

## Preparation of gelatin microspheres containing lactic acid – Effect of cross-linking on drug release

RASSOUL DINARVAND<sup>1\*</sup>  
SANAZ MAHMOODI<sup>1</sup>  
EFFAT FARBOUD<sup>1</sup>  
MASSOUD SALEHI<sup>2</sup>  
FATEMEH ATYABI<sup>1</sup>

<sup>1</sup> Department of Pharmaceutics  
Faculty of Pharmacy, Tehran University  
of Medical Sciences, Tehran PO Box  
14155-6451, Iran

<sup>2</sup> Faculty of Public Health  
Tehran University of Medical Sciences  
Tehran 14174, Iran

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In this study, gelatin microspheres containing lactic acid were prepared by the polymerization technique using glutaraldehyde as the cross-linking agent. Dried microspheres were loaded by immersing them in an aqueous solution of lactic acid. In order to prepare microspheres with an appropriate drug release profile, the effect of time of cross-linking and the amount of cross-linking agent on the swelling properties of microspheres and their release profile were investigated. The microencapsulation efficiency, microspheres appearance, particle size, swelling ratio and drug release profile were also studied. Microspheres prepared with a larger amount of cross-linking agent, or after longer cross-linking time, showed a reduced swelling ratio in aqueous media. *In vitro* release pattern of lactic acid from gelatin microspheres showed a biphasic profile and the release rates were reduced upon increasing the amount of cross-linking agent and prolonging the cross-linking time.

**Keywords:** lactic acid, microspheres, gelatin, cross-linking

Encapsulation of drug molecules in particulate carriers as a method of controlled delivery of molecules has been studied extensively. In recent years, a number of different particulate systems, such as microcapsules and microspheres, have been proposed and used in topical formulations as drug carrier vehicles. It has been claimed that these new drug vehicles can improve and control drug release from conventional topical formulations (1). Furthermore, the massage effect of these particles on the skin can have cleansing and stimulating effects (2). With such devices, drugs can be effectively delivered to the upper skin layers (viable epidermis) without penetration into deeper layers. Therefore, microencapsulation reduces systemic uptake of active agents and supplies drug molecules to the skin over a prolonged period of time (3). It has been shown that reduction of either the applied dose or the frequency of administration give better pharmacological results compared to administration of conventional doses of drugs (3).

This study deals with microencapsulation of lactic acid as an anti-ageing agent. Lactic acid, an  $\alpha$ -hydroxy acid (AHA), is used to combat the visible effects of ageing. It produ-

\* Correspondence, e-mail: [dinarvand@tums.ac.ir](mailto:dinarvand@tums.ac.ir)

ces more skin hydration than other  $\alpha$ -hydroxy acids and smoothes the lines and wrinkles of the skin (4). Natural polymers such as gelatin have been widely used for the preparation of particulate drug delivery systems. Use of gelatin in pharmaceuticals is particularly attractive by virtue of its biocompatibility and biodegradability along with a total absence of toxicity or allergic problems (5). It has been utilized in the fabrication of both injectable and oral drug delivery systems (6–10). Being a soluble polymer, gelatin has to be chemically cross-linked to become insoluble at 37 °C. Aldehyde derivatives such as formaldehyde, glutaraldehyde or other bifunctional reactants have been used to produce insoluble biodegradable gelatin microspheres (11). Glutaraldehyde is used as a cross-linking agent to obtain rigid microspheres. This method has been widely studied in various formulations by different researchers (9, 12). Glutaraldehyde is expected to produce cross-links between gelatin molecules and thereby to slow down the rate of drug release from microspheres (13). In this method, it is important to remove excess oil by washing the particles with solvents such as acetone. Otherwise, the oil retained in the microspheres may cause aggregation and alter the morphological properties of the microspheres. This washing procedure is also said to remove excess of the cross-linking agent (14). Acetone, used to remove excess glutaraldehyde, extracts the water content of microspheres to obtain hardened microspheres that are easily filtered and dried.

In a previous study, we investigated the effect of different formulation variables on the particle size of gelatin microspheres containing lactic acid (15). In this study, the effect of cross-linking on the release profile of gelatin microspheres is reported. Impact of both the duration of cross-linking reaction and the amount of cross-linking agent on the swelling and drug release properties of microspheres is reported.

