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Controllable Synthesis of Horseradish Peroxidase Loaded Poly(D,L-lactide) Nanospheres

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In this work, horseradish peroxidase (HRP), a model protein, was encapsulated by the precipitation method with additional step of homogenization, using polymer poly(D,L-lactide) (PDLLA). The purpose of this study was to expand the application of a modified precipitation method to HRP-loaded PDLLA spheres and to find optimal conditions for preparing HRP-loaded PDLLA spheres with the best properties, with size under 1000 nm. Influence of process variables on the size and morphology of the particles has been studied. Size of the spheres depends on homogenization speed, co-solvent selection, chloroform to water ratio and PVA concentration. Particle size was ranging between 460 nm and 1130 nm, according to the preparation conditions. The optimized conditions yielded nanospheres with 46% encapsulation efficiency and average diameter of 530 nm. A particle size under 1000 nm enables intravascular injection and is also desirable for intramuscular and subcutaneous administration, minimizing possible irritant reactions. It was found that modified precipitation method was widely applicable to HRP-loaded PDLLA spheres, by choosing optimal preparation conditions.

Keywords:

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1. INTRODUCTION

Nanotechnology is a fast-growing area, involving the fabrication and use of nano-sized materials. Recent advances in nanoscience have allowed researchers to apply revolutionary new approaches in their research at molecular and biological cellular levels, thereby advancing the understanding of process in host of areas which up to now had not been possible to study, in particular nano-bio-technology.[1–6] In recent years, significant effort has been devoted to develop nanotechnology for drug delivery since it offers a suitable means of delivering small molecular weight drugs, as well as macromolecules such as proteins, peptides or genes by either localized or targeted delivery to tissue of interest.[7–9] A number of different polymers, both synthetic and natural, have been utilized in formulating biodegradable nanoparticles. Synthetic polymers have the advantage of sustaining the release of the encapsulated therapeutic agent over a period of days to several months compared to natural polymers which have relatively short duration of drug release. They can be prepared according to different methods, like emulsification/solvent evaporation,[10–12] dialysis,[13] spray-drying,[14] precipitation[15,16] and phase separation.[17] Poly(ε-caprolactone) (PCL), poly(D,L-lactide) (PLA) and poly(D,L-lactide-co-glycolide) (PLGA) have gained attention for the preparation of a wide variety of delivery systems due to their biodegradable and biocompatible properties.[18] The degradation of PLA occurs primarily through hydrolysis and metabolism in the citric acid cycle. For use as a drug delivery system, the polymer degradation (polymer chain scisson) and erosion (mass loss of polymer matrix) are important processes for a controlled drug release. Numerous peptides, proteins and genetic materials are being looked upon as therapeutic agents.[4,19,20] However, most of these materials possess short *in vivo* half-lives due to physical and chemical instability or enzymatic degradation. As a consequence, frequent administration is required to achieve desirable therapy. The incorporated proteins must remain stable in the polymers and should be released continuously. Further requirements for the carrier itself are no negative effects on metabolism and complete absorption without any side-effects. So, it has been necessary to control the delivery of these biotechnology

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products. One approach to the development of such new drug delivery systems involves the use of biodegradable nanoparticles, taking advantage of their extremely small particle size and large specific surface area.[9]

In recent years, the preparation of protein-loaded microspheres has attracted much attention.[4, 7, 20] Peroxidases are reported to be one of the most stable enzymes in plans.[21] Horseradish peroxidase (HRP) is an important heme-containing enzyme that has been studied for more than a century, belonging to class III of the plant peroxidase superfamily, and its structure and biochemical properties have been characterize very well. It is not surprising therefore that so little is known about their specific functions in the plant due to high redundancy found in genes, the broad spectrum of substrates accepted by these enzymes, and the very similar immunological properties of different isozymes.[22, 23]

One primary concern with protein encapsulation in PDLLA nano- and microparticles is the loss of therapeutic efficacy of the protein due to degradation/denaturation of the protein. Inactivation of protein could occur through two different mechanisms. First protein is exposed to organic solvents during the formulation procedure that caused denaturation of the protein. Second, the acidic environment generated during the degradation of PDLLA could produce similar inactivation.[18] The drug release from poly(D,L-lactide) occurs in two periods: primarly by diffusion without mass loss, followed secondarily by erosion of the matrix. The fast initial release of incorporated protein HRP could be altered by a modification of the coating of PDLLA and PDLLA coating could extend the release kinetics.[24, 25] Some authors showed that the initial burst effect can be reduced by adding a surfactant to the internal emulsion.[19]

Modified precipitation method[26, 27] has been used to prepare biodegradable polymeric micro and nanospheres for protein delivery. This is a simple and inexpensive method of incorporating protein with biodegradable polymer, and protein-loaded microspheres prepared by this method have many advantages such as improved encapsulation efficiency (EE), reduced toxicity, and protection proteins from biologically inactivation. HRP was chosen as a model protein in this study, since it is very sensitive to solvents and elevated temperature.[19] Our work focuses on the effect of process and formulation parameters in the preparation of HRP-loaded PDLLA spheres in the specific size range of 500–1000 nm. In this study, the applicability of modified precipitation method for obtaining PDLLA particles was further tested for obtaining HRP-loaded PDLLA particles. Here, we report characterization of horseradish peroxidases, using SDS-Page electrophoresis, one of the class III peroxidases. Differential process parameters were varied in order to test their influence on the particle size and shape, and to obtain the smallest and most uniform particles.

2. EXPERIMENTAL DETAILS

2.1. Materials

Poly(D,L-lactide) with an average molecular weight of 50 000 g/mol was purchased from Sigma (Sigma-Aldrich, Germany). Organic solvent, chloroform, was obtained from Lachema (Lachema, Czech Republic). Alcohols, ethanol (EtOH) and methanol (MeOH), were obtained from Superlab (Superlab, Serbia). Horseradish peroxidase (MW = approximately 44 kDa) was obtained from Fluka. Polyvinyl alcohol (PVA) (Sigma-Aldrich, Aldrich Chemie, Steinheim, Germany) with 89% hydrolyzation degree was used as stabilizing agent. All reagents were of reagent grade and used as received.

