difference between them as P value < 0.05 is reported.

RESULTS AND DISCUSSION

Validation of the assay method

Validation of the assay method designed was based on polymeric matrix structure and release profile of drug from the *in situ* forming system. Also, it was applied for measurement of leuprolide acetate due to UV–vis spectrophotometric method which was reported by Lowry and Hartree.[21–23] Three-point calibration graph for leuprolide acetate was linear over the range (2–200 μg/mL). The linear regression equation for calibration plots was $y = 4.8094x + 32.875$, where y denotes absorbance and x the analyte concentration [μg/mL]; the correlation coefficient was 0.9972. Linearity of the calibration graph was tested by plotting residuals (δy) versus concentration. Residuals are distributed at random around the zero-line, without any trend; the calibration function can therefore be regarded as linear. The accuracy of the method was determined for three concentrations (92.4, 94.3, and 93.8, respectively) with recovery percentage and reproducibility was reported in terms of relative standard deviation (RSD) as reflected in Table I. As reflected in Table I, the small values of RSD obtained showed that the determination of the analyte in dilute release-media samples was adequately reproducible and recoveries obtained (105.6–106.3) speaks for acceptable accuracy with the proposed analytical method.

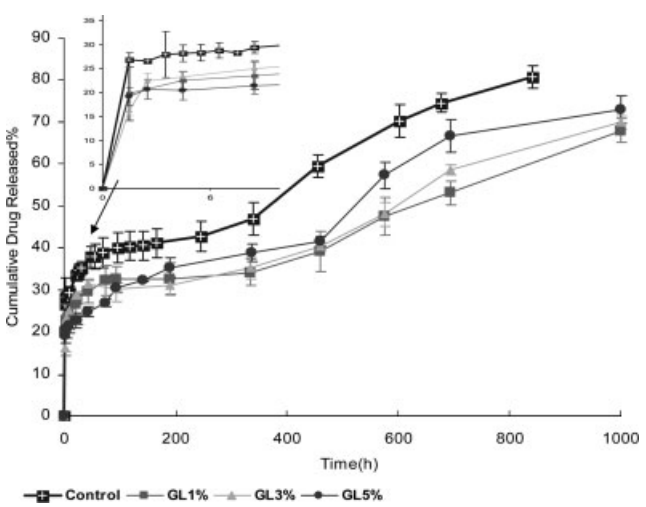

Figure 2 The release of leuprolide acetate from PLGA solution without additive and with 1, 3, and 5% (w/w) glycerol.

Leuprolide acetate release studies

Control samples

The release profile of leuprolide acetate from polymer porous media without additive is shown in Figure 2. From this figure it is evident that the release profile in the control sample, of leuprolide acetate loaded PLGA sol–gel systems included three distinct stages:

1. The burst release was too rapid during the first 24 h (28.2%) (Table II). At this stage, all drugs from the surface and other nearby drugs were washed by the aqueous environment and were removed through the pores and channels of the matrix.
2. The drug release continued slowly for 9 days (12.3%). The mechanism of release was by diffusion from polymeric system.
3. Drug was released by degradation of polymers to oligomers and monomers; therefore, the amount of released drug increased until 27 days (40.2%). At this stage, PLGA-H degraded completely.

Glycerol effects

The effect of glycerol on release profile of leuprolide acetate from polymer porous structure is shown in Figure 2. As this figure shows, in samples with glycerol, the amount of released drug was less than control sample. It is evident that in the release profile of the glycerol containing samples, three distinct stages are noticed as in control samples:

1. The burst release was too rapid during the first 24 h (27.8, 28.8, and 22.8% from systems with 1, 3, and 5% glycerol, respectively) (Table II, Fig. 2).