## EXPERIMENTAL

### *Materials*

Gelatin (food grade, NF), lactic acid (90%), glutaraldehyde, toluene, acetone, span 80, sulfuric acid and copper sulphate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) were purchased from Merck (Germany). Sesame oil was purchased from Aseel (United Arab Emirates). 4-Hydroxy biphenyl was obtained from Fluka (Switzerland).

### *Preparation of gelatin microspheres*

Gelatin microspheres were prepared using an overhead stirrer with a five-blade paddle (diameter 50 mm) (15). Five mL of gelatin solution (20%, *m/V*, in water) was preheated to 80 °C and added drop-wise to 70 mL of sesame oil (viscosity 43.4 mPa s at 20 °C) containing 1% (*m/m*) Span 80 (with respect to the mass of the oil phase) warmed to the same temperature. The biphasic system was stirred under turbulent flow conditions using an overhead stirrer (RW20DZM.n, IKA Labortechnik, Germany) to form a w/o emulsion. Glutaraldehyde-saturated toluene was prepared by mixing equal volumes of glutaraldehyde and toluene in a decantation funnel. After shaking for 10 minutes, the mixture was allowed to separate. The upper toluene layer saturated with glutaraldehyde was separated

and added to the w/o emulsion. The dispersion was mixed for various time intervals at an appropriate speed (1200 rpm).

Microspheres were then separated by decantation and washed free of oil with 20 mL of toluene for 2 min at 1500 rpm. The microspheres were then washed and dehydrated 3 times with 20 mL of acetone at 2000 rpm. Finally, microspheres were allowed to dry at room temperature (25 °C). Upon drying, a yellow to yellowish orange colored free flowing, fine powder was obtained. The gelatin microspheres were observed by both optical microscopy (B3050 Prior, Prior Scientific, UK) and scanning electron microscopy (Leica Manuf. Cambridge S 360, UK).

### *Loading of lactic acid*

Lactic acid loading was carried out using the drug uptake method. One gram of dried microspheres was allowed to swell in 40 mL solution of 90% lactic acid at  $37 \pm 0.2$  °C for 30 min under continuous magnet stirring. Microspheres were then rapidly collected using paper filtration, washed with water and acetone, and dried at room temperature. Microencapsulation efficiency or the total drug content, expressed as percentage, is defined as the ratio of the mass of drug encapsulated within the microspheres to the total mass of microspheres.

### *Particle size measurement*

Microspheres diameter and size distribution were measured by laser light diffractometry equipment (Mastersizer X, Malvern Instrument, UK). The average particle size was expressed as the volume mean diameter in micrometers.

### *Determination of the swelling ratio*

The swelling ratio of gelatin microspheres was determined using a Laser particle sizer (Analysette 22 Fritsch, Germany). Doubly distilled water was used as the swelling medium. Microspheres were left in swelling medium for at least 60 min to reach maximum swelling. Volumetric measurements were made by measuring the diameters of microspheres placed in the swelling medium at appropriate time intervals. The swelling ratio was calculated from the ratio of the volume of swollen particles to that of dry particles.

### *Drug content in microspheres*

Microspheres (100 mg) were crushed in a porcelain mortar and then suspended in 50 mL of doubly distilled water. The amount of lactic acid released after 24 hours was evaluated as the amount of the drug loaded in microspheres. No more drug release was observed after 24 hours. Colorimetric method was used to measure the amount of lactic acid (16); acetaldehyde produced by the reaction between lactic acid and hot sulfuric acid was measured. Acetaldehyde reacted with copper and 4-hydroxy biphenyl and gave a chromogen with an absorption peak at 570 nm (Spectronic plus, Milton Roy, USA). The amount of lactic acid was calculated from the standard calibration curve. All experiments were carried out in triplicate.

### *In vitro drug release*

Microspheres (100 mg) were placed in a container and immersed in 100 mL of doubly distilled water at  $37 \pm 0.5$  °C under continuous magnet stirring. Filtrate (5 mL) was withdrawn at appropriate time intervals and the concentration of lactic acid was determined as stated above. To maintain a constant volume, 5 mL of water were returned to the container.

### *Statistical analysis*

The effect of cross-linking time and the amount of cross-linking agent on the lactic acid release from gelatin microspheres were analyzed separately using Repeated Measures Analysis of Variance. When significant differences between the formulations were observed, multiple comparisons by the Duncan test were applied.

