Preparation and Characterization of Poly-DL-lactide–Poly(ethylene glycol) Microspheres Containing λDNA

Xianmo Deng, Yu Liu, Minlong Yuan, Xiaohong Li, Li Liu, W. X. Jia

Chengdu Institute of Organic Chemistry, Academia Sinica, P.O. Box 415, Chengdu 610041, People's Republic of China

Received 26 September 2001; accepted 1 March 2002

ABSTRACT: Polylactide (PLA) and a block copolymer, poly-DL-lactide–poly(ethylene glycol) (PELA) were synthesized by bulk ring-opening polymerization initiated by stannous chloride. A linear DNA molecule, λDNA, was used as the model DNA. PLA, PELA, λDNA-loaded PLA and PELA microspheres were prepared by the solvent-extraction method based on the formation of multiple $w_1/o/w_2$ emulsion. The particle-size distribution, surface morphology, and DNA loading characterized the microspheres. The mean diameter of λDNA-loaded PELA microspheres was proved to be 3.5 μm. The integrity of the λDNA molecules, after preparing the microspheres, was determined by agarose gel electrophoresis. The result suggested that most of the λDNA molecules could retain their integrity after being encapsulated by PELA. The PELA microspheres could also prevent λDNA from being degraded by DNase. The *in vitro* degradation and release of PLA, PELA, and λDNA-loaded PELA microspheres were carried out in a pH 7.4 buffer solution at 37°C. Quantitatively, evaluating the molecular weight reduction, the mass loss, the particle-size changes, and the particle-size distribution changes also monitored the degree of degradation. The release profile was assessed by measurement of the amount of λDNA present in the release medium at determined intervals. The degradation profiles of the PELA microspheres were quite different from those of the PLA microspheres. The introduction of the hydrophilic poly(ethylene glycol) domain in PLA and the presence of λDNA within the microspheres exhibit the apparent influence on the degradation and release profiles. A biphasic release profile was proved, that is, an initial burst release during the first days, then a gradual release. It was demonstrated that the PELA microspheres could be used potentially as a controlled release-delivery system for λDNA.
 J Appl Polym Sci 86: 2557–2566, 2002

Key words: preparation; microspheres; morphology; degradation

INTRODUCTION

One of the applications of polymers having the most potential is in drug-delivery systems. In recent years, gene-delivery systems have become of great interest among drug-delivery systems, because gene therapy is becoming an attractive method for treating or preventing acquired and inherited genetic disease.[1,2] Among those gene-delivery systems, biodegradable polymeric systems have the most potential owing to their biodegradability, biocompatibility, low toxicity, and minimal immune response.[3–6] Particularly, poly-DL-lactide (PLA) and poly-DL-lactide-*co*-glycolide (PLGA) have been investigated extensively as matrices for releasing genes.[7–11] Due to their hydrophobilicity, however, the polymers show some limitations[12]: The molecules of DNA are hydrophilic and the difference in their physicochemical properties with the hydrophobic polymeric matrix has a profound influence on the encapsulation efficiency during the preparation procedure and DNA stability during manufacture, storage, and release processes. For example, during the initial release phase *in vivo*, the hydrophobic polymer prevents the penetration of water into the center of the microspheres, thus forming an acidic microenvironment due to the accumulated acidic breakdown products, such as lactic and glycolic acid. This acid condition, combined with elevated temperature and hydrophobic surfaces, may provide conditions in which DNAs are unable to survive for a long time.

To improve the hydrophilicity of PLA, a second component, poly(ethylene glycol) (PEG), which has been widely used to improve the biocompatibility of the blood-contacting materials, was introduced into PLA to form the block copolymer PELA.[13] The biodegradability and biocompatibility of PELA make it a suitable candidate polymer for the development of controlled delivery systems of water-soluble molecules. The hydrophilic domain of PELA, acting as a surface modifier of the hydrophobic PLA network, can promote the stability of water-soluble molecules, increase their loading efficiency, and

Correspondence to: Y. Liu (Poly1634@hotmail.com).
Contract grant sponsor: National Project 973 of China.
Contract grant sponsor: National Natural Science Foundation of China; contract grant number: 20004009.

Journal of Applied Polymer Science, Vol. 86, 2557–2566 (2002)

decrease the amount of emulsifier used in the microsphere preparation process.

In our previous investigations, PELA was studied as a protein drug-delivery system. We encapsulated human serum albumin and outer-membrane proteins (OMP) of *Vibrio cholera* and *Leptosira interrogans* with PELA successfully.[13–15] In this study, we attempted to use PELA as a nonviral gene-delivery system to encapsulate λDNA, a linear double-stranded DNA molecule, evaluated the characters of λDNA-loaded microspheres, and investigated the degradation behaviors of the microspheres and the release profile of the λDNA-loaded microspheres.

