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Polymer 45 (2004) 1583–1589
polymer
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Preparation of poly[DL-lactide-*co*-glycolide]-based microspheres containing protein by use of amphiphilic diblock copolymers of depsipeptide and lactide having ionic pendant groups as biodegradable surfactants by W/O/W emulsion method

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Received 3 July 2003; received in revised form 18 December 2003; accepted 25 December 2003

Abstract

To develop the preparative method for poly(DL-lactide-*co*-glycolide)-based microspheres containing proteins, we prepared microspheres from mixture of poly(DL-lactide-*co*-glycolide) and polydepsipeptide-*block*-poly(DL-lactide) having cationic or anionic pendant groups. Since the latter amphiphilic copolymers consisting of hydrophobic poly(DL-lactide) segment and hydrophilic polydepsipeptide segment with amino or carboxyl groups could be converted to cationic or anionic block copolymers, they could act as biodegradable surfactants on the preparation of poly(DL-lactide-*co*-glycolide)-based microspheres by water-in-oil-in-water emulsion method. The amphiphilic block copolymers were established to stabilize primary emulsions on the preparation of microspheres by scanning electron microscopy. We investigated the effects of the addition of the block copolymers on the entrapment efficiency of protein, the release behavior of protein from microspheres and the stability of protein.

Keywords: Polydepsipeptide; Amphiphilic diblock copolymer; Biodegradable surfactant

1. Introduction

In recent years, proteins and DNAs have become valuable as potent drugs. However, such water-soluble macromolecules are very unstable in body because of their enzymatic hydrolyses. It is a very important research target to develop delivery systems for proteins and DNAs with high stability by protecting them from enzymatic hydrolysis. Polylactide (PLA), polyglycolide (PGA) and their copolymers have been used as such biodegradable biomedical materials as absorbable suture [1–4]. PLA and its copolymer have been also used as degradable drug release depot of microsphere (MS)s [5,6]. The preparation of MSs from such PLA-based copolymers as poly(lactide-*co*-glycolide) (PLGA), poly(lactide-*block*-ethylene oxide) and their degradation mechanisms have been reported [7–17]. In general, water-in-oil-in-water (W/O/W) emulsion methods were used to prepare MSs containing water-soluble drugs from PLA copolymers. To apply this method for delivery of protein drugs, the high entrapment efficiency and the prevention of protein denaturation would be key points. Moreover, the achievement of homogeneous dispersion of water inner phase for primary W/O emulsion in MSs and the controlled release of protein drugs without initial burst should be very important. Recently, we reported the synthesis of biodegradable amphiphilic AB-type diblock copolymers, poly(depsipeptide-*block*-lactide) having ionic pendant groups, poly[(Glc-Lys)-*b*-LA], PGK(+)-*b*-PLA having cationic pendant groups and poly[(Glc-Asp)-*b*-LA], PGD(−)-*b*-PLA having anionic pendant groups [18]. Moreover, we reported these AB-type diblock copolymers acted as biodegradable surfactants when PLA-based MSs were prepared by oil-in-water emulsion methods [19]. By using these diblock copolymers as surfactants in W/O/W emulsion system for preparation of MSs containing protein drugs, the homogeneous dispersion of primary water inner

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doi:10.1016/j.polymer.2003.12.067

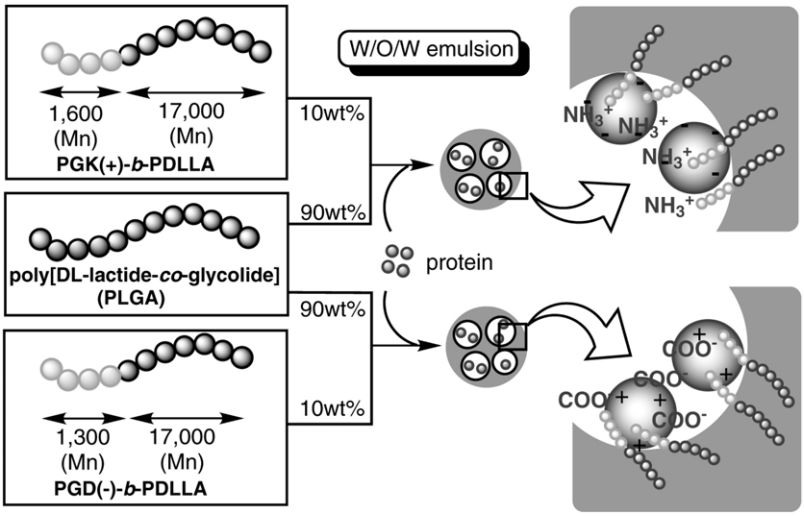


Fig. 1. Schematic illustration of the effects of PGK(+)-*b*-PDLLA and PGD(−)-*b*-PDLLA on the formation of MSs of PLGA.