2.2. Preparation of PDLLA Spheres with Encapsulated Horseradish Peroxidase

Briefly, spheres were prepared as follows: 40 mg of commercial PDLLA granules were dissolved in chloroform, and 1 mg of horseradish peroxidase was dissolved in 1 ml of water and this solution was added drop-wise to polymer solution while mixture was stirred at 1200 rpm using magnetic stirrer, to make an emulsion. This mixture was added drop-wise to ethanol or methanol and after that dispersion was instilled to PVA solution containing 1 wt% PVA or 5 wt% of PVA while the mixture was stirred at 1200 rpm by a magnetic stirrer. The resultant mixture was then stirred at high speed at 21 000 rpm using a homogenizer (Ultra-Turrax T25, IKA, Germany). The solution was then centrifuged for 2 h on 4000 rpm, decanted and dried under vacuum for a few days at room temperature. The supernatant solution was stored for the analysis of HRP content using spectrophotometry. (Schematic diagram of manufacturing process for HRP-loaded PDLLA spheres (PDLLA/HRP 97.5/2.5%) is shown in Fig. 1). All process parameters are listed in Table I. Individual preparations were repeated at least three times.

2.3. Yield in Preparation, Loading Efficiency and Loading Amount

Particles, dried at room temperature, were weighed, and the yield was calculated in percentages using equation:

$$\text{Percentage yield} = [(\text{weight of particles})/(\text{weight of polymer} + \text{weight of HRP})] \times 100$$

HRP absorbs light at wavelength of 403 nm. Based on measuring absorbance of the solution with a known concentration of HRP (Fig. 2(a)) at 403 nm, a calibration curve was prepared (Fig. 2(b)). The linear relationship between light absorbance at 403 nm and HRP concentration is shown according to the Beer-Lambert Law: $A = \varepsilon c l$ (where A is absorbance at sample concentration c (in

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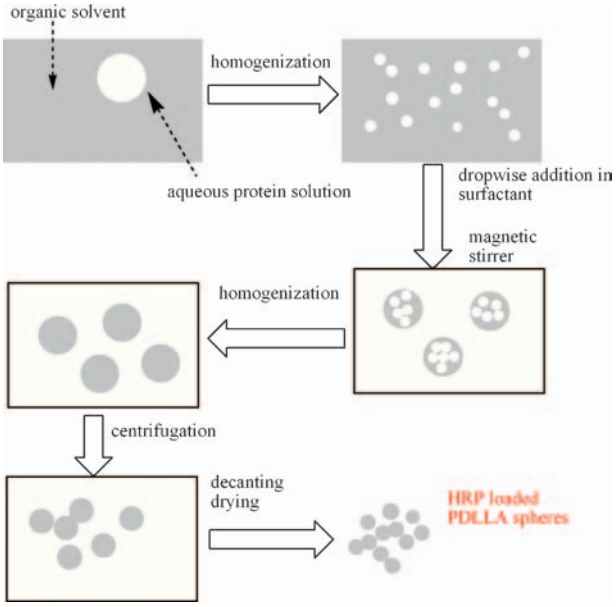

Fig. 1. Shematic diagram of manufacturing process for HRP-loaded PDLLA spheres.

this case concentration of the HRP (mg/ml)), l is path length of quartz cell and ε is the absortivity). By applying this standardized relationship, supernatant obtained during the synthesis was analyzed to determine the concentration and amount of non-encapsulated HRP. Knowing the initial amount of HRP used in PDLLA-HRP synthesis, the percentage of HRP loaded into the particles was obtained (loading efficiency). Loading amount was calculated by means of equation:

$$\text{Loading amount} = [\text{Loading efficiency } (\%)/100] \times \text{Total amount of HRP added}$$

The UV measurements were performed on GBC, Cintra 101 UV spectrophotometer in the frequency interval of 300–600 nm.

2.4. Morphology Studies

The surface morphology of HRP-loaded PDLLA particles were studied by a JEOL JSM-6460LV scanning electron microscopy (SEM) and SUPRA 35 VP Carl Zeiss field emission scanning electron microscope (FESEM). A bit of HRP-loaded PDLLA powder was deposited on a metal plate and sputtered for a few minutes with gold using the physical vapor deposition (PVD) process. The samples were sputtered (SCD 005 sputter coater), using 30 mA current from the distance of 50 mm during 180 s.

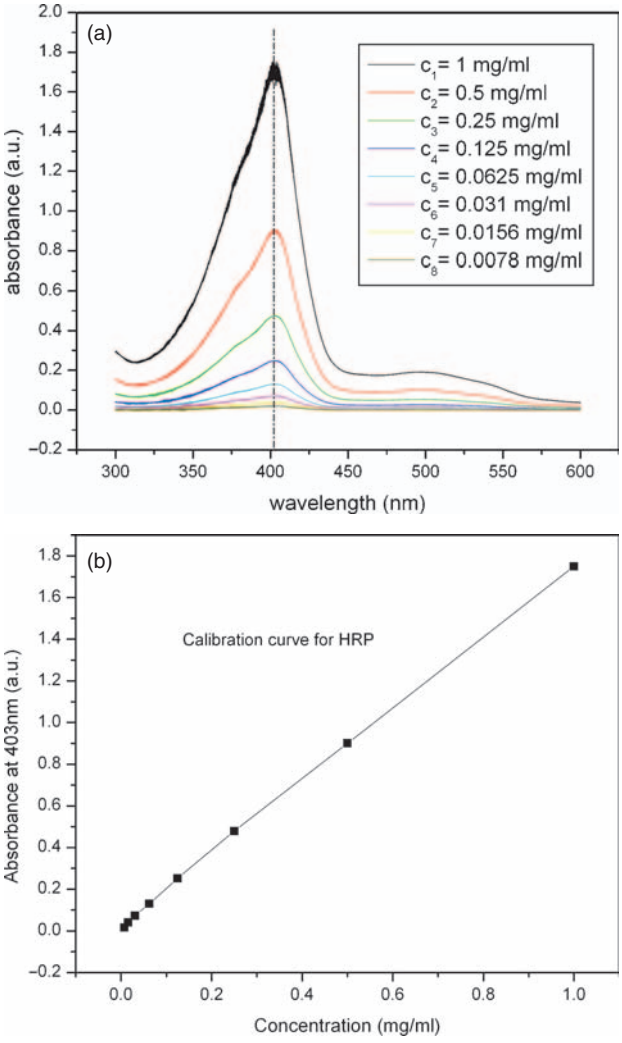

Fig. 2. (a) UV spectra of the solution with a known concentration of HRP and (b) graph of the linear relationship between HRP concentration (mg/ml) and absorbance at 403 nm as obtained from the samples with known HRP concentration.