## RESULTS AND DISCUSSION

The mean particle size of microspheres prepared in this study was in the range of 10 to 1000  $\mu\text{m}$ . Fig. 1 shows a typical particle size distribution. The prepared microspheres were a free flowing, yellow colored powder. The color of the microspheres changed from yellow to yellowish orange and to brown when cross-linking time and concentration of glutaraldehyde-saturated toluene increased. This phenomenon may be caused by increasing the degree of gelatin cross-linking.

Fig. 2 shows the SEM photographs of the gelatin microspheres treated with glutaraldehyde. As can be seen, spherical microspheres with a very smooth and uniform surface were formed. No physical pores on the surface of the microspheres are seen. Appearance of the microspheres surface and their mean particle size were only slightly affected by the experimental cross-linking conditions (time and amount of the cross-linking agent).

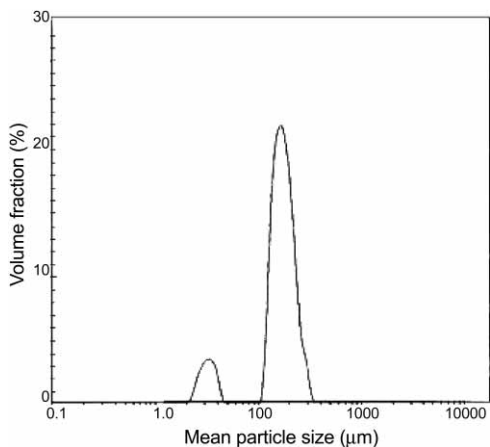


Fig. 1. Particle size distribution of gelatin microspheres.

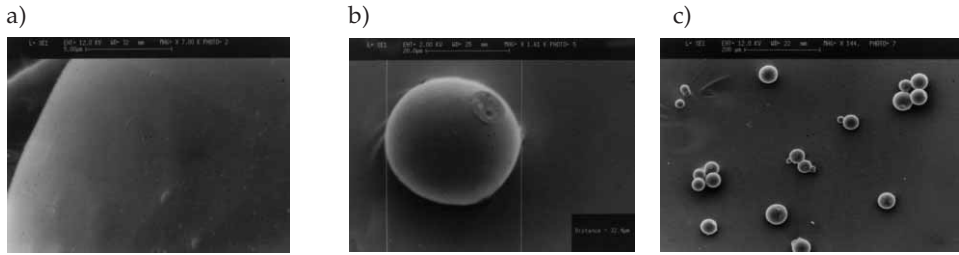


Fig. 2. Scanning electron micrographs of gelatin microspheres. Magnification: a) 7000, b) 1610, c) 144 $\times$ .

The swelling ratio of different formulations of gelatin microspheres was investigated as a function of the glutaraldehyde content and cross-linking time. As shown in Fig. 3, the swelling ratio of microspheres increased dramatically when a smaller amount of cross-linking agent was used. The same pattern was observed when the duration of cross-linking was altered, *i.e.*, the longer the time of cross-linking the lower was the ratio of swelling. When microspheres were prepared during the cross-linking time of 1, 4 and 12 hours, their mean swelling ratio ( $\pm$  SD) was  $8.34 \pm 0.42$ ,  $4.51 \pm 0.21$  and  $1.44 \pm 0.09$ , respectively. The mean swelling ratio ( $\pm$  SD) of microspheres was  $6.31 \pm 0.29$ ,  $4.51 \pm 0.23$  and  $3.09 \pm 0.18$  when concentrations of 12, 21 and 35% (V/V) of glutaraldehyde-saturated toluene were used, respectively.

In the presence of lactic acid, gelatin cross-linking using glutaraldehyde is not possible. Therefore, gelatin microspheres were first prepared and cross-linked and then loaded with lactic acid. Drug loading after microspheres preparation is also advantageous since impurities associated with the microsphere preparation process may be removed prior to loading.