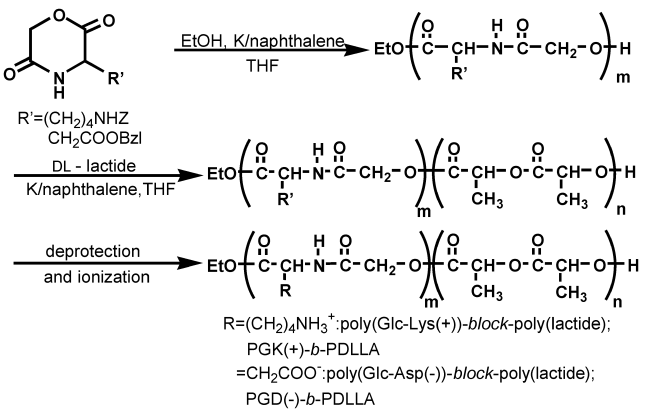
phases containing protein in MSs because of electrostatic repulsion among cationic pendant groups or anionic pendant groups would be expected.

This article concerns the preparation of MSs entrapping hydrophilic model protein drugs from the mixture of PLGA and the diblock copolymers by W/O/W emulsion method (Fig. 1), the effect of addition of the diblock copolymers on the entrapment efficiency of the protein drugs, the dispersion state of primary W/O emulsion in MSs, release behavior and the stability of the protein drugs.

2. Experimental

2.1. Materials

L-Lactide (L-LA) purchased from boehringer GmbH Ingelheim was recrystallized three times from ethyl acetate before use. Dry tetrahydrofuran (THF) purchased from Wako Pure Chemical Co. was used as a polymerization solvent without purification. Bovine serum albumin (BSA), lysozyme as hydrophilic model protein drugs and poly(DL-lactide-*co*-glycolide) (PLGA) with a lactide: glycolide ratio of 85:15 were purchased from Sigma Chemical Co. The other reagents were commercial grade and used without further purification.



Scheme 1. Synthetic route of polydepsipeptide-*block*-poly(DL-lactide).

Poly[depsipeptide-*block*-(DL-lactide)]s having cationic pendant groups (PGK(+)-*b*-PDLLA) and having anionic pendant groups (PGD(−)-*b*-PDLLA) were synthesized by the method reported previously in Scheme 1 [18]. Protected cyclodepsipeptide synthesized by the methods reported previously were polymerized in THF using potassium ethoxide as an initiator to obtain the corresponding protected polydepsipeptides. L-Lactide was then polymerized using the potassium alcoholates of the obtained polydepsipeptides as macroinitiators to give the protected block copolymers. The obtained protected polymers were analyzed by ^{1}H NMR and gel permeation chromatography (column, TSK-GEL Multipore HXL-M × 2; eluent, THF; detector, RI and UV_{252}; standard, polystyrene). The deprotections of Z and OBzl groups were carried out by the hydrogen bromide/acetic acid treatment and the trifluoromethansulfonic acid-thioanisole/trifluoroacetic acid treatment, respectively.

^{1}H NMR (DMSO-d_6) (PGK(+)-*b*-PDLLA): $\delta = 1.1$ (t, CH_3CH_2), 1.3–2.0 (br, $CH(CH_2)_3$), 1.5 (s, $CHCH_3$), 2.7 (s, CH_2NH), 3.8 (s, CH_2O), 4.1 (s, $COCHNH$), 4.6 (s, CH_2CO), 5.1 (s, $CHCH_3$).