Table I. The preparation conditions for precipitation method for obtaining HRP-loaded PDLLA microspheres.

Batch no.	Protein in 1 ml of water	Cosolvent	PVA	Homogenizer speed (rpm)	Chloroform to water ratio
1	2 mg HRP	EtOH	30 ml 1% w/v	10 min, 21 000	1:20
2	2 mg HRP	(solvent toluol) EtOH	30 ml 1% w/v	10 min, 21 000	1:20
3	2 mg HRP	MeOH	20 ml 5% w/v	10 min, 21 000	1:13.33
4	2 mg HRP	EtOH	20 ml 5% w/v	10 min, 21 000	1:13.33
5[a]	2 mg HRP	EtOH	20 ml 5% w/v	10 min, 21 000	1:13.33

[a]The fifth experiment is done under the same preparation conditions as the fourth experiment, with only one difference that in the fifth experiment dropwise addition of PDLLA-HRP loaded doispersion in PVA solution is done using homogenizer (without use of magnetic stirrer).

2.5. Stereological Analysis

The mean diameter and the size distribution of the particles were measured by stereological analysis.[28] About 200–300 particles in the SEM were measured and the following parameters were determined: area section A_A, perimeter L_p, maximal diameter of the particle D_{max}, feret X, feret Y, and form factor (FL). The particles size was examined by semi-automatic image analyzer (Leica Q500MC with Leica QWin software).

2.6. Differential Scanning Calorimetry

Blank PDLLA spheres and PDLLA spheres with encapsulated HRP were analyzed using differential scanning calorimetry (DSC-2, Perkin Elmer Pyris 6). A total of 5–10 mg of sample was weighted and placed into a sealed aluminum pan. The sample was purged with 40 ml/min of dry nitrogen and heated from 0 °C to 140 °C at a rate of 10 °C/min (depending on the melting temperature of the sample) and thermograms were registered (1st scan). The samples were held at the end temperature for 2 min, and after that cooled to −10 °C at the rate of 10 °C/min. After this cooling the samples were re-heated and the new thermograms were registrated (2nd scan).

2.7. Protein stability

The structural integrity of proteins extracted from spheres was characterized by sodium dodecyl sulfate poly(acryl amide) gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out in the presence of 0.1% SDS using a 9% slab gel prepared by a gel casting and electrophoresis unit (Mini-Protean® electrophoresis system; Bio-Rad, Hercules, CA). Protein samples and standards were treated with SDS-PAGE sample buffer containing SDS and dihiothreitol for 3 min at 95 °C, and electrophoresis was performed at a constant voltage of 120 V. Protein bands on the gel were stained with Coomassie Brilliant Blue.

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3. RESULTS AND DISCUSSION

3.1. Percentage of Yield in Preparation, Loading Efficiency and Loading Amount

The modified precipitation method, recently applied for obtaining PLGA[28] and PDLLA[26] particles was used for obtaining HRP-loaded PDLLA spheres with various properties. For the encapsulation of proteins (HRP) into polymer spheres it is better to use PDLLA[26] than PLLA,[29] because of shorter period of degradation. Copolymers of L-lactide with D,L-lactide are synthesized to reduce the degree of crystallinity of L-lactides and to fasten that way the process of degradation.

The results of the determination of the PDLLA/HRP particle yield were similar for each of the samples and in all cases greater than 38.1%.

Table II. Loading efficiency and loading amount of HRP-loaded PDLLA particles.

PDLLA/HRP %	Supernatant absorbance (403 nm)	Amount of HRP in supernatant (mg)	Loading efficiency (%)	Loading amount (mg)
97.5/2.5	0.0597	0.54	46	0.46

The loading efficiency and the physicochemical properties of spheres depend on the interactions between polymer, drug and solvent. The characteristic of the modified precipitation method consists of the combination of two water miscible organic solvents, chloroform and alcohol (methanol or ethanol). Chloroform has higher affinity to PDLLA than to PVA and alcohol has higher affinity to PVA than to PDLLA. Alcohol is poor solvent for PDLLA and alcohol accelerates the phase separation rate of PDLLA during the dispersion process.

The activity of proteins present in the polymer particle was confirmed, and also was calculated the loading efficiency and loading amount (Table II) of particles with optimal characteristics (Batch 4 Table I). The loading efficiency of HRP was determined using standard curve (Fig. 2(b)) of data obtained by assaying known concentrations of HRP solutions. The amount of HRP in the supernatant has been calculated from the product of the supernatant's absorbance at 403 nm (Fig. 3) and its measured volume (25.5 ml). The results are shown in Table II. Assuming that all of the HRP concentration not found in the supernatant was encapsulated by PDLLA spheres, the loading efficiency was determined. The loading efficiency of HRP in PDLLA spheres is 46%. One of the main reasons why there appears a difference between theoretical and experimental values of enzyme loading are usually obtained due to enzyme loss during the encapsulation process which, mainly, results from enzyme adsorption to the vessel.[19] Many authors showed that the presence of PVA can influence the drug encapsulation efficiency and release profiles of proteins from micro and nanospheres.[30]

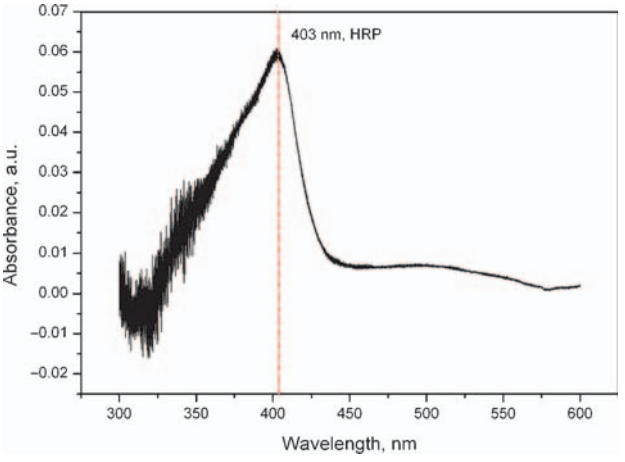

Fig. 3. UV spectra of HRP from the supernatant.