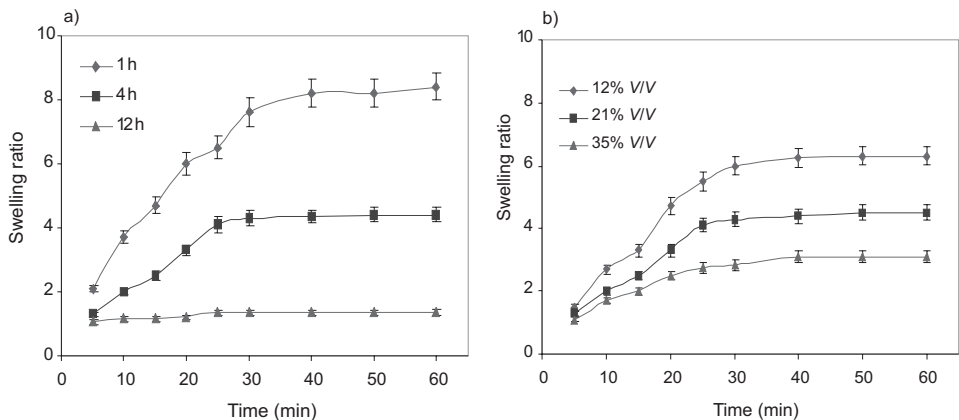


Fig. 3. Effect of: a) cross-linking time, b) concentration of cross-linking agent (glutaraldehyde) on mean swelling ratio ( $\pm$  SD) of gelatin microspheres. The experiment was repeated 3 times. SD is represented as error bar.

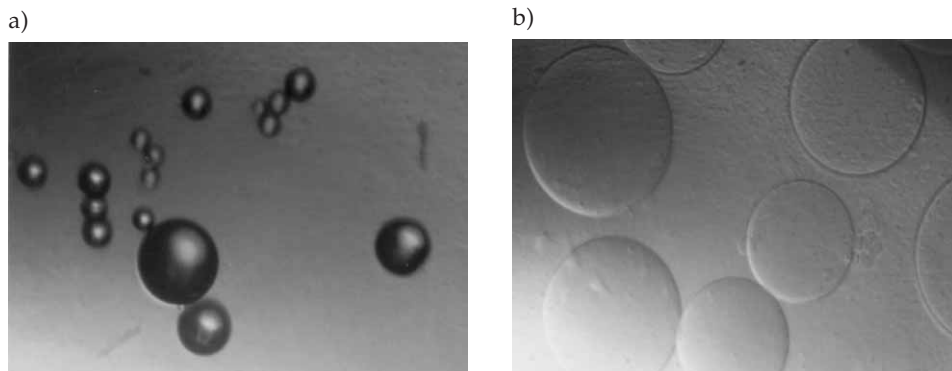


Fig. 4. Optical micrograph of gelatin microspheres: a) without lactic acid, not swelled, b) with lactic acid, swelled during drug loading. Magnification: 400 $\times$ .

Fig. 4 shows the optical micrograph of gelatin microspheres swelled in lactic acid. Drug loading may be carried out under aqueous conditions when needed (17).

Table I shows the effect of the amount of acetone, used as washing agent, on the microspheres, particle size and drug loading. As can be seen, the mean particle size for drug loaded microspheres washed with a smaller quantity of acetone was larger than for the others. This increase in mean particle size can be attributed to agglomeration of the small sized microspheres, as observed under the microscope. Use of smaller quantities of acetone as dehydrating solvent, increased the drug loading efficiency of microspheres; low microencapsulation efficiencies were observed when a large quantity of acetone was used. This phenomenon may be caused by the high solubility of lactic acid in hydrophilic solvents such as acetone. It may also be assumed that lactic acid molecules are not fully encapsulated by the gelatin walls of microcapsules, but they are attached to the gelatin surface of microspheres. Since lactic acid is freely soluble in acetone, a small amount of acetone is capable to dissolve a substantial amount of lactic acid. By increasing the quantity of acetone, microencapsulation efficiency was decreased. Reduced microencapsulation efficiency due to the solvent effect was earlier reported for alginate microspheres using isopropanol as the dehydrating solvent (18), for apomorphine hydrochloride gelatin microspheres using acetone as the dehydrating and drug washing solvent (19), and for collagen-gelatin microspheres containing deoxyuridine during washing with water (20).

Table I. Effect of the volume of acetone<sup>a</sup> on drug loading and the mean particle size of microspheres<sup>b</sup>

Acetone (mL)	Drug loading (%)	Mean particle size ( $\mu\text{m}$ )
30	63	54
50	44	32
100	23	18

<sup>a</sup> Used as washing solvent for gelatin microspheres.

<sup>b</sup> Cross-linked with glutaraldehyde-saturated toluene (35%, V/V, glutaraldehyde) for 4 hours.