EXPERIMENTAL

Materials

λDNA (48,502 bp) was supplied by the Sino-American Biotechnology Co. (Luoyang, China); PEG, molecular weight: 6000 daltons, was purchased from the Guangzhou Chemical Reagents Department (Guangzhou, China); and DL-lactide was purchased from Aldrich (Milwaukee, WI). The copolymer PELA was prepared by bulk ring-opening polymerization of lactide and PEG initiated by stannous chloride; DNase I (RNase free) was purchased from the Takara Biotech. Co. (Dalian, China). All other chemicals and solvents were reagent grade or better.

Preparation of microspheres

λDNA-loaded microspheres were prepared by an emulsion/evaporation procedure, which was based on the formation of a modified double-emulsion $w_1/o/w_2$. Briefly, an aqueous solution of λDNA, as the w_1 phase, was dispersed into the organic solution (as the o phase), consisting of the polymer dissolved in methylene chloride. The mixture was emulsified with a magnetic stirrer for 2 min at room temperature to form the primary w_1/o emulsion. This emulsion was then added to the external water phase containing stabilizers, and a high-speed homogenizer emulsified the mixture again. The organic solvent was extracted by adding 100 mL of 6% isopropanol; then, the mixture was stirred at a moderate speed at room temperature for 5–6 h. The solidified microspheres were collected by centrifugation (Tomy Seiko Co. Ltd) at 8000 rpm for 10 min. The obtained microspheres were rinsed with distilled water and centrifuged three more times, then dried for at least 24 h in a vacuum oven at room temperature and stored at −20°C

Particle characterization

Particle-size distribution

Particles were sized by a laser diffractometer using a Malvern2000 laser sizer (Malvern Instruments PC6300, Malvern, England). The average particle size was expressed as the volume mean diameter in micrometers.

Surface morphology

The surface morphology of the microspheres was evaluated by scanning electron microscopy (SEM, Amray). A monolayer of dry microspheres was mounted on an aluminum stub using double-sided carbon tape. The sample was coated with a 10-nm-thick palladium/gold film using a sputter coater. The coated samples were examined using an electron acceleration voltage of 5–10 kV.

Determination of DNA loading of microspheres

PELA microspheres, 200 mg, were dissolved in 1500 μL methylene chloride, and 500 μL of 10 mmol/L Tris–EDTA buffer (pH 7.4) was added to extract λDNA. After centrifugation at 3000 rpm for 5 min, the aqueous layer was transferred into a fresh tube. The amount of λDNA was determined by an ultraviolet spectrophotometer (Beckman), and the stability of λDNA was assessed by 0.8% agarose gel electrophoresis.

In vitro degradation study

The microspheres, 300 mg, were suspended in 10.0 mL of 0.1*M* phosphate buffered saline (PBS, pH 7.4). The mixtures were kept in a thermostated shaker water bath (Jiangsu Taicang Medical Apparatus Co., China) at 37°C, 60 rpm. The degradation medium was removed at certain intervals, by centrifugation, from the vessel containing the microspheres, and the removed degradation medium was replaced with the same volume of fresh PBS. At regular intervals, triplicate samples for each kind of microsphere were recovered, rinsed with distilled water to remove any residual buffer salts, and dried to a constant weight in a vacuum desiccator at room temperature.

The degree of degradation was estimated from the decrease of molecular weight and from the mass loss and the morphological changes of the microspheres. Each point of the plots describing the degradation data represents the mean of triplicate samples.

The molecular weight of the polymers was determined by gel permeation chromatography (GPC) in tetrahydrofuran (THF) at 30°C with an Ultrastyragel lineal column (Waters, USA) and a refraction index detector (R401, Waters) and calibrated with polystyrene standards. The mass loss was calculated by comparing the net weights of the microspheres at predetermined intervals with the initial net weight of the microspheres. SEM (Amary) was used to observe the surface morphological changes. Samples of fresh mi-

TABLE I
Characteristics of λDNA-loaded Microspheres

Polymer	Molecular weight[a] (kDa)	Diameter[b] (μm)	Concentration of polymer solution (%, w/v)	DNA entrapment (wt %)	Loading efficiency (%)
PELA	15	13.8	6	0.08	18.5
	36	8.4	6	0.08	20.5
	63	3.5	6	0.08	31.7
	63	2.1	6	0	—
	83	3.4	6	0.08	33.4
	83	3.2	8	0.06	37.2
	100	3.3	6	0.08	34.7
	100	2.9	8	0.06	41.8
PLA	58	4.8	6	0.08	29.4
	58	2.4	6	0	—

[a] Measured by GPC.
[b] Measured by laser diffractometer.

crospheres and microspheres from the degradation experiments were dissolved in THF to eliminate DNA.