TABLE II
The Amounts of Burst Release of Leuprolide from Systems with Additives

Percentage of additive	0 (%)	1 (%)	3 (%)	5 (%)
Formulation consists of glycerol	28.2	27.8	28.8	22.8
Formulation consists of ethyl heptanoate	28.2	23.3	18.3	11.3

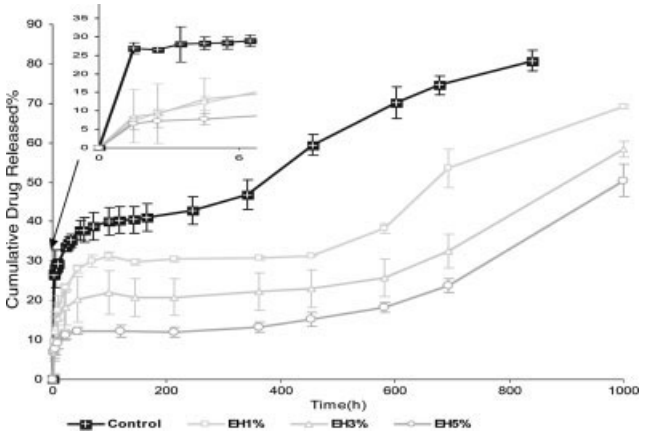

Figure 3 The release of leuprolide acetate from PLGA solution without additive and with 1, 3, and 5% (w/w) ethyl heptanoate.

2. The drug release continued until 14 days for systems with 1 and 3% glycerol and slowly until 20 days for 5% glycerol (34.1, 32.2, and 44.1%, respectively).
3. The amount of released drug increased until 37 days. The evaluation of the system was conducted until 1000 h. Therefore, it may be asserted that the release has proceeded further than the recorded time length. The sudden release of drug has, however, happened due to degradation of polymer.

The initial burst release in sample with 5% glycerol was lower than the samples of 1 and 3%. Least significant difference (LSD) test showed a difference of drug release between formulations 3 and 5% at the first 24 h (P value < 0.05) (Fig. 2).

Ethyl heptanoate effects

The release profile of leuprolide acetate of formulations with ethyl heptanoate is illustrated in Figure 3. The performance and evaluation of system were carried out up to 1000 h. It is shown that the release profile in all samples with ethyl heptanoate was characterized by three distinct stages:

1. The burst release was rapid during the first 8.5 h and by increasing ethyl heptanoate percentage concentration from 1 to 5% (w/w) the burst effect was slowed down. Therefore, 5% (w/w) ethyl heptanoate was more effective on burst reduction than the formulation with 1 and 3% (w/w) ethyl heptanoate. In addition, the advantages of LSD test showed that the drug release from three formulations demonstrated significant differences two by two together in the first 24 h (Table II).
2. In second stage the drug release continued until 600 h with little amount of drug. Ethyl heptanoate due to its hydrophobic character showed minimum tendency for diffusion and reduced the porosity of polymer matrix.
3. Because of polymer degradation, the amount of drug release was suddenly increased. Therefore, oligomers formed at this stage were removed from the matrix rapidly and release of drug occurred as a secondary burst.

In Figure 4, the results show that all the systems with glycerol present as additives at different loadings (1, 3, and 5% w/w) displayed a release pattern similar to system with no additive (control). But, as it was desired all the systems of leuprolide acetate release pattern showed a considerable reduction in the initial burst of drug release in presence of additive. These trends show close similarities with our previous works in our group that reported by Bakhshi et al.[4] on naltrexone release of PLGA system and Astaneh et al.[10] on leuprolide acetate release of PLGA system. Figure 4 shows that although there is no difference observed between rates of release profiles after 400 h in all the samples, in case of ethyl heptanoate, however, the amount of drug released was lower than samples with the same percentage of glycerol added as well as the control sample.

Morphological studies

The surface and cross section morphologies of porous structures with and without additives after 24 h in release media with two different magnifications are illustrated in Figure 5. The Figure 5(A) of SEM micrographs shows relatively large size pores on the implant surface. Furthermore, the polymer porous media without additives demonstrate interconnected

