Acta Pharm. 54 (2004) 215–229 Original research paper

Poly(lactide-co-glycolide) microparticles as systems for controlled release of proteins – Preparation and characterization

ALEKSANDRA PORJAZOSKA[1]
KATERINA GORACINOVA[2]
KRISTINA MLADENOVSKA[2]
MARIJA GLAVAŠ[2]
MAJA SIMONOVSKA[2]
EMILIJA IVANOVSKA JANJEVIĆ[3]
MAJA CVETKOVSKA[1]*

[1] *Institute of Organic Technology Faculty of Technology and Metallurgy University »Ss Cyril and Methodius« Skopje, R. Macedonia*

[2] *Institute of Pharmaceutical Technology Faculty of Pharmacy, University »Ss Cyril and Methodius« Skopje, R. Macedonia*

[3] *Institute of Nuclear Medicine and Pathophysiology, Faculty of Medicine University »Ss Cyril and Methodius« Skopje, R. Macedonia*

Poly(DL-lactide-co-glycolide) (PDLLGA) and poly(L-lactide-co-glycolide) (PLLGA) copolymers were prepared by bulk ring opening polymerization of lactide and glycolide and characterized by GPC, FTIR, ^{1}H NMR and DSC. Copolymers with different molar masses at a constant lactide/glycolide ratio were used for preparation of bovine serum albumin (BSA)-loaded microparticles by the double emulsion w/o/w method. The influence of the copolymer molar mass and composition on the microparticle morphology, size, yield, degradation rate, BSA-loading efficiency and BSA release profile were studied. For microparticles prepared from PDLLGA copolymers, a biphasic profile for BSA release was found and for those made from PLLGA copolymers the release profile was typically triphasic; both of them were characterized by high initial burst release. Possible reasons for such behavior are discussed.

Keywords: poly(lactide-co-glycolide), microparticles, protein, *in vitro* release

Received February 9, 2004
Accepted July 16, 2004

The treatments for which proteins and peptides are prescribed as therapeutic agents require stable levels of active components over prolonged periods. One way to meet these requirements are sustained release systems, generally based on biodegradable polymers, of which polylactide (PLA) and its copolymers (PLGA) with glycolide (GA) are commonly used. An advantage of the lactide/glycolide copolymers is the well documented versatility in polymer properties (via manipulation of the comonomer ratio, molar mass, polymer crystallinity) and the corresponding performance characteristics (*e.g.*, predictable *in vivo* degradation rates) (1, 2). In addition to the polymer chemistry, drug

* Correspondence, e-mail: majac@ereb1.mf.ukim.edu.mk; majac@ian.tmf.ukim.edu.mk

release rates can be controlled through variation of the microparticle formulation parameters, and thus the physical characteristics of the resulting particles (3–9). For proteins with low or negligible solubility in polymers such as PLGA, the diffusional transport through the polymer phase will be prevented. In this case, polymer degradation will play a crucial role in the mechanism of protein release (2, 10–12). Based on this finding tailoring of PLGA devices with suitable degradation properties that will allow a controlled release of high molar mass, water-soluble protein for long term therapeutic application has become an important area of research. Biodegradable microparticles have been the most studied devices due to their relatively simple fabrication and facile administration to a variety of locations *in vivo*. Several methodologies for microencapsulation of proteins and peptides have been developed. Most of them are essentially based on the phase separation technique, such as the solid-in-oil-in-water (s/o/w) method (6, 12) or emulsion-evaporation technique, such as the water-in-oil-in-water (w/o/w) method (7–9), that enable production of particles with a wide range of mean diameters.

The aim of this study was to evaluate the influence of copolymers' (PDLLGA and PLLGA) molar mass and composition on the properties of bovine serum albumin (BSA)-loaded microparticles prepared by the w/o/w emulsion technique. The copolymers were prepared by bulk ring-opening polymerization (13) and characterized by GPC, FTIR, ^{1}H NMR and DSC analyses. The influence of the copolymer molar mass and composition on the drug release rate and the rate of degradation of polymeric microparticles were also investigated.

EXPERIMENTAL

Materials

Poly(DL-lactide-co-glycolide) (PDLLGA) and poly(L-lactide-co-glycolide) (PLLGA) were synthesized by bulk ring-opening polymerization (13) and characterized.

Albumin, bovine, fraction V, min. 96% was purchased from Sigma Chemical Co. (UK). Radiolabelling of BSA was performed by ^{131}I obtained from Biointernational (France). Poly(vinyl alcohol) (PVA) (M_w 72 000 g mol^{-1}, 98%, hydrolyzed) was supplied by Merck (Germany). All solvents were of HPLC grade and were supplied by Merck.

Synthesis of poly(lactide-co-glycolide) copolymers

PLGA copolymers were prepared by ring opening polymerization of lactide (LA) and glycolide (GA) in the presence of stannous octoate as catalyst (13). A solution of stannous octoate in dry chloroform and a mixture of monomers were added to a reaction tube (molar ratio monomer to catalyst was 1000). The molar ratio of the feed DL-LA/GA was 85/15, and L-LA/GA was 75/25. The solvent was removed in vacuo, and the tube was sealed and immersed in a silicon oil bath at 115 °C. At the end of polymerization (24 h), the product was dissolved in a small amount of chloroform and precipited in an excess of methanol. Actual copolymer composition, that is the molar ratio of monomers (DL-LA/GA or L-LA/GA) in copolymers, was found from ^{1}H NMR analysis. For PDLLGA, it was 80/20, and for PLLGA, 68/32.

Copolymer characterization

The synthesized copolymers were characterized by FTIR, ^{1}H NMR, GPC and DSC.