In vitro release study

The release profile of λDNA from PELA microspheres was determined in 0.2*M* PBS (pH 7.4). The microspheres, 500 mg, were incubated in 10.0 mL of PBS and shook at 37 ± 0.5°C at 60 rpm. At predetermined intervals, 2.0 mL of the release medium was removed after centrifuging (5 min, 3000 rpm) and another 2.0 mL fresh PBS was added to replace the removed sample. The concentration of released DNA was determined by an ultraviolet spectrophotometer. All the release tests were performed three times independently and the results were reported as the mean of these triplicate assays.

DNase I digestion study

The study of DNase I digestion was according to Sisay et al.[16] The microspheres, 299 mg, were suspended in 4 mL of 10 mmol/LTris–HCl buffer (pH 8.0) containing 3.2 mL of 10 m*M* $MgSO_4$, and 2 μL of 5μg/μL DNase I in 0.9% NaCl was added. The mixture was incubated for 30 min at 37°C. After digestion, the samples were washed three times with 1000 μL of fresh Tris–EDTA buffer (pH 8.0) and assessed by 0.8% agarose gel electrophoresis.

RESULTS AND DISCUSSION

PELA was used as a protein-delivery system during our previous studies.[13–15] From these investigations, the simplified preparative procedures of microspheres, the controlled degradation velocity of a polymer, and the higher encapsulated efficiency of protein were obtained. According to the features of PELA, we attempted to use PELA as a gene-delivery system.

It is well known that bacteriophage λ, which infects *E. coli* cells, is one of the best studied bacteriophage, which also has been much studied as a model for the regulation of gene expression. Derivatives are commonly used as cloning vectors, Therefore, in the studies, λDNA was chosen as the model genes for evaluating the delivery of linear, double-stranded DNA with a higher molecular weight (48.5 kb).

Characterization of microspheres

PLA and PELA with different molecular weights were used as microsphere matrices. The results of the characteristics of the microspheres are provided in Table I. In comparing the microspheres made of PLA and PELA, with similar weight-average molecular weights, it was found that the diameters of the PELA microspheres are smaller than those of the PLA microspheres, and λDNA entrapment of 0.08%, with a loading efficiency of 31.7% was achieved for the PELA microspheres, whereas a loading efficiency of 29.4% was obtained for the PLA microspheres prepared by a similar process. This is the result of the increased phase compatibilities between λDNA and the PELA block polymer, which are due to the hydrophilic domains in PELA. The loading efficiency of PELA microspheres increases with increase of the molecular weight, while the average size of the microspheres decreases with increase of the molecular weight, and increase of the polymer concentration is related to decrease of the particle size and increase of the loading efficiency. As we know, PELA, with a higher molecular weight, contains less PEG content, so that it is less hydrophilic, which appears to be a disadvantage in forming microspheres. On the other hand, PELA is a somewhat negatively charged block copolymer for the PEG domains. The more PEG that contains PELA, the more negative are the copolymers. Also, the molecules of DNA are also negative for the phosphate

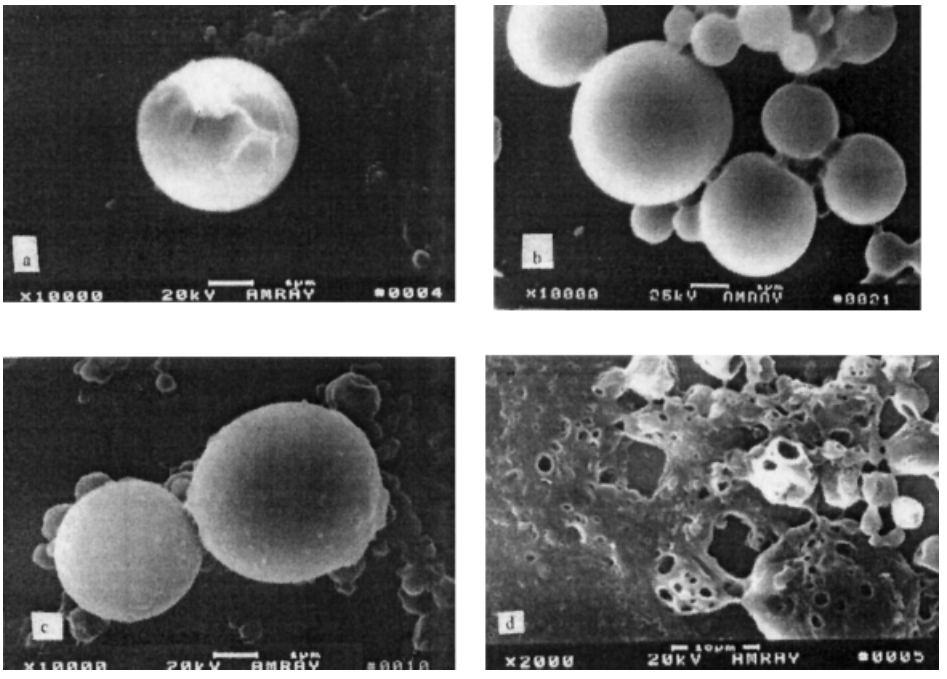

Figure 1 Surface morphology of microspheres.

group. Because of electrostatic interaction, more negative charges of the copolymers interfere with the formation of microspheres, so that increase of the PELA molecular weight contributes to a decreasing particle size and an increasing loading efficiency. But if the molecular weight is so high that the composition of PEG is too limited, then the hydrophilic property is only slightly improved. As a consequence, the polymer did not advantage the DNA, just like PLA.