The drug encapsulation efficiency increased with increasing PVA concentrations in the continuous phase. Higher viscosity achieved by increasing PVA concentrations could minimize diffusion of protein from the disperse phase to continuous phase during the fabrication of microspheres.[30]

Gander et al. have stated a hypothesis that for the efficient encapsulation of medicines in the polymer, interactions type medicine-polymer must dominate over interactions medicine-solvent or polymer-solvent.[14] The encapsulation efficiency of the medicine presents one of the crucial criteria for the optimal technique of microencapsulation. Gander et al. have shown that encapsulation efficiency depends much on the solvent which is used. Acetone and dichloromethane have themselves as good solvents, with the encapsulation efficiency of 65% and 75% (w/v), whereas toluene, nitroethane and dimetilcarbonate result in medium efficiency of 41%–58%. In our experiments chloroform was used as a solvent, which has showed itself as a good solvent whereas toluene, because of immediate diffusion of proteins from the polymer matrix didn't show itself desirable. Facts so far show that for the majority of systems based on solvents, the erosion of the matrix is inversely proportional compared with the encapsulation efficiency. The systems of the solvents which have the most intense encapsulation efficiency incline to the least erosion from the matrix which is one of the inevitable conditions for efficient incorporation of proteins in the polymer matrix.

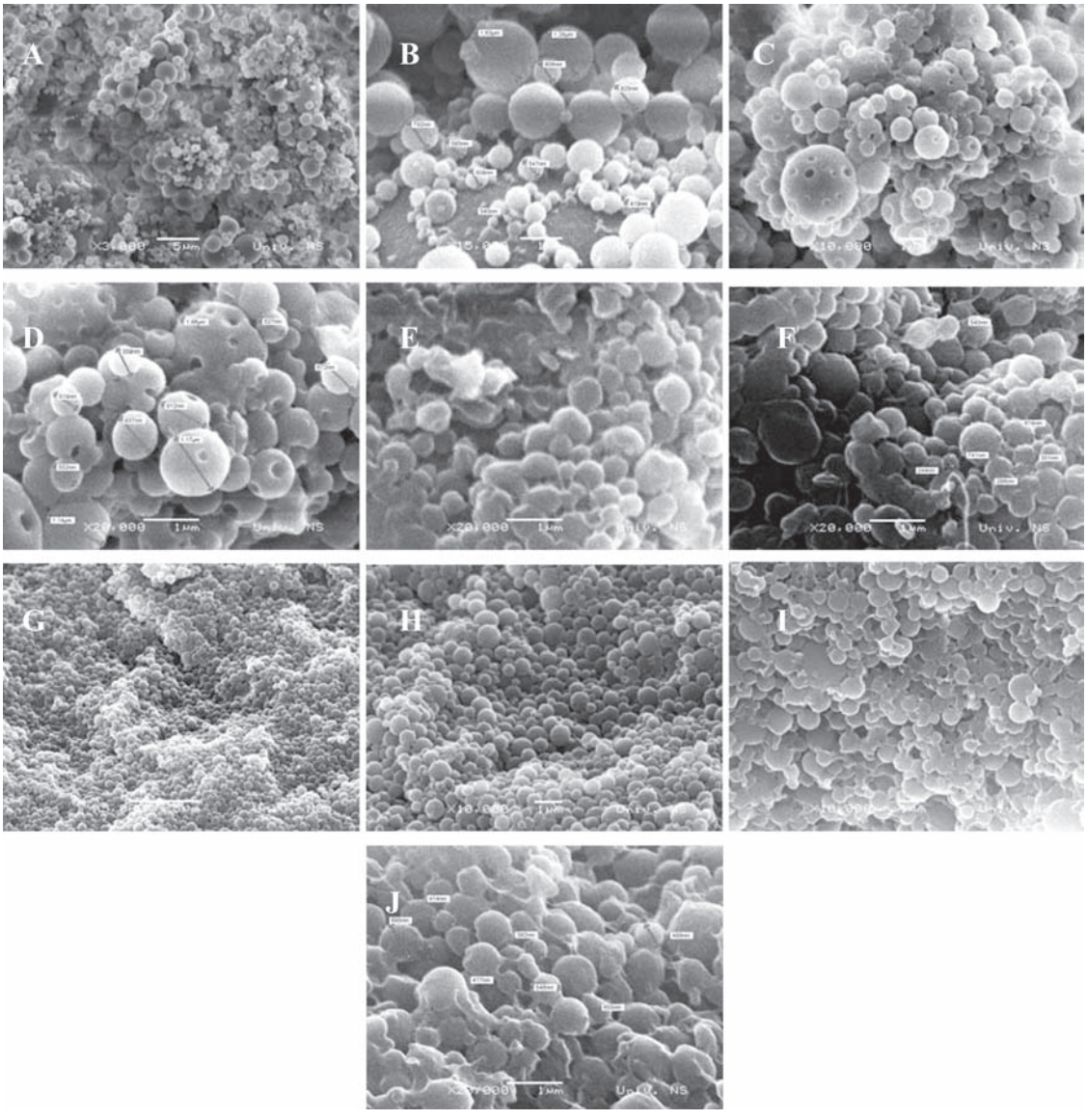

Fig. 4. SEM images of HRP-loaded PDLLA spheres prepared by precipitation method. The size of the bar is shown on the images. Images A and B—the first batch, images C and D—the second batch, images E and F—the third batch, images G and H—the fourth batch, images I and J—the fifth batch. All batches are listed in Table I.

3.2. Morphology Studies

On the basis of SEM and FESEM images of the obtained powders (Figs. 4 and 5), it can be seen that by incorporating proteins HRP in the PDLLA spheres, there was no achievement of significant changes in the morphological characteristics of suitable PDLLA particles. The size of the PDLLA spheres in which the protein is incorporated is smaller compared with the PDLLA particles without proteins, obtained in our previously experiments.[26] The