The infrared spectra of copolymers were recorded on a Perkin Elmer 983 IR spectrometer (Perker Elmer, USA) at room temperature. The following IR peaks assigned to PLGA were recorded (ν, cm^{-1}): 2997–2965 (CH_2, CH_3), 1759 (C=O), 1360–1450 (CH_3), 750 (CH).

^{1}H NMR spectra of the copolymers were obtained on a Bruker AC 200L spectrometer (Bruker, USA) at 200 MHz, in deuterated chloroform at 20 °C. ^{1}H NMR data were as follows: ($CDCl_3$, δ, ppm): 1.56 (3H,C**H**$_3$), 4.77 (2H,C**H**$_2$), 5.18 (H, OC**H**CH_3). The composition of copolymers was calculated from the ratio of absorbances at 4.77 and 5.18 ppm.

The molar masses of copolymers (mass average, M_r and number average, M_n) were determined by gel permeation chromatography (Waters, USA) using the Waters styragel column HT6F and Waters 410 differential refractometer detector. THF was used as the eluting solvent at a flow rate of 1 mL min^{-1} and polystyrene standards were used for calibration purposes.

Thermal characterization of the copolymers was performed using a DuPont DSC 910 Model (DuPont, USA) device. The samples were scanned from –140 °C to +140 °C, at a heating rate of 10 °C min^{-1}. DSC samples were first heated under nitrogen to +140 °C, then quenched to –140 °C using liquid nitrogen. This heating/cooling cycle was repeated twice. The data were analyzed from the second heating run.

The main characteristics of the prepared copolymers are presented in Table I.

Table I. Main characteristics of copolymers

Copolymer	Feed ratio of LA/GA (mol/mol)	Composition on the basis of ^{1}H NMR (mol/mol)	M_w	*PDI*	T_g (°C)
PDLLGA					40–41
I	85/15	81/19	21 850	1.65	
II		80/20	44 700	1.68	
III		80/20	83 800	2.94	
PLLGA					47
1	75/25	68/32	44 050	2.30	
2		68/32	88 000	1.95	

M_w – weight average molar mass; *PDI* – polydispersity or heterogeneity index ($PDI = M_w/M_n$, where M_n is the number average molar mass); T_g – glass transition temperature

Preparation of PLGA microparticles containing bovine serum albumin

Microparticles were prepared using the w/o/w emulsion technique (14, 15) according to the following procedure. PLGA (0.05 g) was dissolved at room temperature in 1 mL of dichloromethane (DCM) and cooled at 4 °C. Aqueous solution of ^{131}I-BSA (2%, *m/V*, 0.5 mL) was sonicated into 1 mL solution of PLGA in DCM (5%, *m/V*) for 15 min to obtain the first (w/o) emulsion, which was then vortexed into 10 mL of cooled (4 °C) aqueous PVA solution (2% *m/V*) to prepare the w/o/w emulsion. Stirring for 3 h at 600 rpm

was applied to remove the solvent. The isolated microparticles were successively washed with deionized water, collected by filtration (Sartorius Type 16692, Sartorius, Germany) and freeze-dried (200 Pa, –40 °C, Christ α 2–4, Bioblock Scientific, France). Microparticles without BSA were referred to as »blank« ones.

Microparticle characterization

Size distribution analysis. – The mean geometric diameter and size distribution of the population of BSA-loaded PLGA microparticles (before and after freeze-drying) dispersed in doubly-distilled water were determined using laser diffractometry (Particle size analysette DLAB/22, Fritsch, Germany). Three independent analyses consisting of 100 repetitive measurements were performed.

Morphology studies. – Microparticles were placed on metal grids using double-sided tape and coated with gold (thickness 2 nm) under vacuum (BAL-TEC MED 020 Coating system, Balzers, Liechtenstein). The shape and surface characteristics of blank and BSA-loaded PLGA microparticles were determined by scanning electron microscopy (Hitachi S-450, Hitachi, Japan).

Yield and BSA-loading determination. – The microparticle yield was determined as ratio between the mass of isolated, freeze-dried microparticles to the total initial mass of the polymer and BSA. The content of radiolabelled ^{131}I-BSA entrapped in PLGA microparticles was calculated as the percent of total radioactivity in the supernatant and in the filtrate after microparticles isolation (»well« counter Scaler Type N529 D, EKCO Electronics, UK) (16, 17).

In vitro *degradation of PLGA microparticles.* – Dispersion of blank microparticles (1.5 mg mL^{-1}) was used to assess the *in vitro* degradation rate during incubation in phosphate-buffered saline (PBS, pH 7.4, 37 °C, 75 strike min^{-1}, horizontal shaker; Haake SWB 20, Fisons, Germany). At regular time intervals, the dispersion was centrifuged at 8000 rpm for 30 min (Jouan MR 22i, Jouan S. A., Centrifuge, France), the total mass of microparticles isolated and freeze-dried to a constant mass was determined and the percentage of remaining microparticles was calculated.

In vitro *release of BSA from PLGA microparticles.* – *In vitro* release studies were performed by suspending the microparticles in isotonic PBS (mg microparticles per mL buffer: 1.5–2.0 mg mL^{-1}) and by simulating the *in vivo* conditions as in biodegradation studies. At each time interval, the ^{131}I-BSA release was determined as the percent of total radioactivity present in the microparticles and the supernatant. In order to determine the initial and the last time release, the *in vitro* release profiles were described using the exponential function: $M_\infty - M_t/M = Ae^{-\alpha t} + Be^{-\beta t} + \ldots$, where M_∞ is the total amount of BSA released, M and M_t is the amount of BSA at zero time and the amount of BSA released at time t, A and B are system-characteristic constants, α and β are rate constants for the initial and later time release, obtained by semilogarithmic plots (16–18).