The amount of drug loaded was calculated using the standard calibration curve ( $R = 0.9995$ ). The percentage loading of lactic acid into microspheres was found to be  $44 \pm 3\%$  when 50 mL of acetone was used for final washing of drug loaded microspheres. The drug content of microspheres was slightly affected by the amount of the cross-linking agent, whereas no difference in drug loading was observed as the cross-linking time period increased. The release profiles of lactic acid from gelatin microspheres cross-linked using different amounts of glutaraldehyde-saturated toluene or during different cross-linking time periods are displayed in Fig. 5. Microspheres maintained their spherical shape even after 24 hours of release period. A biphasic pattern of drug release was observed for all samples. Thus, regardless of the conditions of the cross-linking of gelatin microspheres, an initial burst release was observed. Within the first hour, 40–80% of the loaded drug was released. This burst release was followed by a prolonged period, during which slower drug release took place. For batches cross-linked with 35% (V/V) glutaraldehyde-saturated toluene and 4 hours of cross-linking time period,  $t_{50\%}$  and  $t_{85\%}$  of drug release were 40 and 480 minutes, respectively. This type of release profile is of interest because the initial burst release can provide the initial penetration of lactic acid, and the sustained release phase supplies the skin with the drug over a prolonged period of time (21). The initial burst drug release may be attributed to the release of drug molecules held loosely into or just beneath the surface of microspheres. Such a burst effect was reported previously for gelatin microspheres (16, 19).

As shown in Fig. 5, by increasing the concentration of glutaraldehyde-saturated toluene from 12 to 35% (V/V), the amount of drug release decreased from 30 to 10% in the first 30 minutes of the drug release experiment. It is also shown that by decreasing the cross-linking time from 12 to 1 hour, the amount of drug release in the first 30 minutes of drug release experiment increased by 12 to 42%. In the second phase, practically all the drug was released from the microspheres cross-linked with either a smaller amount of cross-linking agent or at various cross-linking time periods. However, only 82% of lactic acid was released within 12 hours from gelatin microspheres cross-linked with 35% (V/V) glutaraldehyde-saturated toluene.

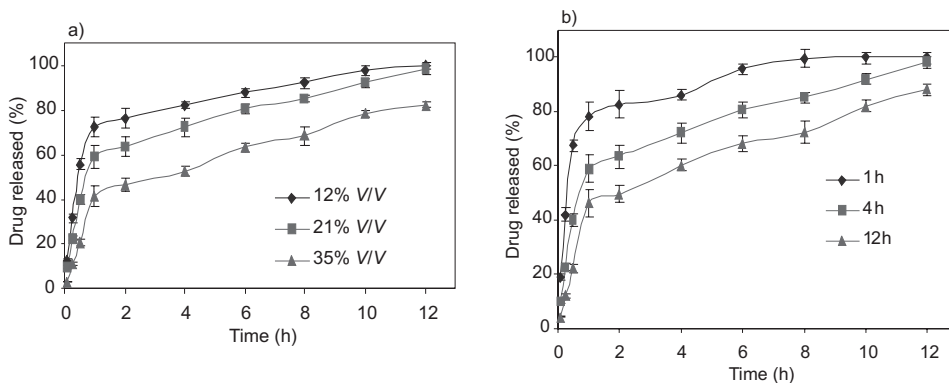


Fig. 5. *In vitro* release profile of lactic acid from gelatin microspheres prepared by: a) different concentrations of cross-linking agent (glutaraldehyde), b) different cross-linking time periods. The experiment was repeated 3 times. SD is represented as error bar.

Analysis by the Repeated Measures Analysis of Variance showed that the mean percentage of drug released *in vitro* from gelatin microspheres prepared with different amounts of glutaraldehyde and during different cross-linking time periods differed significantly ( $p < 0.005$ ). This was confirmed by the Duncan test multiple comparison.

The rate constants for drug release were then computed for both the fast (first hour) and slow release phases (Table II). It can be seen that the rate of drug release decreased with increasing the glutaraldehyde content and cross-linking time for both the fast and slow release phases. These results clearly indicate that the release of therapeutic agents from gelatin microspheres can be controlled by varying these parameters.