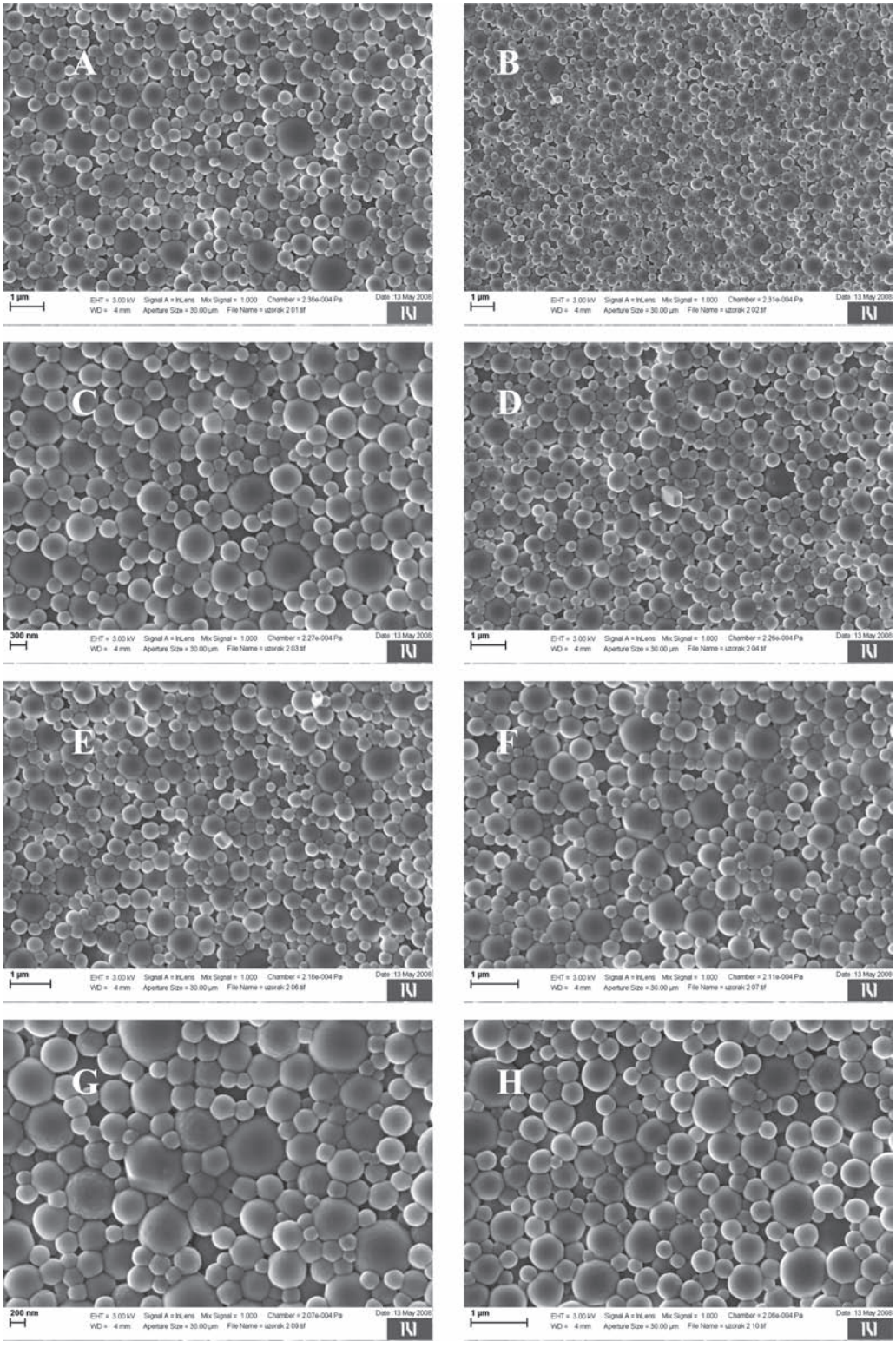

Fig. 5. FESEM images of HRP-loaded PDLLA spheres from the fourth batch (Table I) prepared by precipitation method.

explanation is found in the optimization of all conditions (chloroform to water ratio, speed and time of homogenization, concentration of surfactant) under which the spheres of pure PDLLA were processed. Based on the results obtained from the particles of PDLLA powder without proteins there is noticed an inclination of behavior of the particles under different conditions of sphere processing. There were performed additional modifications of processing parameters (the volume of water is increased to 30 ml or the concentration of PVA is increased up to 5%) with the aim to obtain even smaller and more uniform PDLLA spheres in which the protein is incorporated. In all the experiments there was performed the homogenization 10 min on 21000 rpm.

From the SEM images of PDLLA powders in which is incorporated HRP (Fig. 4) it can be seen that modified precipitation method which is conducted under above mentioned conditions (Table I) as a result gives spheres with the mean diameter in the range of 460–1130 nm (Table III) and very narrow distribution of sizes. Particles are very uniform and non-agglomerated. The SEM images of HRP-loaded PDLLA particles prepared under different preparation conditions (such as co-solvent selection, PVA concentration, chloroform to water ratio) are presented in Figure 4: A, B, C, D, G, H, I and J represent batches 1, 2, 4 and 5 prepared with ethanol at 10 min 21 000 rpm, with 30 ml 1% PVA w/v (images A–D) and 20 ml 5% PVA w/v (images G–J). Images E and F represent batch 3 prepared with methanol and 20 ml 5% PVA w/v. All batches are listed in Table I. Images presented in Figure 4 are representative from at least three preparations.

From the SEM images, we can see that all HRP-loaded PDLLA particles are spherical with smooth surface, except the particles obtained with toluol as solvent (Fig. 4, images C and D). Particles obtained with toluol as solvent have small holes on the surface. Our explanation for this phenomenon is as follows: protein from the microspheres is removed by fast diffusion to toluol, which can affect the small pores on the surfaces of the polymeric spheres to be formed (Fig. 4, images C and D). Porous spheres have large surface area and hence have high initial burst. On the basis of the results it can be concluded that in our experiments toluol has not shown as a desirable solvent.

As shown in Figure 4, no matter what kind of co-solvent was used, spherical HRP-loaded PDLLA particles can be obtained. However, the size of HRP-loaded PDLLA spheres showed dependence on the initial co-solvents. When MeOH was used as initial co-solvent, particle size was relatively smaller than those of ethanol. In addition, particles obtained with ethanol have more spherical shape than those obtained with methanol. The different sizes of HRP-loaded PDLLA spheres prepared from different initial co-solvents might be caused by the differences in physicochemical properties between polymer and co-solvents such as solubity and viscosity of the polymer to the co-solvent and miscibility of co-solvent and water.[13] The average diameter of the particles prepared by modified precipitation method is smaller with methanol (Fig. 4, images E and F) then ethanol (Fig. 4, images A–D and images G–J). Methanol caused spheres to become slightly irregular. This observation was attributed to the higher water solubility of methanol, which caused those microparticles to be formed faster.[26]

During the emulsion formation, the droplets get smaller and smaller under the strong shear stress, while the droplets tend to coalesce again to reduce their surface energy. The presence of surfactant molecules can stabilize the emulsion by forming a protective layer around droplets thus impending droplet coalescence and coagulation.[31] Important properties of stabilizing agent for optimal stabilization of the droplets during microencapsulation are
(a) high surface activity,
(b) a high viscosity in the external phase,
(c) an adequate electrical charge, and
(d) the existence of a film adsorbed on the droplet surface.