In Figure 1, (a) shows a scanning electron micrograph of λDNA-loaded PELA microspheres, (b) shows that of blank PELA microspheres, and (c) shows the PLA microspheres. The figure shows that the λDNA-loaded PELA microspheres are mainly spherical and the surface of them appears pitted and irregular particles still exist. Since the molecules of PELA are somewhat negative, and so are those of λDNA, it is difficult to form perfect spherical microspheres for the electrostatic repelling interaction, while the molecular weight of λDNA is larger and the linear structure makes the molecular chain longer. These factors are also harmful to forming microspheres. Consequently, the surface of λDNA-loaded microspheres is not as smooth as that of blank PELA microspheres. However, the surface of PELA microspheres is smoother than that of the PLA microspheres. This is also caused by the increased hydrophilic property of PELA, which leads to the increased phase compatibilities between PELA and the outer water phase in the preparation process.

According to the laser diffraction assay, the mean particle diameter of λDNA-loaded PELA (the molecular weight of PELA is 63 kDa) microspheres is 3.5 μm, which is very important to DNA-loaded microspheres. One of the reasons is that a smaller particle size can efficiently prevent capillary clogging. Also, the microspheres are expected to be taken up by phagocytes. It has been reported that, in order to be taken up by phagocytes, the particle size of the microspheres must be in the 10-μm range.[16] Another reason is that microspheres with a size of 1–5 μm can be targeted to certain tissues, such as the intestinal Peyer's patches, liver, and spleen after oral administration.[17] According to these, this particle is suitable for being taken up by phagocytes and for reaching certain tissues.

It is well known that the homogenization speed is crucial to the particle size, but the molecules of DNA are sensitive and easily damaged by a shearing force. In our investigations, the initial homogenization speed was less than 2000 rpm for 2 min, and the residual methylene chloride was extracted at a speed of 500 rpm. It was found that the particle size of the microspheres would be poorly distributed with a higher extraction speed and the morphology of the microspheres would be influenced also by the extraction speed. The integrity of the λDNA molecules after being extracted from the microspheres was analyzed by agarose gel electrophoresis.

Figure 2 shows the result of agarose gel electrophoresis. The agarose gel electrophoresis shows that the λDNAs extracted from the microspheres have two forms (lane 3). The two forms of λDNA are separated into distinct bands by electric voltage. The damaged λDNAs, being smaller than the integral λDNA molecules, move faster than do the integral λDNAs through the agarose polymer network, so that two

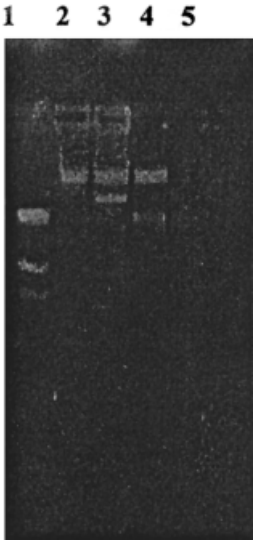

Figure 2 Agarose gel electrophoresis of λDNA.

distinct bands are shown. But the band of smaller molecules is narrower than that of the integral one, which indicates that most of the λDNAs keep their integrity through the preparation of the microspheres and extraction from the microspheres. But the molecular weight of λDNA is larger and the molecular chain is longer, so that the λDNA molecules are more sensitive to shearing and more easily damaged. Moreover, the λDNA is a linear molecule. Therefore, further works are needed to investigate whether the smaller, supercoiled DNA molecules can be encapsulated in PELA microspheres successfully. The residual methylene chloride present in the microspheres was below 20 ppm evaluated by gas chromatography, which was lower than the limited value according to the USPXXIII requirement (i.e., 500 ppm for methylene chloride).[18]

DNase I degradation

As we expected, PELA can protect λDNA from being degraded by DNase I. In Figure 2, lane 5 shows free λDNA after being degraded by DNase I, and lane 4 shows λDNA encapsulated in microspheres after exposure to DNase I. The electrophoresis shows that free λDNA treated by DNase I degraded completely, while λDNA encapsulated in the microspheres was able to keep its integrity after exposure to DNase I.