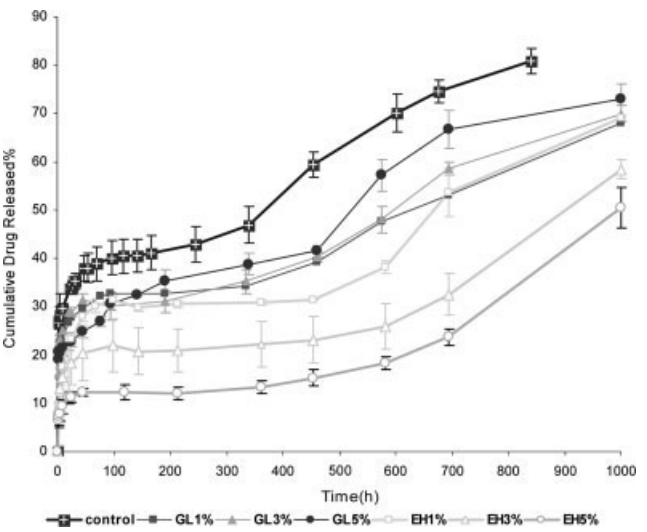

Figure 4 The comparison of release of leuprolide acetate from PLGA solution without additive and with 1, 3, and 5% (w/w) ethyl heptanoate and glycerol.