A wide range of substances, such as PVA, methyl cellulose, sorbitan monooleate (spans), sodium alginate, gelatin, and sodium dodecyl sulfate, have been used for the stabilization of polymeric particles produced by precipitation method. In this study, PVA was used as stabilizing agent and emulsifying agent. By increasing the concentration of PVA, the mean particle size of microspheres decreased (Fig. 4 and Table III). When the concentration of PVA was varied from 1% w/vol to 5% w/vol particle size decreased from 1130 nm to 530 nm (Table III). The effect was probably mainly due to the increasing viscosity of the PVA solution.

Table III. Results of the stereological analysis of HRP-loaded PDLLA microspheres.

Powder HRP-loaded PDLLA (batch)	L_p (μm)			D_{max} (μm)			Feret X (μm)			Feret Y (μm)			Perimeter form factor		
	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean
1	1.42	9.85	3.78 ± 1.74	0.39	2.83	1.13 ± 0.52	0.24	1.98	0.78 ± 0.34	0.30	2.23	0.80 ± 0.40	0.63	1.00	0.88 ± 0.08
2	1.65	10.97	3.36 ± 1.50	0.43	2.92	0.96 ± 0.41	0.29	1.88	0.72 ± 0.27	0.25	2.38	0.62 ± 0.32	0.63	0.94	0.81 ± 0.05
3	0.63	3.18	1.62 ± 0.45	0.14	0.94	0.46 ± 0.14	0.08	0.60	0.30 ± 0.09	0.11	0.72	0.35 ± 0.11	0.58	0.88	0.77 ± 0.04
4	0.67	3.93	1.83 ± 0.62	0.18	1.03	0.53 ± 0.17	0.12	0.82	0.36 ± 0.12	0.10	0.81	0.36 ± 0.13	0.63	1.00	0.78 ± 0.05
5	0.90	3.79	2.05 ± 0.62	0.25	1.29	0.59 ± 0.18	0.15	0.92	0.42 ± 0.13	0.15	0.90	0.41 ± 0.13	0.71	0.95	0.81 ± 0.04

From the SEM images, we can see that incorporation of proteins (HRP) in PDLLA spheres hasn't changed dramatically morphological characteristics of the particles. No drug crystals outside the particles could be seen in the SEM images. The particles with the best properties (spherical, non agglomerated, most uniform) are obtained in batch 4. (Table I, Fig. 4 images G and H and Fig. 5 images A–H) with ethanol as co-solvent, 5% PVA, 1:13.33 chloroform to water ratio and 10 min of homogenization on 21,000 rpm.

3.3. Stereological Analysis

By stereological analysis, the mean diameter and the size distribution of the particles were determined (Figs. 6, 7 and Table III). Based on the obtained results of the stereological analysis of HRP loaded PDLLA particles, it is visible that the average mean size of the particles from the first batch, varies from 0.24 to 2.83 μm depending on the stereological parameter taken in consideration (feret X, feret Y or D_{max}). (Table III). Feret X values range from 0.24 to 1.98 μm with its mean size 0.78 μm. The average mean size of the particles from the second batch varies from 0.25 to 2.92 μm. Feret X values range from 0.29 to 1.88 μm with its mean size 0.72 μm. The average mean size of the particles from the third batch varies from 0.08 to 0.94 μm and feret X values range from 0.08 to 0.60 μm with its mean size 0.30 μm. The average mean size of the particles from the fourth batch varies from 0.10 to 1.03 μm and feret X values range from 0.12 to 0.82 μm with its mean size 0.36 μm. The average mean size of the particles from the fifth batch varies from 0.15 to 1.29 μm. Feret X values range from 0.15 to 0.92 μm with its mean size 0.42 μm.