In vitro degradation

Figure 3 shows the changes of the molecular weight of λDNA-loaded PELA and PLA microspheres *in vitro*. The molecular weight decreases with the incubation time. As to the PELA microspheres, the decrease is more rapid initially, but slower afterward. The molecular weight of λDNA-loaded PELA microspheres decreases more rapidly than that of the blank PELA microspheres. In contrast, decrease of the molecular weight of the PLA microspheres is at a constant velocity. In the investigations, the molecular weight showed about a 57.3% loss of blank PELA microspheres after 80 days, while a 76.0% loss of molecular weight was observed for the λDNA-loaded PELA microspheres; the PLA microspheres only lost approximately 28.6% of their molecular weight. Figure 4 shows the mass loss of the PELA microspheres during degradation. After the first 40 days, no distinct mass loss can be detected for the PLA and PELA microspheres, and λDNA-loaded PELA microspheres show

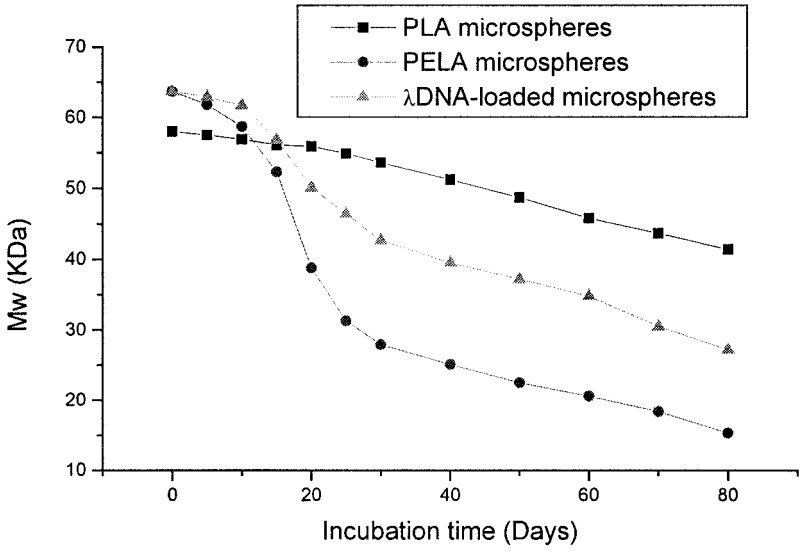

Figure 3 Molecular weight loss of PELA microspheres *in vitro*.

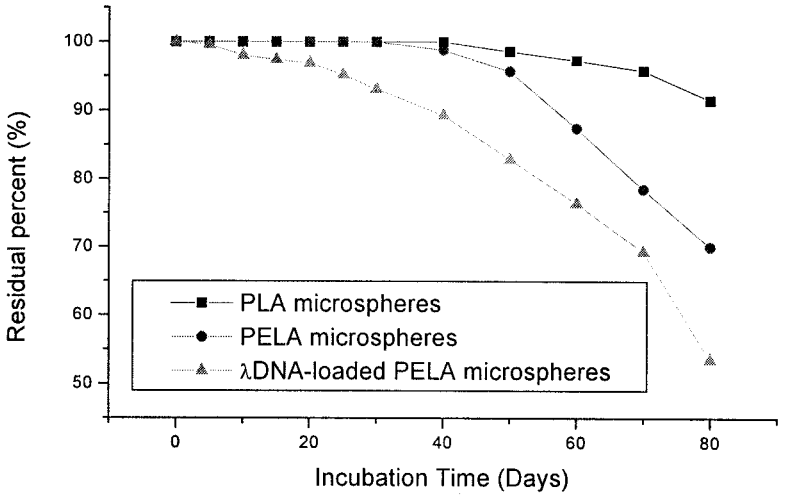

Figure 4 Mass loss of PELA microspheres *in vitro.*

a slow mass loss. Afterward, the mass of λDNA-loaded PELA microspheres is lost rapidly, whereas the PELA microspheres show a slow loss until 50 days, but the PLA microspheres exhibit a very slow mass loss. After 80 days, PLA, PELA, and λDNA-loaded microspheres maintained 91.5, 70.0, and 53.5% of their mass, respectively. From Figures 3 and 4, we found that the mass loss lags behind the molecular weight loss, which suggests that the mass loss occurs when the fragments of oligomers that are soluble in the degradation medium are formed, but the molecular weight loss begins when the microspheres are dispersed in PBS, that is, the scission of bonds begins as soon as the microspheres are exposed to PBS. When PEG, a soluble group in water, is introduced into PLA, the affinity between the water and the polymer is increased, the hydration of the polymer is promoted, and the permeation of water into the microsphere matrix is easier.

The presence of λDNA in the PELA microspheres leads to faster degradation. This might be attributed to the hydrophilic property of the matrix being increased after λDNAs are encapsulated by the microspheres. Another reason for that was the forming of passages in the microsphere matrices after the λDNA diffusion out of the microspheres. When the passages are formed, water permeates into the microspheres more easily, and then the hydration of the polymer is promoted.