^{1}H NMR (DMSO-d_6) (PGD(−)-*b*-PDLLA): $\delta = 1.1$ (t, CH_3CH_2), 1.5 (s, $CHCH_3$), 3.0 (s, $CHCH_2$), 4.7 (s, OCH_2CO), 5.0 (s, $COCHNH$), 5.2 (s, $CHCH_3$).

As a control surfactant, non-ionic amphiphilic AB-diblock copolymer, MeO-PEG-*b*-PDLLA, was also synthesized by the same method.

2.2. Preparation of microspheres containing protein

The MSs from PGK(+)-*b*-PDLLA/PLGA blend containing BSA (MS[PGK(+)-*b*-PDLLA/PLGA:BSA]) and the

Table 1
Material compositions for preparation of MSs containing protein

Composition	MS[polydepsipeptide-*b*-PDLLA/PLGA]	MS[MeO-PEG-*b*-PDLLA/PLGA]	MS[PLGA]
Polydepsipeptide-*b*-PDLLA (mg)	60	–	–
MeO-PEG-*b*-PDLLA (mg)	–	60	–
PLGA (mg)	540	540	600
Methylene chloride (ml)	7	7	7
Protein (mg)	15	15	15
PBS (μl)	300	300	300
PBS containing 0.1% PVAaq (ml)	300	300	300

MSs from PGD(−)-*b*-PDLLA/PLGA blend containing lysozyme (MS[PGD(−)-*b*-PDLLA/PLGA:lysozyme]) were fabricated by the water-in-oil-in-water (W/O/W) emulsion method and solvent evaporation method reported previously [20]. Briefly, 540 mg of PLGA and 60 mg of PGK(+)-*b*-PDLLA or PGD(−)-*b*-PDLLA were dissolved in 7 ml of methylene chloride, whereas 15 mg of protein were dissolved in 300 μl of phosphate buffer solution (PBS) [1/15 M−KH_2PO_4−Na_2HPO_4] (pH 7.4). Polymer/methylene chloride solution added in a test tubes containing of protein/PBS, and then the sonication was carried out with a bath-type sonicator (Bransonic 220, Yamato) for 5 min and a probe-type sonicator (UD-200, Tomy) of 100 W for 1.5 min. The primary emulsion obtained was added slowly into 300 ml of a 0.1 wt% PVA aqueous solution containing PBS, and stirred at room temperature. After 30 min, this solution was stirred at 35 °C for 60 min, and then methylene chloride was completely evaporated under reduced pressure. The suspension was centrifuged at 10,000 rpm for 10 min to obtain the precipitated MSs. The obtained MSs were washed five times with water and then lyophilized.

MSs from MeO-PEG-*b*-PDLLA/PLGA blend containing BSA (MS[MeO-PEG-*b*-PDLLA/PLGA:BSA]) or lysozyme (MS[MeO-PEG-*b*-PDLLA/PLGA:lysozyme) and MSs from homoPLGA containing BSA (MS[PLGA:BSA]) or lysozyme (MS[PLGA:lysozyme]) were prepared according to the same method as described above. The compositions of these polymers for preparing MSs are shown in Table 1.

2.3. Shape, inner porosity and surface of the MSs

The average diameter of the MSs obtained was estimated from a JEOL JSM-35 scanning electron microscope (SEM). The distribution of MS size was estimated by sampling 100 MSs randomly. The MSs were rendered electrically conductive with a 20 nm coating of gold on the surface after drying for the observation by means of SEM. The shape, inner porosity and surface of the MSs were also observed with SEM.

2.4. Entrapment efficiency of protein into microspheres

By measuring the absorbance at 290 nm of the solution of 40 mg of the MSs dissolved in 2 ml of 1N NaOH on a spectrophotometer (SHIMAZU UV-2400PC), the amounts of protein entrapped into MSs were determined from a calibration curve of standard solutions [21].

2.5. In vitro protein release study

One hundred and fifty milligrams of the protein-loaded MSs were placed in a test tube and immersed in 5 ml of PBS at 37 °C. The protein amount released from the MSs was regularly monitored by measuring the absorbance at 280 nm for protein using a calibration curve obtained from standard solutions. In each measurement, 1 ml of supernatant was taken out from the release medium, and placed in a 1 mm cell. After the addition of 1 ml of fresh medium, the test tube was continuously incubated.