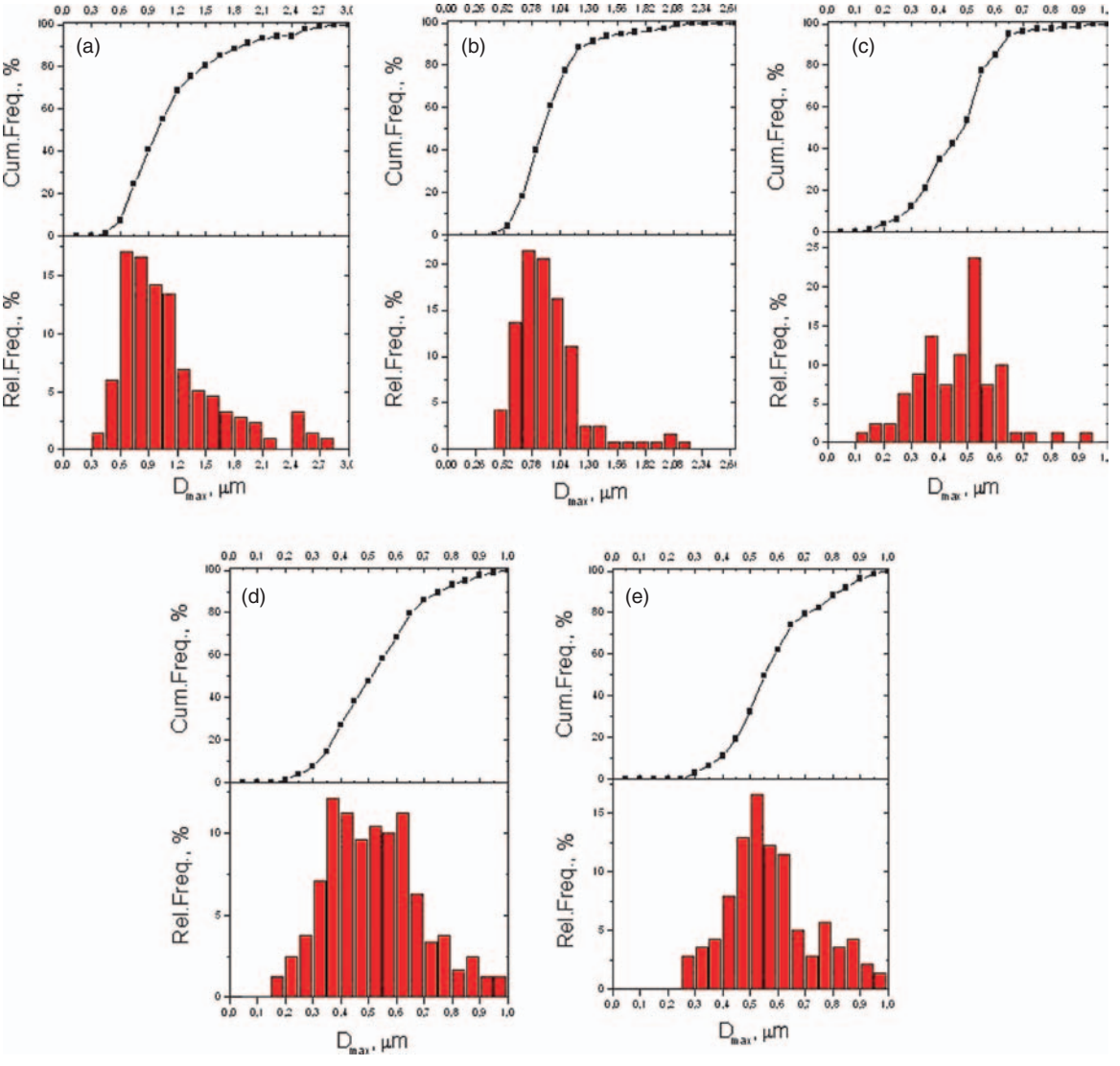

Fig. 6. Stereological analysis of PDLLA-HRP spheres prepared by precipitation method, based on D_{max}. Batch 1 (a), batch 2 (b), batch 3 (c), batch 4 (d) and batch 5 (e). All batches are numbered as listed in Table I.

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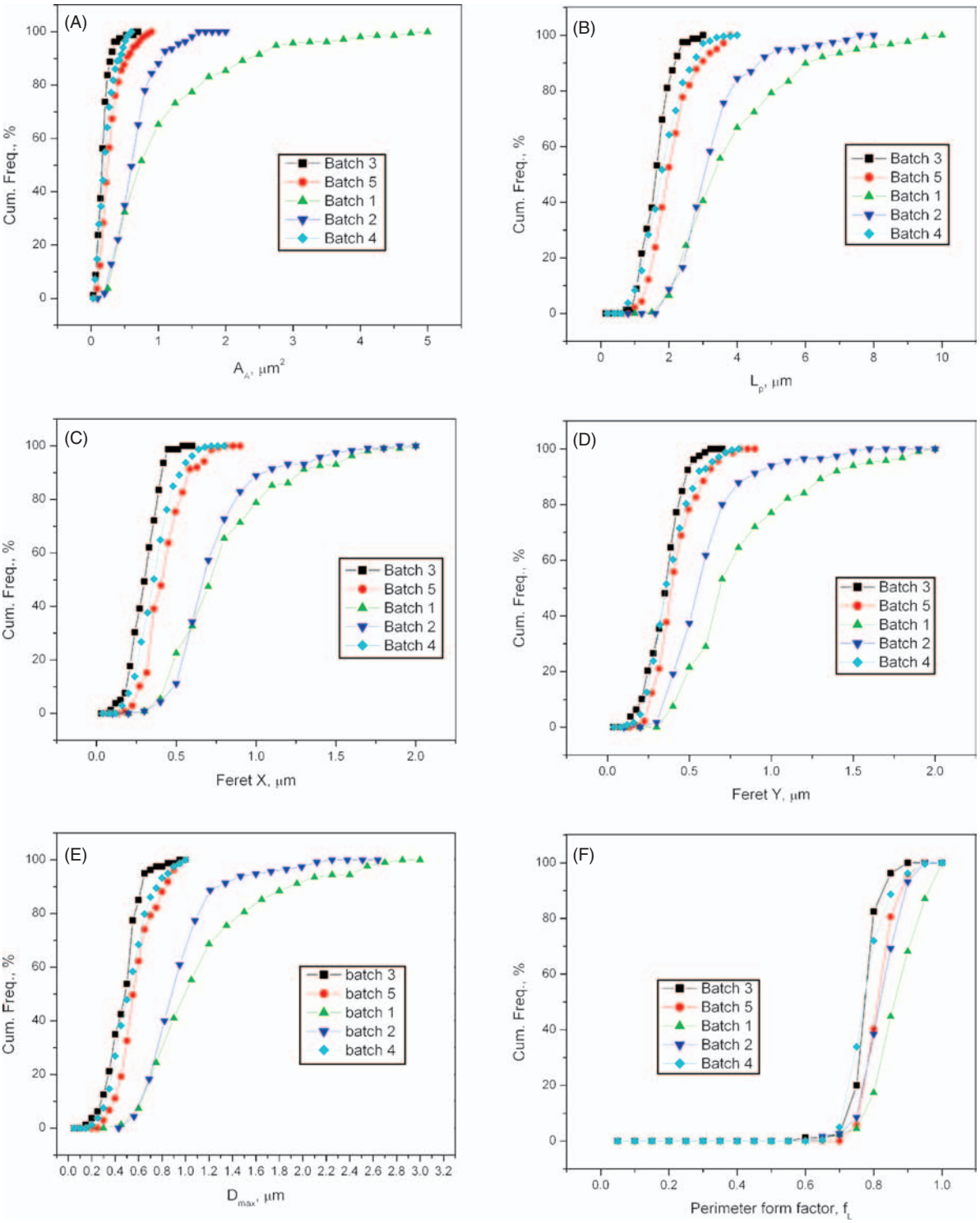

Fig. 7. Compared results of stereological measurements of all batches of HRP-loaded PDLLA particles: A_A (A), L_p (B), Feret X (C), Feret Y (D), D_{max} (E) and fL (F), prepared by precipitation method. All batches are numbered as listed in Table I.