It is well known that acid is harmful to the scission of the ester bond. The degradation of PELA will generate an acid medium for the part of PLA in the structure of PELA. At the beginning, the medium condition is neutral. The scission of ester bonds occurs as soon as microspheres begin dispersing in the PBS. With degradation of the polymer, the medium near the polymer becomes acidic and the scission of the ester bonds starts slowing. However, the cleavage of the ether bonds of PEG will accelerate in an acidic medium. Therefore, the degradation of PELA continues even more rapidly than at the beginning when two kinds of bonds in the molecular main chain of PELA both degrade. When the soluble fragments of the microsphere matrix are formed, there is a loss of mass of the microspheres

Figure 5 shows the changes of particle size during degradation. Because of the increase of hydration or swelling with the degradation, the diameters of the microspheres become larger and larger, but the changes are very slow at the beginning 30–40 days. During solidifying of the polymer matrix, with extraction of the solvent, the polymer molecules in one microsphere, generally, entangle; when the solvent is extracted out completely, the polymer molecules' entanglement networks are formed at the end. Consequently, the shape of most of the microspheres does not change roughly as long as the frame of the polymer entanglement network of the microspheres is not damaged, despite more and more pits and cavities forming on the outside surface. With degradation, the entanglement networks are generally damaged and the shape of the microspheres is changed little by little. After that, water enters the microspheres and the entanglement network, so the polymer swells, and then the particle size of the microspheres becomes larger and larger. Thereby, the velocity of the degradation is increased. Following that, the entanglement network is damaged so seriously that the microspheres collapse. Some collapsed microspheres form the fragments of polymers or oligomers; others gather to form membranes of the matrix, which is shown in Figure 1(d). Then, the particle size becomes larger and larger.

One reason for the formation of the matrix membranes is that, when the microspheres collapse, the fragments spread to form small membranes. Another reason is due to the molecular forces among the mol-

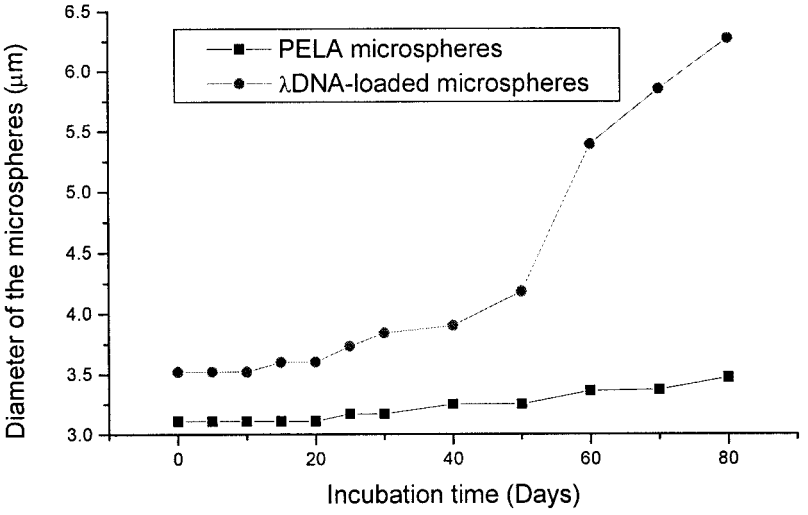

Figure 5 Particle-size changes of microspheres *in vitro*.

ecules of the collapsed microspheres and polymer fragments. Theoretically, the hydrolysis of chemical bonds (ester and ether bonds) occurs simultaneously with the formation of new bonds, but, in fact, the hydrolysis is much faster than is the bond formation; the bond formation cannot even be observed. However, the trend of gathering exists between molecules of the polymer. This offers the possibility for the collapsed microspheres and fragments to gather together. Also, the Brownian movement of the particles enhances the collision opportunities among the collapsed microspheres and fragments. When the collapsed microspheres or fragments collide with each other, the macromolecules of different fragments of the collapsed microspheres entangle by the molecular force. The molecular interlocking makes the membranes of the matrix form gradually.

Figure 6 shows the changes of the particle-size distribution of λDNA-loaded PELA microspheres. From the figure, it is seen that, with the incubation time, the amount of larger-size particles increased gradually. We know that the change of the particle size is very little for the initial 30–40 days. But the distribution of the size changes. The reason is that the large microspheres and irregular particles will contact with the water molecules more easily because of the large surface area and the pits and cavities on the surface, and then the large microspheres will swell first and form the larger particles. Moreover, we perceived that the loss of molecular weight was obvious during the initial 40 days; at the same time, the mass loss of the microspheres was not remarkable and neither was the change of the particle size. However, the particle-size distribution was changed distinctly. These results indicate that the bonds of polymer molecules cleave as soon as the microspheres are exposed to PBS. The consequences of bond cleavage are the loss of molecular weight and the formation of some polymer fragments, which are not soluble in the degradation medium; thus, no mass loss of microspheres is observed. Also, the size of these fragments may be larger than those of the original microspheres, which is the result of the spreading of some parts of the microspheres' surface. Moreover, then, the particle-size distribution is changed. But the larger fragments and irregular particles are in the minority, so the change of the particle-size distribution has little effect on the mean particle size.