2.6. Circular dichroism measurement

Circular dichroism (CD) measurements of BSA and lysozyme released from MS[PGK(+)-*b*-PDLLA/PLGA:BSA] or MS[PGD(−)-*b*-PDLLA/PLGA:lysozyme] immersed in a pH 7.4 PBS for 21 days were carried out in PBS at room temperature using the CD spectropolarimeter (JASCO J-820).

2.7. Evaluation of enzymatic activity of lysozyme

The enzymatic activity of lysozyme was colorimetrically evaluated using *p*-nitoriphenyltetra-*N*-acetyl-β-chitotetraoside (NP-$(Glc\text{-}NAc)_4$) as a substrate at 25 °C [22]. Substrate solution of 0.014 μmol/ml in PBS was prepared. The substrate solution and the released lysozyme solution or native lysozyme solution were separately incubated at 25 °C for 30 min before the activity evaluation. The substrate solution was mixed with an equal volume of native or released lysozyme solution to start the enzymatic reaction. The change in the absorbance at 340 nm due to the hydrolysis of the nitrophenyl ester group of the substrate was monitored at 25 °C.

Table 2
Results of polymerization of DL-lactide with polydepsipeptide or MeO-PEG-OH

Macroinitiator		DL-Lactide g (mmol)	M_n [a]	M_w/M_n [a]	code-[*m*,*n*] [b]
Abbreviation	mg (μmol)				
Poly[Glc-Lys(Z)] ($M_n = 1600$)	80 (50)	1.1 (7.5)	19,000	1.9	PGKZ-*b*-PDLLA-[5,119]
Poly[Glc-Asp(OBzl)] ($M_n = 1300$)	65 (50)	1.1 (7.5)	18,000	1.8	PGD(OBzl)-*b*-PDLLA-[5,116]
MeO-PEG-OH ($M_n = 2000$)	200 (100)	2.2 (15)	10,000	1.3	MeO-PEG-*b*-PDLLA-[44,56]

Polymerization was carried out in THF at r.t. for 30 min. Molar ratio of DL-lactide to macroinitiator (m/I) = 150. Initial concentration of DL-lactide was 1.0 or 2.0 M for polydepsipeptide or MeO-PEG-OH, respectively.

[a] Estimated by GPC.

[b] *m*, *n*: Degree of polymerization of depsipeptide unit (or ethyleneglycol unit) and LA unit, respectively.

3. Results and discussion

3.1. Characterization of PGK(+)-b-PDLLA and PGD(−)-b-PDLLA

The obtained protected polymers were analyzed by ^{1}H NMR and GPC elution profiles were analyzed with UV and RI. The elution profiles from the RI detector were fitted with those from the UV detector. The degree of polymerization of the poly(DL-lactide) segment calculated by subtraction of the degree of polymerization of protected depsipeptide segment from the total M_n of protected block copolymer agreed with molar ratio of lactide to depsipeptide segment estimated from ^{1}H NMR spectra. These results suggest that they did not contain the corresponding unreacted protected polydepsipeptide and homopolylactide. The results of polymerization of DL-lactide using polydepsipeptide or MeO-PEG as macroinitiator are summarized in Table 2; PGKZ-*b*-PDLLA-[5,119], PGD(OBzl)-*b*-PDLLA-[5,116] and MeO-PEG-*b*-PDLLA-[44,56] were synthesized as AB-type diblock copolymers. By ^{1}H NMR measurements, the achievement of complete elimination of the Z and OBzl groups from PGKZ-*b*-PDLLA and PGD(OBzl)-*b*-PDLLA by the acid treatment was confirmed, respectively. The GPC date of the deprotected block copolymers suggested that main chain cleavage did not occur under our deprotection reaction.

In order to apply PGK-*b*-PDLLA and PGD-*b*-PDLLA as cationic and anionic surfactants, amino and carboxyl groups of them were converted to the hydrobromide salt (PGK(+)-*b*-PDLLA) and the sodium salt (PGD(−)-*b*-PDLLA), respectively.