RESULTS AND DISCUSSION

Copolymers of DL- or L-lactide and glycolide with different molar masses (M_w from 21 000 to 88 000 g mol^{-1}) and different content of glycolide (20 to 32%) (Table I) were synthesized, characterized, and used for preparation of blank and BSA-loaded microparticles by the w/o/w method.

Microparticles were used to study the influence of the copolymer molar mass and composition on the microparticle yield, their morphology, size and size distribution, as well as on the BSA-loading efficiency. The data are given in Table II. The influence of the copolymers and microparticles characteristics on the degradation rate and the rate of BSA release were also studied.

Microparticle yield, morphology, size distribution and BSA-loading efficiency

In the case of all copolymers (I–III and 1–2, Table II), the blank microparticles were spherical and non-porous *vs*. the porous structure of loaded microparticles. Representative scanning electron micrographs are given in Figs. 1a–d.

Table II. Main data for blank and loaded microparticles

Copolymer	PLGA (mg)	BSA (mg)	Yield (%)	$d_{geo} \pm SD$ (μm)[c]	BSA found in microparticles (mg)	Loading efficiency (%, *m*/*m*)	$d_{geo} \pm SD$ (μm)[d]
PDLLGA[a]							
M_w							
21 850 (I)	50	5	53.3	11.47 ± 1.25	0.425	8.5	16.01 ± 1.30
44 700 (II)	50	5	51.0	12.89 ± 1.43	0.440	8.8	17.91 ± 1.69
83 800 (III)	50	5	53.1	12.28 ± 1.55	0.635	12.7	19.37 ± 2.36
PLLGA[b]							
M_w							
44 050 (1)	50	5	58.6	10.0 ± 1.33	0.890	17.8	19.35 ± 1.97
88 000 (2)	50	5	44.1	25.51 ± 1.67	1.635	32.7	70.45 ± 2.56

Lactide/glycolide molar ratio: [a] (80/20 mol/mol), [b] (68/32 mol/mol).
Mean geometric diameter n = 3 of: [c] blank microparticles, [d] loaded microparticles.

It was found that there were no significant differences in the particle size distribution of blank and loaded microparticles prepared from copolymers with different molar masses when the DL-LA/GA molar ratio was 80/20. Thus, the size of microparticles expressed as the mean geometric diameter (± SD, n = 3) ranged from 11.47 ± 1.25 μm to 12.28 ± 1.55 μm for blank, and from 16.01 ± 1.30 μm to 19.37 ± 2.36 μm for loaded microparticles (Table II). This was not the case of PLLGA (with L-LA and molar ratio of L-LA/GA = 68/32) microparticles; with the increase of the molar mass, the diameter rose from 10.0 ± 1.33 μm to 25.51 ± 1.67 μm for blank, and from 19.35 ± 1.97 μm to 70.45 ± 2.56 μm for loaded microparticles (Table II).

Influence of the copolymer composition on particle size distribution was observed in the copolymers with the highest M_w. Thus, for copolymers with comparable M_w (~ 80 000 g mol^{-1}) (copolymers III and 2, Table II), the mean geometric diameter for microparticles of 68/32 L-LA/GA copolymer was 70.45 μm *vs*. 19.37 μm for microparticles of 80/20 DL-LA/GA copolymer.

The microparticles yield was similar for all series of PLGA microparticles and nearly 50% of the total initial mass. However, regarding the drug entrapment, a higher loading efficiency was observed in the series with higher M_w and a higher content of GA (Table II).

A possible explanation for the increase of the microparticle size as well as the loading efficiency with the increase of the copolymers molar mass and the content of GA

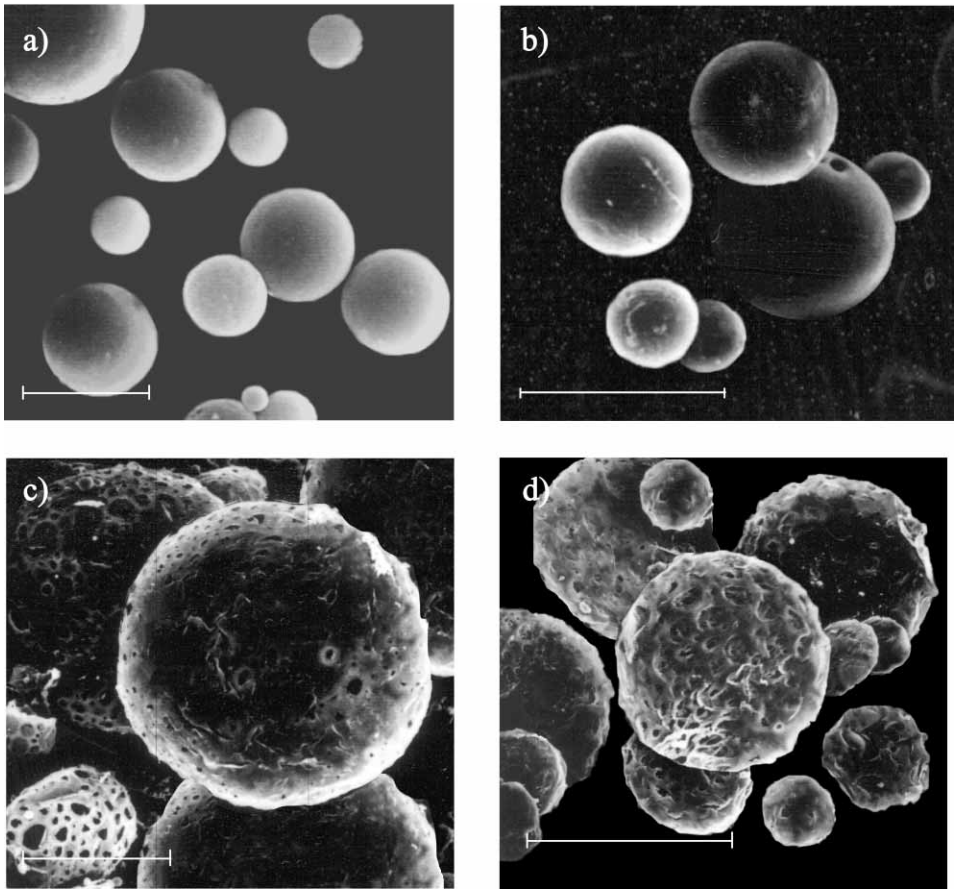

Fig. 1. SEM of blank PLGA microparticles: a) copolymer II and b) copolymer 2 and BSA-loaded PLGA microparticles: c) copolymer II and d) copolymer 2. a) and c): bar stands for 10 μm; b) and d): bar stands for 50 μm). (For microparticles see also Table II).