With degradation, the "membranes" are generally dissolved in the degradation medium, and the particle size must become smaller and smaller, until complete disappearance of the membrane. As mentioned above, the mass loss does not commence until the polymer fragments that are soluble in the degradation medium are generated; hence, the mass loss lags behind the molecular weight loss, which suggests that the PELA copolymer hydrolysis should contribute to the bulk degradation of the polymer, which is identical with the degradation mechanism of PLA.[19] Furthermore, it is required that the surface layer hydrolysis and erosion must precede the molecular weight loss, which is confirmed by Figure 3. In the figure, the rate of the molecular weight loss is relatively lower during the initial days. The phenomenon proves that time is needed for water to permeate into the polymer matrix completely. Figures 3 and 4 also show that the molecular weight loss and mass loss of PELA microspheres are faster than are those of the PLA microspheres, and those of λDNA-loaded PELA microspheres are even more faster than those of the PELA microspheres. The results reflect the improved water uptake and swelling of the polymer and the increased hydrolysis rate of the polymer with the introduction of hydrophilic PEG domains into PLA. The encapsulation of λDNA within

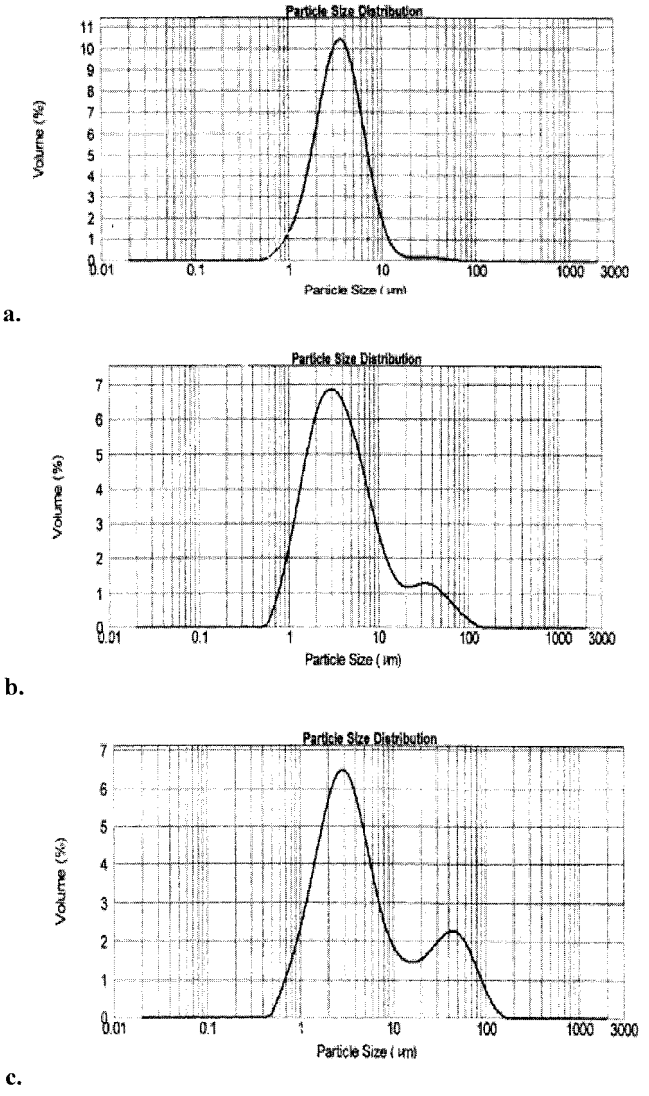

Figure 6 Changes of particle-size distribution of λDNA-loaded PELA microspheres *in vitro*.

the polymer matrix increases the hydrophilicity of the microspheres. These are proved by the difference of the diameters measured by SEM and laser diffractometry, respectively. For instance, the diameters measured by laser diffractometry are 4.8, 2.1, and 3.5 μm for λDNA-loaded PLA microspheres, blank PELA microspheres, and λDNA-loaded PELA microspheres, respectively, while the diameters measured by SEM are 4.3, 1.8, and 2.7 μm, and the increased rates are 10.4, 14.3, and 28.6%, respectively, because the microspheres are dispersed in water in the laser difractometer, while microspheres are required to be the vacuum-dried state for SEM measurement.

From the above, it can be concluded that the degradation of PLA and PELA commences from the permeation of water into the polymer matrix, ensued by the bulk hydrolysis of ester bonds, and followed by the polymer molecular weight loss and then the mass loss. The existence of a hydrophilic PEG domain promotes the permeation of water, and λDNA loading improves the permeation further.

In vitro release

Figure 7 shows the *in vitro* release of λDNA-loaded microspheres. In the initial 0–10 days, the release of λDNA from the PELA microspheres was up to approximately 20%. A slower sustained release was observed over the next days. But with the PLA microspheres, the amount of burst release was approximately 56–58%.