3.2. Characterization of microspheres

The BSA-loaded MSs, MS[PGK(+)-*b*-PDLLA/PLGA:BSA], MS[MeO-PEG-*b*-PDLLA/ PLGA:BSA], MS[PLGA:BSA] and the lysozyme-loaded MSs, MS[PGD(−)-*b*-PDLLA /PLGA:lysozyme], MS[MeO-PEG-*b*-PDLLA/PLGA:lysozyme], MS[PLGA:lysozyme] could be prepared by the application of sonicated W/O/W emulsion and solvent evaporation method. The results of entrapment of BSA and lysozyme as hydrophilic model protein drugs into these MSs are summarized in Table 3. The osmotic pressure suppressed the entrapment of protein into the MSs prepared by W/O/W emulsion method. So, in order to solve the problem of osmotic pressure, PBS (pH 7.4) was used instead of water as outer and inner aqueous phases. Consequently, all kinds of MSs could show high entrapment efficiency for proteins.

3.3. Release behavior of protein

The results of the release behavior of proteins from six kinds of MSs in PBS (pH 7.4) at 37 °C are shown in Fig. 2. Although the proteins loaded in MS[MeO-PEG-*b*-PDLLA/PLGA] and MS[PLGA] were not released even for 77 days at all, the proteins loaded in MS[PGK(+)-*b*-PDLLA/PLGA] and MS[PGD(−)-*b*-PDLLA/PLGA] were released slowly without initial burst. Such difference of release behavior could be explained by the difference of dispersion

Table 3
Entrapment efficiencies of protein into MSs

MS	Yield (%)	Average size (μm)	Entrapment efficiency (%)[a]
MS[PLGA:BSA]	86.3	98	100
MS[PGK(+)-*b*-PDLLA/PLGA:BSA]	80.9	120	103
MS[MeO-PEG-*b*-PDLLA/PLGA:BSA]	85.6	86	100
MS[PLGA:lysozyme]	84.4	111	105
MS[PGD(−)-*b*-PDLLA/PLGA:lysozyme]	77.5	98	105
MS[MeO-PEG-*b*-PDLLA/PLGA:lysozyme]	75.6	116	106

Feed ratio of protein/polymer (w/w) = 2.5%.

[a] [Protein/polymer (w/w) found]/[protein/polymer (w/w) in feed] × 100.

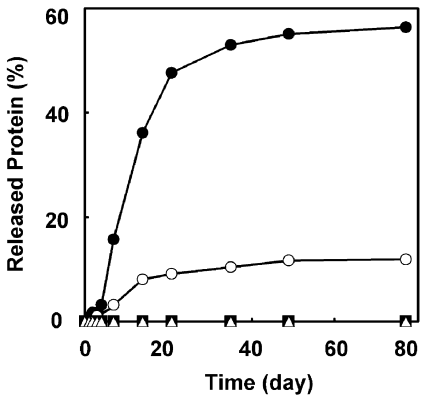


Fig. 2. Release rate of entrapped protein from MSs in PBS at 37 °C; (●) MS[PGK(+)-*b*-PDLLA/PLGA:BSA], (○) MS[PGD(−)-*b*-PDLLA/PLGA:lysozyme], (■) [MeO-PEG-*b*-PDLLA/PLGA:BSA], (□) [MeO-PEG-*b*-PDLLA/PLGA:lysozyme], (▲) MS[PLGA:BSA], (△) MS[PLGA:lysozyme].

of proteins in the MSs. The obtained MSs entrapping proteins were frozen by liquid nitrogen and cracked to observe the dispersion states of their water inner phases. SEM images of cross-section of MSs prepared are shown in Fig. 3. It was found from Fig. 3 that the primary water inner phases of MS[MeO-PEG-*b*-PDLLA/PLGA] and MS[PLGA] were collected only in neighborhood of center areas of MSs to form the large water inner phases. On the contrary, the primary water inner phases of MS[PGK(+)-*b*-PDLLA/PLGA:BSA] and MS[PGD(−)-*b*-PDLLA/PLGA:lysozyme] were homogeneously dispersed in the all areas of MSs because of electrostatic repulsion among quaternary amine groups and carboxylate groups, respectively. That is, the poly(depsipeptide-*block*-lactide)s having cationic and anionic pendant groups were clarified to act as good biodegradable surfactants and to prevented aggregation of