From the results obtained from the stereological examination we can see that the particles from the first and the second batches are larger having a size distribution with mean diameter of 1130 nm and 960 nm, respectively (Fig. 6 and Table III). The particles from the third, the fourth and the fifth batches are smaller with mean diameter of 460 nm, 530 nm and 590 nm, respectively (Fig. 6 and Table III). The Figure 7 represents compared results of the cumulative dependence of sizes of all the series in which is incorporated the protein HRP (Batches 1–5). The size of

the spheres in which the protein is incorporated, as it can be noticed from the results, depends on the concentration of the surfactant and the co-solvent selection.

As shown in Figure 6 and Table III, the size of HRP-loaded PDLLA spheres showed dependence on the initial co-solvents. When methanol was used as initial co-solvent, particle size was relatively smaller (D_{max} is 460 nm) than those obtained with ethanol (D_{max} is 530 nm).

3.4. Protein Stability

The PDLLA spheres without and with encapsulated HRP were investigated by DSC and the result is shown in Figure 8. Only one peak was observed in the thermograms. Pure PDLLA spheres showed the glass transition peak at 51.22 °C and HRP-loaded PDLLA spheres showed glass transition peak at 44.93 °C. These results demonstrated that HRP loaded in PDLLA spheres influenced the flexibility of the polymer chain. Glass transition temperature (T_g) of protein formulations is considered to be one of the major determinants of protein stability. T_g of the polymer is defined as the transition temperature between the rubbery and glassy states. Microscopically, a polymer chain may have cooperative localized motion above T_g and below T_g. The greater mobility above T_g is due to a greater free volume and higher degree of both translational and rotational freedom.[32]

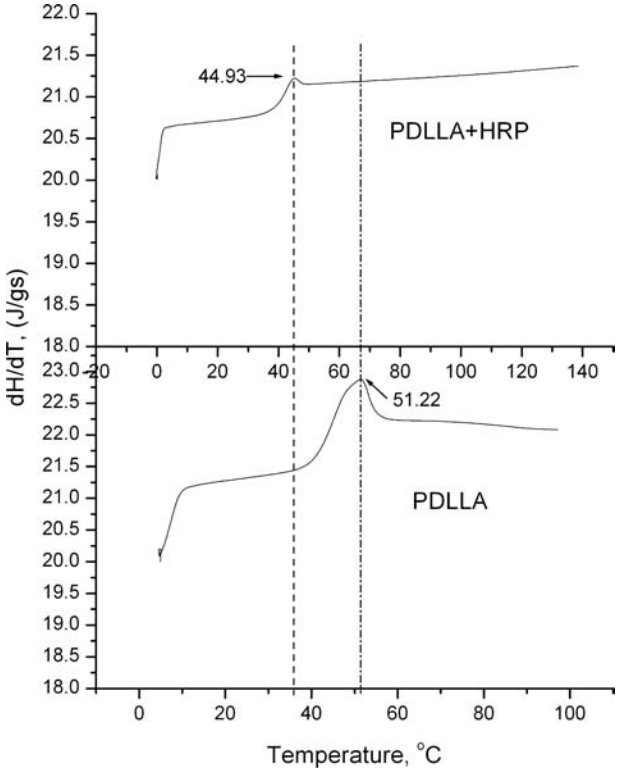

Fig. 8. DSC thermograms of PDLLA spheres and HRP-loaded PDLLA spheres.

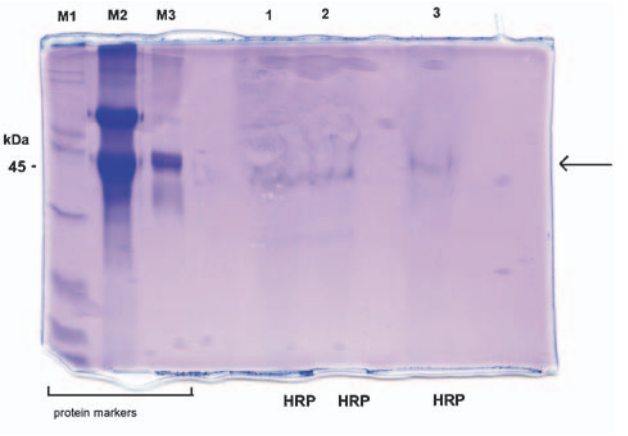

Fig. 9. SDS-PAGE of proteins extracted from HRP-loaded PDLLA microspheres for the assessment of protein stability. (Lanes M1, M2 and M3) Molecular weight standard markers (M1 (Protein molecular weight markers-Amersham 6500-20500 Da), M2 (BSA-66 kDa, Albumin-45 kDa and Carbonic Anhidraze-29 kDa), M3 (Albumin-45 kDa)). Lanes (1), (2) and (3) HRP (45 kDa) extracted from microspheres (Batch 4 Table II).

As shown by SDS-PAGE analysis in Figure 9, the proteins extracted from the HRP-loaded PDLLA microspheres showed good stability without structural integrity changes. The protein extracted from HRP-loaded PDLLA spheres showed same retention time of native HRP without the trace of protein aggregates and degradation process. These results suggest that the preparation process of the HRP-loaded PDLLA microspheres did not affect the structural integrity of proteins. The enzymatic activity changes of HRP were also examined to determine the activity loss of the proteins during the microsphere preparation process. Furthermore, it is well known that encapsulation of the enzyme increases its stability over extended periods of time, compared to the free enzyme in aqueous solutions.[19]

4. CONCLUSION

HRP-loaded PDLLA spheres were successfully prepared by the modified precipitation method that was previously applied for blank PDLLA and PLGA microspheres. HRP-loaded PDLLA particles have been successfully formulated with spherical morphology, suitable particle size, high protein encapsulation efficiency and good protein stability. To synthesize small spheres, the data indicates that is ideal to have a high PVA concentration, a low chloroform-to-water ratio, and high homogenization speed. The best results were obtained when ethanol was used as co-solvent and with 10 min of homogenization on 21000 rpm. The mean diameter of these particles is 530 nm and the encapsulation efficiency is 46%. On the basis of the results it can be concluded that in our experiments toluol has not shown as a desirable solvent. The average diameter of the particles prepared by precipitation

method is smaller when methanol was used then ethanol but methanol caused spheres to become slightly irregular.

This is a simple and inexpensive method of incorporating protein within biodegradable polymer with many potential advantages such as improved encapsulation efficiency (EE), reduced toxicity, extended the duration of drug etc. A novel delivery system could be useful for the sustained delivery of protein drugs.

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