could be as follows. First, the copolymers with a higher molar mass (III and 2, Table II) provide a more viscous solution. Emulsification of a solution of high viscosity is more difficult and leads to a larger size of microparticles prepared by the w/o/w solvent evaporation procedure. Higher viscosity may result in a faster microsphere hardening as well, and thus in a more difficult diffusion of BSA out of the microsphere into the outer water phase. Second, PLLGA has a higher content of the hydrophilic segment in the backbone, which may interact with BSA and prevent BSA from diffusing out, resulting in higher encapsulation efficiency. A similar explanation was proposed by Yang *et al*. (4).

Degradation rate of microparticles/polymers

Typical crystallinities for polyglycolide (PGA) are 46–52% (T_g = 36 °C), while for poly(L-lactide) (PLLA) the crystallinity is around 37% (T_g = 57 °C) (19). Poly(DL-lactide) (PDLLA) is amorphous. It was found (19) that the range of compositions from 25–70% molar ratio of GA results in amorphous L-LA/GA copolymers. In the case of DL-LA in copolymer, the amorphous region extends from 0–70% GA (19). It means that all our samples are amorphous. In this case, water uptake, which is crucial for polymer degradation, will depend on the content of hydrophilic GA in the copolymer and on its molar mass. The influence of particle size, surface available for hydrolytic degradation and porosity on the biodegradation rate has also to be taken in consideration.

The results represented in Figs. 2a,b show that a more rapid mass loss during the *in vitro* degradation study occurred for the microparticles prepared from copolymers with lower molar masses (copolymers I and 1). For the PDLLGA copolymers with the same lactide/glycolide ratio (80/20), the polymer with the lowest molar mass of 21 850 g mol^{-1} degraded most rapidly (Fig. 2a). Similarly in PLLGA copolymers (68/32), the polymer with the lower molar mass of 44 050 g mol^{-1} degraded more rapidly than the polymer with a higher molar mass of 88 000 g mol^{-1} (Fig. 2b). For example, after 60 days, the total mass of PLLGA microparticles with M_w 88 000 g mol^{-1}, was reduced by 6.2%, while the PLLGA microparticles with M_w 44 050 g mol^{-1} showed a 34% mass reduction.

Influence of the difference in particles size on the degradation rate could also be expected. However, comparing the degradation of PDLLGA and PLLGA microparticles with similar diameters and composed of polymers with similar M_w of 44 000 g mol^{-1}, but with a different lactide/glycolide ratio (copolymers II and 1, Table II), it seems that the influence of the content of GA predominates. Microparticles prepared from polymers with a higher glycolide content showed a more rapid mass loss. For example, during 60 days there was an 11% reduction in microparticle mass for PDLLGA microparticles (copolymer II; LA/GA = 80/20) and as high as 34% reduction in mass for PLLGA microparticles (copolymer 1; LA/GA = 68/32). Another reason for such behavior could be a higher heterogeneity or polydispersity index, *PDI* (*PDI* = M_w/M_n) for copolymer 1 (2.30) compared to copolymer II (1.68). It could be expected that the faster degradation of the lower molar mass fraction, present in copolymer 1, increases the local acidity, thereby accelerating the hydrolysis of higher molar mass species.

During the early stages of the degradation of copolymers, there was a period of little or no mass loss, especially for copolymers with higher molar masses. For example, practically no degradation for the PDLLGA copolymer with M_w 83 800 g mol^{-1} (copolymer III, Table II) was noticed in the course of 60 days, while for PLLGA copolymer with

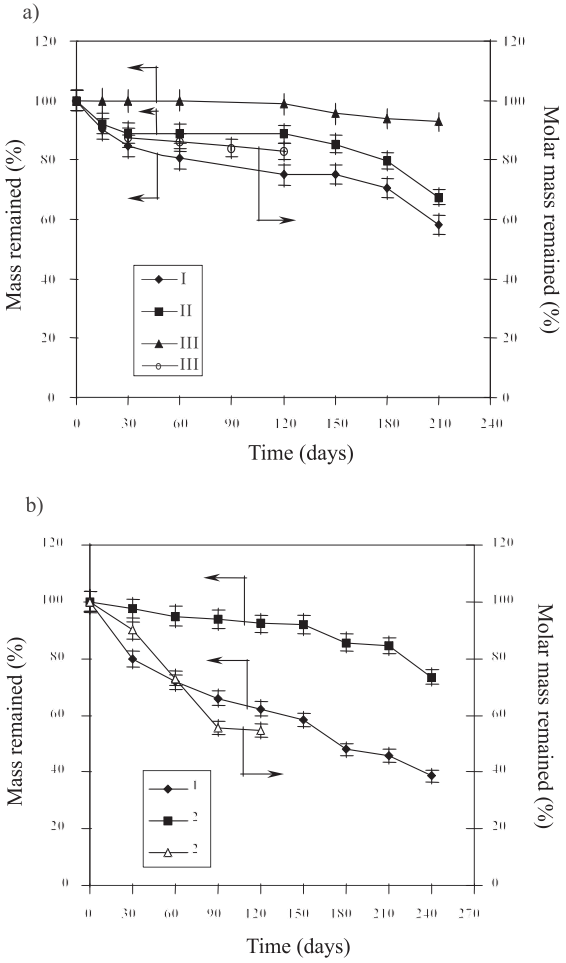

Fig. 2. Mass reduction of different batches of blank freeze-dried microparticles: a) PDLLGA (copolymers I–III, Table II) and b) PLLGA (copolymers 1–2), with time as a result of polymer degradation. SD bars for (n = 3) are given.