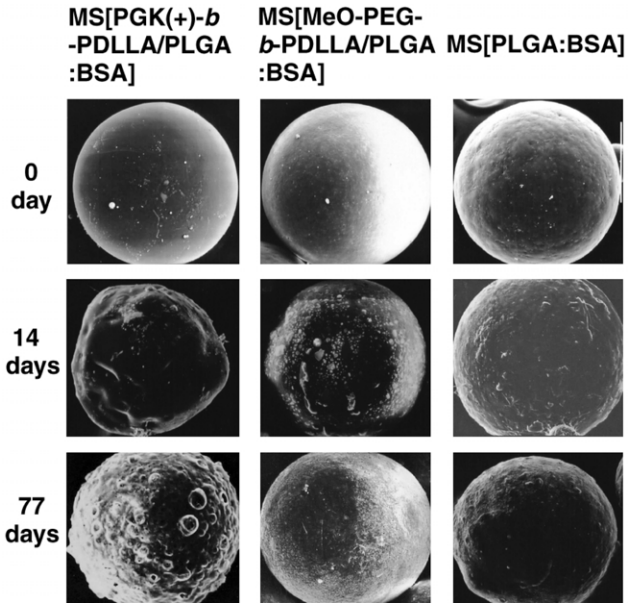


Fig. 4. SEM images of MSs containing BSA during the biodegradation test.

the primary water inner phases during the preparation process of MSs. Since proteins existed in water inner phases of MSs, the difference of location distribution of water inner phases directly reflected on the release behavior of proteins from PLGA-based MSs. SEM images of MSs containing BSA before and after the biodegradation test are shown in Fig. 4. MSs were found to be degraded from their surfaces. Therefore, MS[PGK(+)-*b*-PDLLA/PLGA:BSA] and MS[PGD(−)-*b*-PDLLA/PLGA:lysozyme] showed the slow release of proteins without initial burst.

The release rate of lysozyme from MS[PGD(−)-*b*-PDLLA/PLGA:lysozyme] was more slowly than that of BSA from MS[PGK(+)-*b*-PDLLA/PLGA:BSA]. Difference of