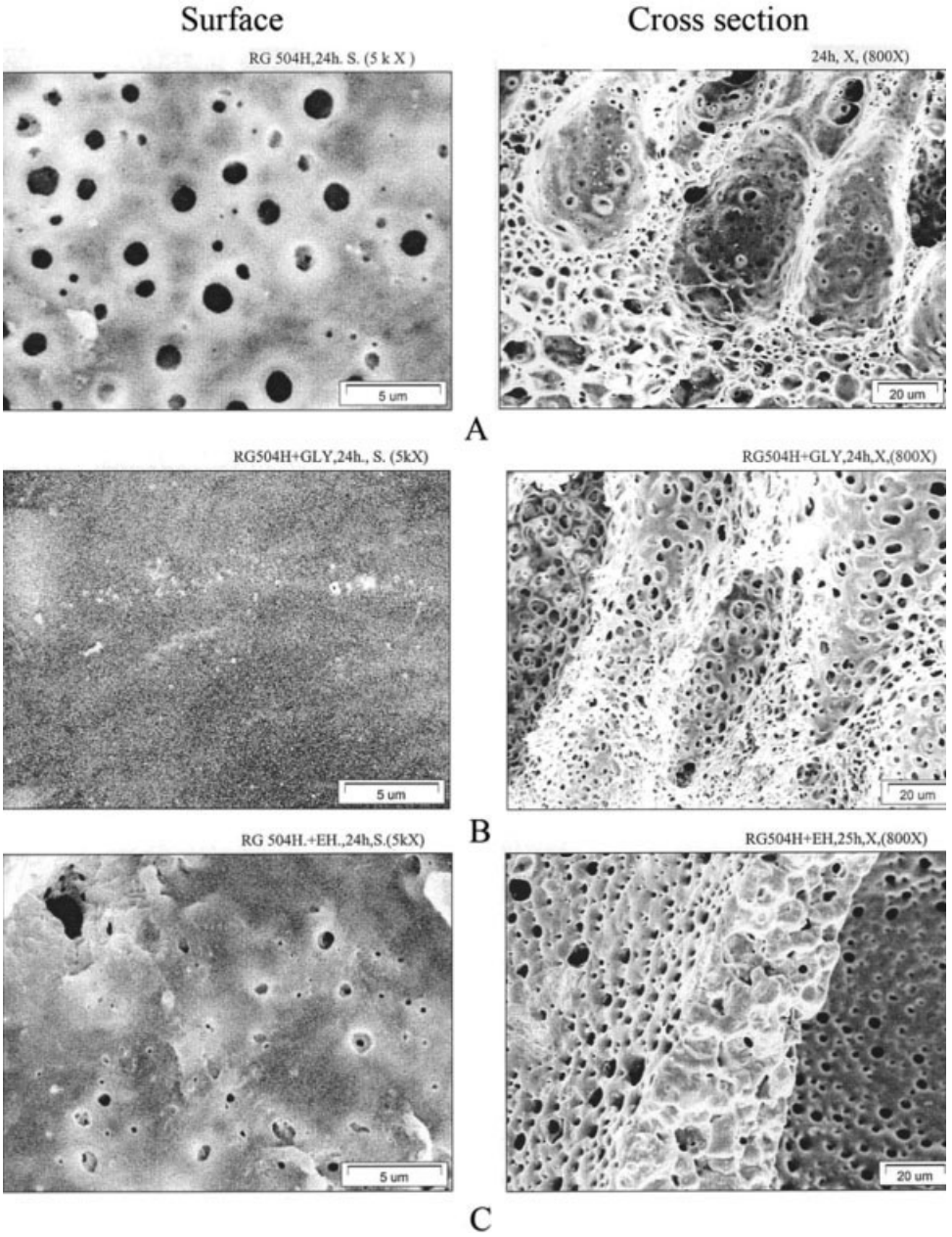

Figure 5 Scanning electron micrographs of PLGA matrix after 24 h storage in PBS: (A) PLGA/NMP/drug; (B) PLGA/NMP/drug/glycerol; (C) PLGA/NMP/drug/ethyl heptanoate. Magnifications of surfaces are ×5000 and magnifications of surfaces are ×800.

pores structures which look like tears. This morphology is named ''tear-like'' structure.[24] With addition of glycerol to polymer solutions both surface and cross section structures have changed sharply. As shown in Figure 5(B), the membrane surface shows a compact structure without any pores compared with formulations without glycerol [Fig. 5(A)] with same magnification. In addition, the presence of glycerol gave rise to big finger- and channel-like structures [Fig. 5(B)] instead of tear-like structures [Fig. 5(A)] of the porous media cross section. To explain this phenomenon, it seems that as glycerol is a hydrophilic additive, with its presence in polymer formulations, the solvent-water exchange rate in the phase separation stage increases in the aqueous coagulation bath. Faster coagulation causes a formation of an asymmetric structure with a dense and compact skin layer and cross sections containing channel- and finger-like structures instead of tear-like structures.[5,25] When ethyl heptanoate is added to polymer formulations both surface and cross section structures are changed as well. As it is shown in Figure 5(C), the membrane surface contains less and smaller pores than the control samples, though

higher than glycerol system. Furthermore, all tear, finger, and channel-like structures disappear and sponge-like structure is observed in porous media cross section. Addition of ethyl heptanoate as a hydrophobic additive decreases the exchange rate of solvent and the nonsolvent in coagulation bath and, therefore, the sponge-like structure takes over the other structures.[5,26,27] In comparison to other formulations, the sponge-like morphology in systems containing ethyl heptanoate causes the lowest level of leuprolide acetate release. It is to be noted that there is a challenge between the surface and cross section morphologies by comparing the control system with that having additives in determining the rate of drug release. Mainly the samples having channel- and finger-like structures exhibit higher release rates compared with samples of tear- and sponge-like structures. It is important to mention that although the system containing glycerol has more compact surface structure than the formulation containing ethyl heptanoate, but the higher release of leuprolide acetate is due to the presence of channel- and finger-like structures of the former. Comparing the control and glycerol containing systems it is evident that due to the presence of channels in the latter the quantity of release is expected to be higher than the former which only constitutes of tear structure. The release study, however, has demonstrated the reverse observation, i.e., the slower release rate in the glycerol system (Fig. 2) is accounted for the presence of dense and very compact surface morphology in the present system. Therefore, there is a competition between the surface behavior and the cross section structure to control the drug delivery system. It clearly shows that in certain stages of morphological changes the cross section morphology or the bulk is a dominant determinant and in some other stages the surface morphology is the controlling dominant.

CONCLUSIONS

In situ forming drug delivery has been successfully prepared by inversion technique using PLGA and leuprolide acetate dissolved in NMP.

The results of release profile of leuprolide acetate loaded PLGA system showed that the presence of additives as rate-modifying agents affect morphology, degradation of PLGA matrix, and release of drug from *in situ* forming systems.

Each additive of ethyl heptanote and glycerol reduced the initial burst release and decreased the release profile at different loadings, while increasing its duration of release.

In addition, the lifetime of the polymeric implants with ethyl heptanoate or glycerol was longer than the system without additive.

It has been found in this study that varying the type and concentration of additive can change the initial amount of drug release and its overall quantitative release profile.

Ethyl heptanoate, however, shows stronger effect than glycerol by reducing the porosity of the surface and tear-like structures disappear and sponge-like structures are being formed.

The accuracy of the method was determined for three concentrations with recovery percentage and reproducibility reported in terms of RSDs. The small values of RSD obtained showed that the determination of the analyte in dilute release-media samples was adequately reproducible and an acceptable accuracy was obtained.

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