M_w 88 000 g mol^{-1} (copolymer 2, Table II) there was only a 6% reduction in microparticle mass. It means that only a decrease of the molar mass can be expected in this period which was confirmed by GPC measurements (Figs. 2a and b).

Deterioration in microparticle surface morphology

With the increase of the incubation time, the deterioration in the microparticle surface morphology of blank microparticles became apparent under SEM (Figs. 3a–f). Smooth and spherical microparticles from the beginning became less spherical as the study progressed. The rate of deterioration of the microparticles appearance was dependent on the rate of polymer degradation, since microparticles prepared from higher molecular mass copolymers exhibited surface deterioration at a much slower rate.

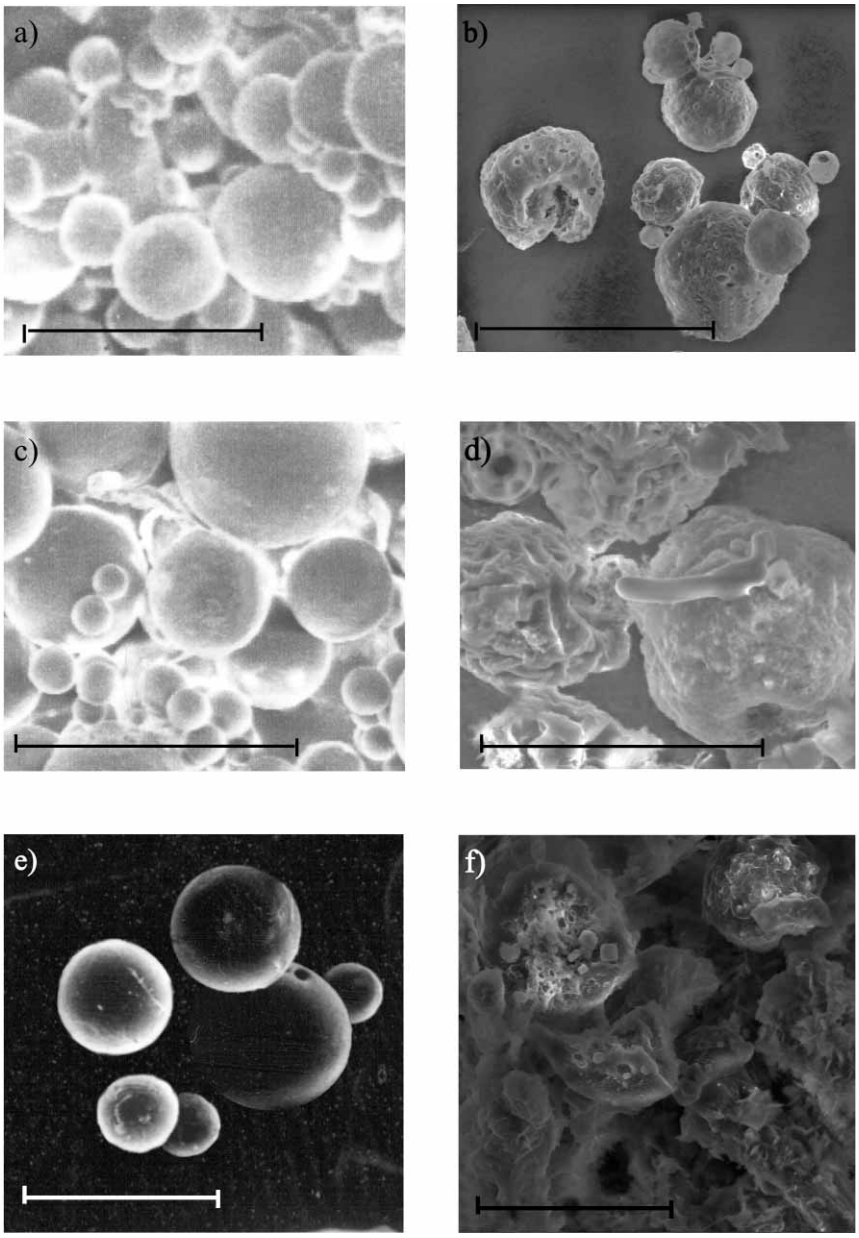

Fig. 3. SEM of PDLLGA microparticles (with copolymer III): a) non-degraded, and b) after 7 months of degradation; PLLGA microparticles (with copolymer 1): c) non-degraded and d) after 7 months of degradation, and PLLGA microparticles (with copolymer 2): e) non-degraded and f) after 12 months of degradation (bars stand for 50 μm). (For microparticles see also Table II).