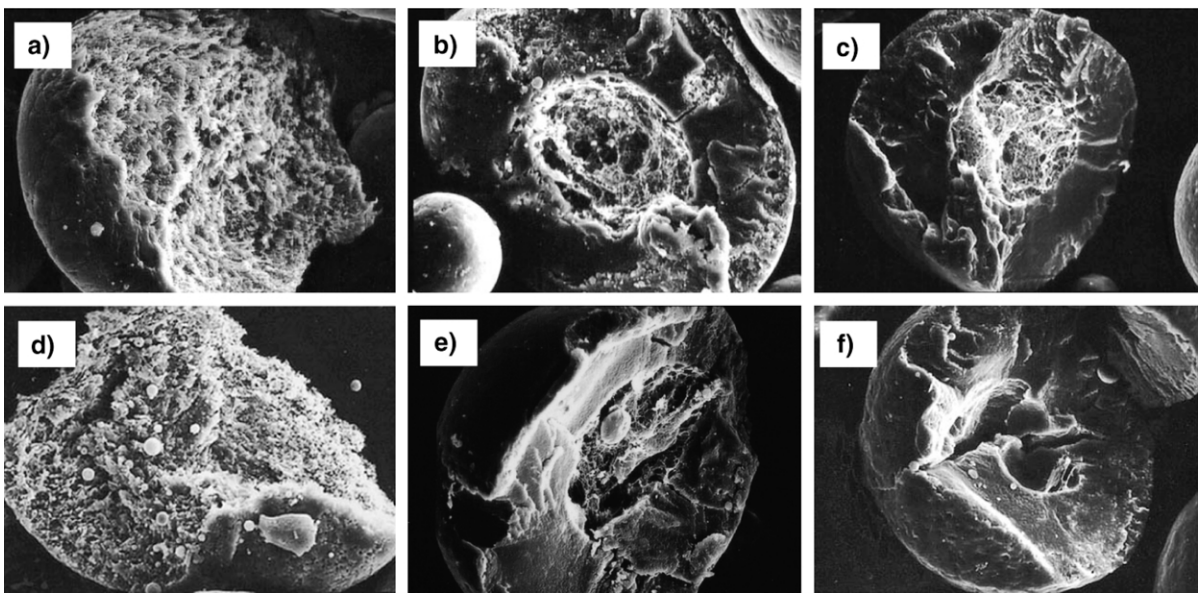


Fig. 3. SEM images of cross-section of MSs entrapping proteins; (a) MS[PGK(+)-*b*-PDLLA/PLGA:BSA], (b)MS[MeO-PEG-*b*-PDLLA/PLGA:BSA] and (c)MS [PLGA:BSA], (d)MS[PGD(−)-*b*-PDLLA/PLGA:lysozyme], (e) MS[MeO-PEG-*b*-PDLLA/PLGA: lysozyme], (f) MS [PLGA:lysozyme].

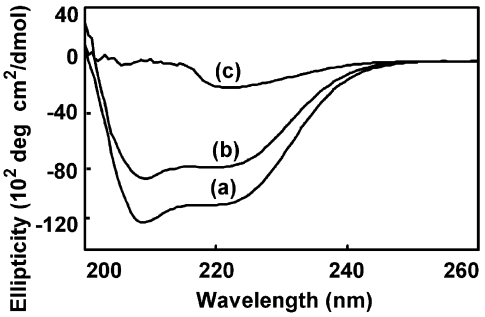


Fig. 5. CD spectra of (a) native BSA, (b) BSA released from MS[PGK(+)-*b*-PDLLA /PLGA:BSA] in pH 7.4 PBS and (c) denatured BSA in 8 M urea.

the release rate might be governed mainly by the strength of electrostatic interaction between protein and charged depsipeptide. Since the interaction of lysozyme (p*I* 11.0) with PGD(−)-*b*-PDLLA was stronger than that of BSA (p*I* 4.9) with PGK(+)-*b*-PDLLA in PBS (pH 7.4), the above results were suggested to be obtained.

3.4. Stabilization of released protein

In order to investigate whether protein molecules were denatured during MS preparation process, the CD measurements were carried out Figs. 5 and 6 show the CD spectra of native proteins and the proteins released from MS[PGK(+)-*b*-PDLLA/PLGA:BSA] and MS[PGD(−)-*b*-PDLLA/PLGA:lysozyme] immersed in pH 7.4 PBS for 21 days, respectively. The CD spectra of proteins released from MSs were almost identical, compared with those of native proteins. Namely, the denaturation of proteins hardly occur in the preparation process of MS[PGK(+)-*b*-PDLLA/PLGA:BSA] and MS[PGD(−)-*b*-PDLLA/PLGA:lysozyme] [23].

3.5. Enzymatic activity of released lysozyme

It is well-known that lysozyme hydrolyzes the β-1,4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine. *p*-Nitrophenol was generated through lysozyme-catalyzed cleavage of β-1,4-glycosidic bond of NP-(Glc-NAc)$_4$. The amounts of generated *p*-nitrophenol

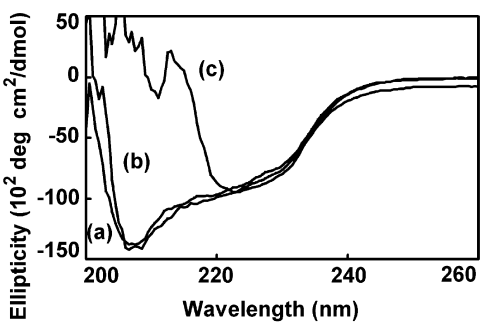


Fig. 6. CD spectra of (a) native lysozyme, (b) lysozyme released from MS[PGD(−)-*b*-PDLLA/PLGA:lysozyme] in pH 7.4 PBS and (c) denatured lysozyme in 8 M urea.

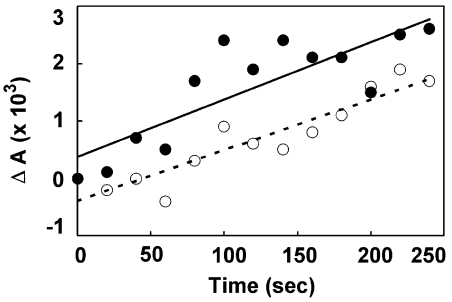


Fig. 7. The change in the absorbance at 340 nm due to the hydrolysis of NP-(GlcNAc)$_4$ by lysozyme; (○) native lysozyme, (●) released lysozyme.

were determined by measuring the absorbance at 340 nm. The initial hydrolysis ability for released lysozyme was almost equivalent compared with that for the native lysozyme (Fig. 7). The released lysozyme was found to keep the enzymatic activity of native lysozyme.