Gelatin microspheres are known to swell in aqueous environments due to hydration. As a new polymeric structure is formed by introducing bridges between polymeric chains during the cross-linking procedure, the extent of the swelling process depends on the degree of cross-linking. Therefore, the denser the cross-linking bridges between the gelatin molecules, the more packed is the structure. Such a structure can be characterized by lower and slower penetration of the solvent through the chain structure of the polymer, suggesting that the swelling ratio and hence the release characteristics of the microsphere can be controlled by varying the content of the cross-linking agent used during the manufacturing process (19). Swelling of microspheres may result in mobility of gelatin chains, facilitating rapid release rates of the drug by diffusion through the polymer. Since glutaraldehyde is responsible for the formation of cross-links, increasing the amount of glutaraldehyde and the cross-linking time will increase the polymer density, resulting in reduction of the macromolecular chains mobility, and the formation of more stable and rigid spheres that show a lower tendency to swell. The finding of this study is in agreement with Reddy and coworkers (7) and Chuo and coworkers (22) findings, in which they reported that drug release from albumin microspheres could be ex-

Table II. Rate constants for drug release for both fast and slow release phases for different formulations

Concentration of cross-linking agent (%, V/V)	Release phase	$k$ (h <sup>-1</sup> )
35	a	0.0040
35	b	0.0008
21	a	0.0062
21	b	0.0019
12	a	0.0090
12	b	0.0035
	Time of cross-linking	
12	a	0.0046
12	b	0.0010
4	a	0.0062
4	b	0.0019
1	a	0.0103
1	b	0.0045

<sup>a</sup> First hour of drug release

<sup>b</sup> After the first hour of drug release



tended by increasing the cross-linking degree of microspheres. Likewise, Sahin and co-workers (14) reported that a decrease in the swelling ratio in terbutaline sulphate loaded albumin microspheres was observed with an increase in the glutaraldehyde concentration. Other researchers such as Raymond *et al.* (13) and Fan and Dash (9) have found similar results. Raymond *et al.* (13) found out that the release of phenytoin sodium from gelatin microspheres can be delayed by the addition of glutaraldehyde to the microsphere formulation. Fan and Dash (9) also reported that increasing the glutaraldehyde concentration decreased the release rate of doxorubicin from gelatin implants. As shown in this study, this effect may be attributed to the fact that the cross-linking process usually hardens the gelatin matrix and could also increase resistance for the penetration of release medium into the implant.

### CONCLUSIONS

Spherical, lactic acid loaded gelatin microspheres were prepared by the chemical cross-linking technique. *In vitro* drug release experiments showed a distinct biphasic release pattern that may be desirable for topical drug delivery, since a therapeutic loading dose can be provided initially and the sustained drug release could maintain the therapeutic drug level. It can be concluded that the desired lactic acid release rate can be achieved by controlling the amount of the cross-linking agent (glutaraldehyde) and the duration of cross-linking time.

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## S A Ž E T A K

### Priprava želatinskih mikrosfera s mliječnom kiselinom – Učinak umrežavanja na oslobađanje ljekovite tvari

RASSOUL DINARVAND, SANAZ MAHMOODI, EFFAT FARBOUD, MASSOUD SALEHI i FATEMEH ATYABI

U radu je opisana priprava želatinskih mikrosfera s mliječnom kiselinom metodom polimerizacije koristeći glutaraldehid kao sredstvo za umrežavanje. Osušene mikrosfere su uronjene u vodenu otopinu mliječne kiseline. Ispitivan je utjecaj vremena umrežavanja i količine sredstva za umrežavanje na bubrenje mikrosfera i njihov profil oslobađanja. Također je proučavan izgled mikrosfera, veličina čestica, bubrenje i profil oslobađanja ljekovite tvari. Mikrosfere pripravljene s većim sadržajem sredstva za umrežavanje ili uz produljeno vrijeme umrežavanja, u vodenom mediju manje bubre. Oslobađanje

mliječne kiseline iz želatinskih mikrosfera *in vitro* je bifazičnog profila, a smanjuje se ako je tijekom pripreme povećana količina sredstva za umrežavanje i produljeno vrijeme umrežavanja. Mikrosfere s odgovarajućim profilom oslobađanja ljekovite tvari mogu se pripraviti podešavanjem ta dva faktora tijekom pripreme mikrosfera.

*Ključne riječi:* mliječna kiselina, mikrosfere, želatina, umrežavanje

*Faculty of Public Health, Tehran University of Medical Sciences, Tehran 14174, Iran*

*Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran PO Box 14155-6451, Iran*