In vitro *release of BSA from microparticles*

Due to the large molecular size of the proteins and their insolubility in polymers, the rate of BSA release from PLGA microparticles is thought to depend mainly on the rate of polymer degradation and BSA diffusion through the microparticle pores. For all batches of the microparticles studied, there was an initial »burst« release of BSA during the first hour of the study. This burst release probably represented the release of poorly entrapped and surface-associated BSA. For the microparticles prepared from the PLLGA (68/32) copolymers, the BSA release showed a typical triphasic release profile (Fig. 4a), in which the initial release of BSA from microparticles prepared from copolymers with the higher molar mass of 88 000 g mol^{-1} (larger mean particle size) was considerably lower (~ 18% during 0.5 hours) than that for the 44 050 g mol^{-1} polymer (~ 52% in the

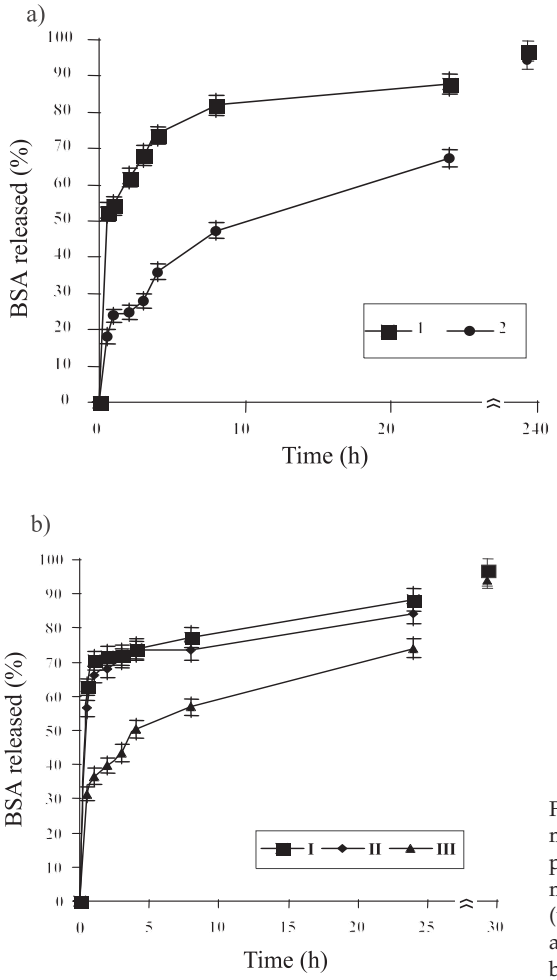

Fig. 4. The effect of the copolymer molar mass on the rate of release of BSA entrapped: a) PLLGA microparticles (with copolymers 1–2) and b) in PDLLGA microparticles (with copolymers I–III). SD bars for n = 3 are given. (For microparticles see also Table II).

same period) (Table III). The same was true for the PDLLGA (80/20) particles, which showed a biphasic release profile (Fig. 4b) where the initial burst of BSA from microparticles prepared from copolymers with lower molar masses of 21 850 and 44 700 g mol^{-1} was considerably higher than that for the 83 800 g mol^{-1} copolymer. Namely, between 31 and 63% of BSA was released in the initial period of 0.5 hours, depending on the copolymer molar mass and/or particle size and porosity (Table III).

Lower initial release from microparticles prepared from polymers with higher M_w, or with a higher content of GA could be correlated with the already discussed influence of the viscosity of the polymer solutions (faster hardening of microparticles with a more compact core) or interactions of the hydrophilic segments with BSA during the encapsulation which prevents the BSA from diffusing out. Thus the microparticles with a higher diameter or prepared from polymers with higher M_w or a higher content of hydrophilic component will show a lower burst effect and slower release.

After the initial burst release from PLLGA (68/32) microparticles, there was a lag phase of low release, followed by a phase of constant BSA release in which, during 25 hours, 87% (copolymer 1) or 65% (copolymer 2) BSA was released (Fig. 4a). In 10 days the BSA was released completely (data not shown graphically) (Table III).

For PDLLGA (80/20) microparticles after the initial release, there was a phase of slow and constant release of BSA for a period of 7 days. Data are shown only for the first 25 hours. Namely, after 25 hours 88% (copolymer I), 85% (copolymer II) or 75% (copolymer III) of BSA was released (Fig. 4b), and in 7 days the BSA was released completely (Table III).

Table III. Kinetic parameters for the BSA release from PLLGA and PDLLGA microparticles

Copolymer	Initial »burst« release		Lag phase of low release		Third phase of constant release	
	R_1[a]	K_1 (h^{-1})	R_2[b]	K_2 (h^{-1})	R_3[c]	K_3 (h^{-1})
PLLGA (68/32)						
M_w (g mol^{-1})						
44 050 (1)	1.000	1.492	0.994	0.165	0.974	0.009
88 000 (2)	1.000	0.397	0.984	0.056	0.910	0.006
PDLLGA (80/20)						
M_w (g mol^{-1})						
21 850 (I)	1.000	1.972	–	–	0.961	0.016
44 700 (II)	1.000	1.665	–	–	0.960	0.022
83 800 (III)	1.000	0.753	–	–	0.963	0.021

[a] Exponential dependence of the semilogarithmic plot on the amount of drug remaining *vs.* time in the initial »burst« release

[b] Exponential dependence of the semilogarithmic plot on the amount of drug remaining *vs.* time in the lag phase of release

[c] Exponential dependence of the semilogarithmic plot on the amount of drug remaining *vs.* time in the phase of constant of release

R_1–R_3 – respective correlation coefficients

K_1 – rate constant for the initial »burst« release, determined from the biexponential function.

K_2 – rate constant for the lag phase release, determined from the biexponential function.

K_3 – rate constant for the phase of constant release, determined from the biexponential function.

In the early phase including the lag phase, the protein release is governed by a diffusion-controlled mechanism through a network of water filled pores and channels. In the later phase, erosion of the polymer matrices is considered to control protein release from the core of microparticles (20). This explanation could be correlated with the hydrophobic/hydrophilic properties of the two series of microparticles, with the microparticle sizes and with the character of molecular mass changes during the blank microparticles degradation process (Figs. 2a,b).