4. Conclusions

MSs entrapping hydrophilic model protein drugs from the mixture of PLGA and poly(depsipeptide-*block*-lactide) having cationic or anionic pendant groups could be prepared. Homogeneous dispersion of water inner phases containing proteins could be achieved in MS[PGK(+)-*b*-PDLLA/PLGA:BSA] and MS[PGD(−)-*b*-PDLLA/PLGA:lysozyme] because PGK(+)-*b*-PDLLA and PGD(−)-*b*-PDLLA acted as good biodegradable surfactants to stabilize the corresponding primary water inner phases having proteins in PLGA-based MSs. These results led the sustained release of the proteins without initial burst. Such PLGA-based MSs prepared using poly(depsipeptide-*block*-lactide) having ionic pendant groups can be expected to be applied as drug release devices for protein drugs. PGK(+)-*b*-PDLLA and PGD(−)-*b*-PDLLA are very interesting biodegradable surfactants from the standpoint of biomedical materials.

References

[1] Frazza EJ, Schmitt EE. J Biomed Mater Res Symp 1970;1:43–58.
[2] Eduardo EA, Juan J. J Surg 1970;131:1–14.
[3] Wise DL, Fellmann TD, Sanderson JE, Wentworth RL. Drug Carriers Biol Med 1979;237–70.
[4] Celli A, Scandola M. Polymer 1992;33:2699–703.
[5] Langer R. Science 1990;249:1527–33.
[6] Makino K, Arakawa M, Kondo T. Chem Pharm Bull 1985;33:1195–201.
[7] Kimura Y, Matsuzaki Y, Yamane H, Kitao T. Polymer 1989;30:1342–9.
[8] Park TG. J Control Rel 1994;30:161–73.
[9] Grizzi I, Garreau H, Li S, Vert M. Biomaterials 1995;16:305–11.
[10] Edulund U, Albertsson A-C. J Polym Sci Part A: Polym Chem 1999;37:1877–84.
[11] Gref R, Monamitake Y, Peracchia MT, Trubetsskoy V, Langer R. Science 1994;263:1600–3.

[12] Ogawa Y, Yamamoto M, Okada H, Yashiki T, Shimamoto T. Chem Pharm Bull 1988;36:1095–103.
[13] Choi YK, Bae YH, Kim SW. Macromolecules 1995;28:8419–21.
[14] Maruyama A, Ishihara T, Adachi N, Akaike T. Biomaterials 1994;15: 1035–42.
[15] Cho CS, Cho KY, Park IK, Kim SH, Sasagawa T, Uchiyama M, Akaike T. J Control Rel 2001;77:7–15.
[16] Ouchi T, Nozaki T, Ishikawa A, Fujimoto I, Ohya Y. J Polym Sci Part A: Polym Chem 1997;35:377–83.
[17] Tabata Y, Ikada Y, Morimoto K, Katsumata H, Yabuta T, Iwanaga K, Kakemi M. J Bioact Compat Polym 1999;14:371–84.
[18] Ouchi T, Miyazaki H, Arimura H, Tasaka F, Hamada A, Ohya Y. J Polym Sci Part A: Polym Chem 2002;40:1218–25.
[19] Ouchi T, Toyohara M, Arimura H, Ohya Y. Biomacromolecules 2002; 3:885–8.
[20] Ouchi T, Hamda A, Ohya Y. Macromol Chem Phys 1999;200: 436–41.
[21] Cleland JL, Mac A, Boyd B, Yang J, Duenas ET, Yeung D, Brooks D, Hsu C, Chu H, Mukku V, Jones AJS. Pharm Res 1997;14:420–5.
[22] Harada A, Kataoka K. J Control Rel 2001;72:85–91.
[23] Molina I, Li SM, Martinez MB, Vert M. Biomaterials 2001;22:363–9.