Another serious problem in delivering protein pharmaceuticals is their inherent physical and chemical instability. Potential sources of irreversible deactivation of proteins encapsulated in PLGA microparticles are the following (12). First, exposure to organic solvents, which is a »stress« factor for proteins, during microparticles formulation; second, increased levels of moisture providing higher protein mobility; third, an acidic environment produced by acidic degradation products and carboxylic acid end groups of PLGA; and fourth, adsorption of the protein to the polymer surface, which may catalyze protein conformation changes from α-helix into β-sheet, resulting in its aggregation, and thus irreversible protein activity loss.

The impact of the encapsulation procedure on the secondary structure of BSA was preliminarily assessed using FTIR spectroscopy, from the amide I region. Technical and theoretical limitations enable determination of only three regions of vibrations, amide I, II and III, of the existing nine (A, B, I–III). The fact that 90% of IR investigations of the secondary protein structure are referred to amide I region (C=O, CN, CCN def.) resulted in an analysis of only this spectral region (21, 22). According to Jackson *et al.* (21), the free amide I vibrations are expected at 1670 cm^{-1}, while vibrations at 1695 cm^{-1} are a result of a strong transitional dipole bonding in β-conformations. Thus, vibrations for the protein amide I region appear between 1700 and 1600 cm^{-1}.

The secondary structure of BSA from the amide I region, according to the methods suggested by Byler and Susi (23), as well as Prestrelski *et al.* (24) and Bramanti and Benedetti (25), was determined after the Fourier-deconvolution of FTIR spectra, using an iterative curve fitting procedure of the Gauss-Lorenzian function. The content of α-helix was selected as a solid parameter for structural integrity, and it was calculated using the peak area in the characteristic frequency region (1660–1653 cm^{-1}). Our preliminary results showed that in the microparticles obtained from all copolymers, the secondary structure of BSA was preserved up to 30%.

Our further research is focused on the investigation of the influence of various stages or various procedures for microparticles preparation on the encapsulated protein activity.

CONCLUSIONS

Poly(lactide-co-glycolide) copolymers with different molar masses and a different molar ratio of DL-lactide or L-lactide to glycolide were used for the preparation of blank and BSA loaded microparticles by the w/o/w method. It was found that the copolymers molar mass and composition influence the mean geometric diameter of blank and loaded microparticles, as well as their loading efficiency. This was correlated with the viscosity of the copolymer solutions and the specific interactions with the hydrophilic segments in the copolymer backbone.

The BSA release profiles, triphasic for microparticles of PLLGA and biphasic for those of PDLLGA, were correlated with the microparticles morphology, size and porosity, as well as with the hydrophobic/hydrophilic properties and molar masses of copolymers and their degradation rates (mass loss and molar mass reduction).

Preliminary analyses of the FTIR spectra of BSA-loaded microparticles, in the amide I region, showed that the secondary protein structure was preserved to about 30%, on the basis of the calculated α-helix content. However, detailed investigations of the magnitude of different stages of the procedure-induced protein structural perturbations during encapsulation are in progress.

Acknowledgements. – This work was supported by the Ministry of Science of the Republic of Macedonia and the Turkish Scientific and Technical Research Council (TUBITAK)-Marmara Research Center, Turkey.

Abbreviations. – DL-LA – DL-lactide, L-LA – L-lactide, PLA – poly(lactide), where lactide is L- or DL, PDLLA – poly(DL-lactide), PLLA – poly(L-lactide), PGA – polyglycolide, PLGA – poly(lactide-co-glycolide), where lactide is L- or DL, PDLLGA – poly(DL-lactide-co-glycolide), PLLGA – poly(L-lactide-co-glycolide), PVA – poly(vinyl alcohol)

REFERENCES

1. C. Berkland, K. K. Kim and D. W. Pack, Fabrication of PLG microspheres with precisely controlled and monodisperse size distributions, *J. Control. Rel.* **73** (2001) 59–74.
2. S. Cohen, T. Yoshioka, M. Lucarelli, L. H. Hwang and R. Langer, Controlled delivery systems for protein based on poly(lactic/glycolic acid) microspheres, *Pharm. Res.* **8** (1991) 713–720.
3. S. K. Sahoo, J. Panyam, S. Prabha and V. Labhasetwar, Residual polyvinyl alcohol associated with poly(D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake, *J. Control. Rel.* **82** (2002) 105–114.
4. Y. Y. Yang, T. Sh. Chung, X. L. Bai and W. Kh. Chang, Effect of preparation conditions on morphology and release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion method, *Chem. Eng. Sci.* **55** (2000) 2223–2236.
5. C. Berkland, M. King, A. Cox, K. K. Kim and D. W. Pack, Precise control of PLG microsphere size provides enhanced control of drug release rate, *J. Control. Rel.* **82** (2002) 137–147.
6. Sh. Takada, Y. Yamagata, M. Misaki, K. Taira and T. Kurokawa, Sustained release of human growth hormone from microcapsules prepared by a solvent evaporation technique, *J. Control. Rel.* **88** (2003) 229–242.
7. X. L. Bai, Y. Y. Yang, T. Sh. Chung, S. Ng and J. Heller, Effect of polymer composition on the fabrication of poly(*ortho*-ester) microspheres for controlled release of protein, *J. Appl. Polym. Sci.* **80** (2001) 1630–1642.
8. F. Boury, H. Marchais, J. E. Proust and J. P. Benoit, Bovine serum albumin release from poly-(α-hydroxy acid) microspheres: effect of polymer molecular weight and surface properties, *J. Control. Rel.* **45** (1997) 75–86.
9. K. J. Zhu, H. L. Jiang, X. Y. Du, J. Wang, W. X. Xu and S. F. Liu, Preparation and characterization of hCG-loaded polylactide or poly(lactide-co-glycolide) microspheres using a modified water-in-oil-in-water (w/o/w) emulsion solvent evaporation technique, *J. Microencapsul.* **18** (2001) 247–260.
10. R. E. Eliaz and J. Kost, Characterization of a polymeric PLGA-injectable implant delivery system for the controlled release of proteins, *J. Biomed. Mater. Res.* **50** (2000) 388–396.

11. D. T. O' Hagan, H. Jefery and S. S. Davis, The preparation and characterization of poly(lactide-co-glycolide) microparticles: III. Microparticle/polymer degradation rates and in vitro release of a model protein, *Int. J. Pharm.* **103** (1994) 37–45.
12. I. J. Castellanos, K. G. Carrasquillo, J. de Jesus Lopez, M. Alvarez and K. Griebnow, Encapsulation of bovine serum albumin in poly(lactide-co-glycolide) microspheres by the solid-in-oil-in-water technique, *J. Pharm. Pharmacol.* **53** (2001) 167–178.
13. A. Porjazoska, N. Kayaman-Apohan, O. Karal-Yilmaz, M. Cvetkovska, K. Baysal and B. M. Baysal, Synthesis and characterization of glycolide, L-lactide, and PDMS-based terpolymers as a support for cell cultures, *J. Biomater. Sci. Polym. Ed.* **13** (2002) 1119–1134.
14. J. W. Kostanski and P. P. deLuca, A novel in vitro release technique for peptide-containing biodegradable microspheres, *AAPS Pharm. Sci. Tech.* **1** (2000) 1–16.
15. I. Soriano, E. Carmen and M. Labres, Preparation and evaluation of insulin-loaded poly(DL-lactide) microspherers using an experimental design, *Eur. J. Pharm.* **2** (1996) 135–142.
16. K. Mladenovska, Lj. Klisarova, E. I. Janjevic and K. Goracinova, BSA loaded gelatin microspheres: Preparation and drug release rate in the presence of collagenase, *Acta Pharm.* **52** (2002) 91–101.
17. K. Mladenovska, E. I. Janjevic, M. D. Glavas, E. E. Kumbaradzi and K. Goracinova, Biodistribution of ^{131}I-BSA loaded gelatin microspheres after peroral application to BALB/c mice – Particle size study, *Acta Pharm.* **53** (2003) 187–197.
18. M. I. Ugwoke, N. Verbeke and R. Kinget, Microencapsulation of amorphine HCl with gelatin, *Int. J. Pharm.* **148** (1997) 23–32.
19. D. K. Gilding and A. M. Reed, Biodegradable polymers for use in surgery-polyglycolic/poly-(lactic acid) homo- and copolymers: 1. *Polymer* **20** (1979) 1459–1464.
20. T. Morita, Y. Horikiri, T. Suzuki and H. Yoshino, Applicability of various amphiphilic polymers to the modification of protein release kinetics from biodegradable reservoir-type microspheres, *Eur. J. Pharm. Biopharm.* **51** (2001) 45–53.
21. M. Jackson and H. H. Mantsch, The use and misuse of FTIR spectroscopy in the determination of protein structure, *Crit. Rev. Biochem. Mol. Biol.* **30** (1995) 95–120.
22. K. G. Carrasquillo, J. C. Aponte Caro, A. Alejandro, D. Diaz Toro and K. Grebenow, Reduction of structural perturbations in BSA by non-aqueous microencapsulation, *J. Pharm. Pharmacol.* **53** (2001) 115–120.
23. D. M. Byler and H. Susi, Examination of the secondary structure of proteins by deconvolved FTIR spectra, *Biopolymers* **25** (1986) 496–487.
24. S. J. Prestrelski, N. Tedischi, T. Arakawa and J. F. Carpenter, Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers, *Biophys. J.* **65** (1993) 661–671.
25. E. Bramanti and E. Benedetti, Determination of the secondary structure of isomeric forms of human serum albumin by a particular frequency deconvolution procedure applied to Fourier transform IR analysis, *Biopolymers* **38** (1996) 639–653.

S A Ž E T A K

Poli(laktid-ko-glikolid) mikročestice kao sustavi za kontrolirano oslobađanje proteina – Priprava i karakterizacija

ALEKSANDRA PORJAZOSKA, KATERINA GORACINOVA, KRISTINA MLADENOVSKA, MARIJA GLAVAŠ, MAJA SIMONOVSKA, EMILIJA IVANOVSKA JANJEVIĆ i MAJA CVETKOVSKA

Poli(DL-laktid-ko-glikolid) (PDLLGA) i poli(L-laktid-ko-glikolid) (PLLGA) kopolimeri priređeni su polimerizacijom laktida i glikolida uz otvaranje prstenova i karakterizirani pomoću GPC, FTIR, ^{1}H NMR i DSC. Kopolimeri različitih molarnih masa i stalnog omjera laktida i glikolida upotrebljeni su za pripravu mikročestica s govođim serumskim albuminom (BSA) metodom dvostruke emulzije tipa voda/ulje/voda. Proučavan je utjecaj molarne mase i sastava kopolimera na oblik, veličinu, iskorištenje i stupanj razgradnje mikročestica, uklapanje i oslobađanje BSA. Za mikročestice pripravljene s PDLLGA kopolimerom utvrđen je bifazični profil oslobađanja BSA, a za mikročestice s PLLGA kopolimerom trifazičan profil. Za obje vrste karakteristično je brzo početno oslobađanje. Razmatrani su mogući uzroci takvog ponašanja.

Ključne riječi: poli(laktid-ko-glikolid), mikročestice, protein, *in vitro* oslobađanje

Institute of Organic Technology, Faculty of Technology and Metallurgy, University »Ss Cyril and Methodius«, Skopje, R. Macedonia

Institute of Pharmaceutical Technology, Faculty of Pharmacy, University »Ss Cyril and Methodius«, Skopje, R. Macedonia

Institute of Nuclear Medicine and Pathophisiology, Faculty of Medicine, University »Ss Cyril and Methodius«, Skopje, R. Macedonia