It is well known that systems based on PLA and its copolymers in an aqueous environment undergo hydration followed by bulk erosion. During erosion, the porosity of the matrix increases and the release of macromolecules by diffusion occurs, that is, the release of λDNA from a microsphere-delivery system depends on both erosion and diffusion. A variety of factors can influence DNA release from the microspheres, including the chemical structure and molecular weight of the polymer, the particle size and morphology of the microspheres, the loading amount and the solubility of the DNA, and the concentration of the release medium, which suggests that no simple mathematical model can be applied to predict or quantify the release rates in these systems because many factors can control DNA release from the microspheres.

In this investigation, the release of λDNA-loaded PELA microspheres revealed a similar biphasic trend, that is, an initial burst of release followed by a period of slow, but sustained release. These phenomena occur because of the electrostatic interaction and the dispersion pattern of DNA within the microspheres. Some DNA molecules are located close to the surface of the microspheres and are released by diffusion before significant degradation of the polymers occurs. Also, the electrostatic repelling forces, which are both between the DNA molecules and the polymer and among the DNA molecules, promote the diffusion of

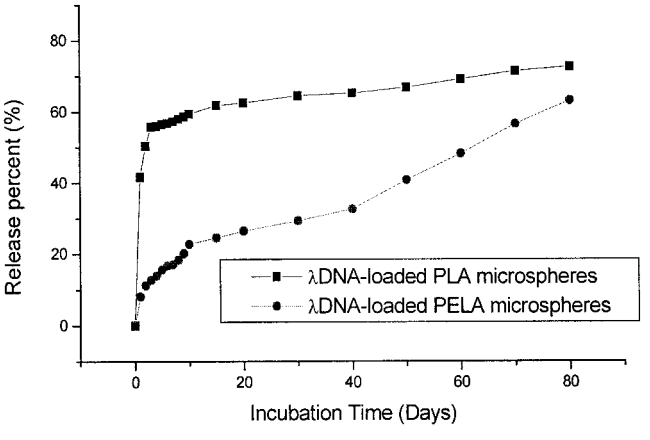

Figure 7 *In vitro* release of λDNA from microspheres.

the DNA molecules into the degradation medium. So, burst release is observed. When the DNA molecules pass through the polymer matrix and degradation occurs, the passages are formed in the microsphere matrix gradually. After the passages are formed, the sustained release begins.

In Figure 7, it can also be perceived that the first phase of release is approximately from 0 to 10 days; after 10 days, it is the second release phase. At the initial 5 days, it shows a 15.6% release is shown; until 10 days, a 22.7% release is shown. But the λDNA-loaded PLA microspheres show a 56.5% release in only 5 days. It is known that the initial burst release is the result of that some λDNA molecules are located close to the surface of the microspheres and release by diffusion before significant degradation of the polymer. The polymer, PLA, is hydrophobic, so that the affinity between the polymer and the DNA molecule is very weak. In the preparative process of the microspheres, most of DNA molecules scatter close to the surface of the microspheres. Also, then, when the microspheres are dispersed in the PBS, burst release is observed before the mass loss and sustained release.

The introduction of PEG in PLA results in the DNA molecules being located preferentially within the deeper sections of the microsphere polymeric matrix, as the improved hydrophilicity of the polymer leads to improvement of the affinity between the polymer and water-soluble macromolecules. For this reason, the burst amount of the PELA microspheres is not as much as that of the PLA microspheres. In Figure 7, it is observed that around 15.6% of λDNA is located close to the surface of the PELA microspheres. These λDNA molecules are released, obviously, before the molecular weight loss and mass loss are observed. After the λDNAs located on the surface of the microspheres leave the microspheres, more pits and cavities are formed on the surface of the microspheres. The specific surface of the microspheres is increased; thus, the contact between the water and the microspheres increases. But the swelling of the polymer is limited in such a small amount of time; the small decrease of the particle size caused by the DNA release and the small increase of the particle size caused by the polymer swelling make the apparent particle size to have no notable change. The λDNA molecules in the deeper section release gradually; nevertheless, most DNA molecules are entrapped within the inner microspheres. They will not be released until the passages passing through the microspheres are formed and water enters the inner microspheres. After water enters the inner microspheres, which is about 10 days in the figure, the swelling of the polymer increases, the degradation of the polymer increases, and the size of the apparent particle also increases. All the increases are correlated with the degradation profiles, which are proved in Figures 3–5.

As to the DNA molecules in the inner microspheres, the formation of passages marks the commencement of sustained release. After that, gradually, more and more polymers are degraded, more and more passages are formed, more and more water enters the inner microspheres, and the particle size becomes larger and larger. Up to 40–50 days, the diameter of the microspheres increases more rapidly; at the same time, the release of DNA also increases, which is in close agreement with the breakdown of the mass loss of the polymer. Therefore, when the molecular weight loss becomes significant and the mass loss is remarkable, an increase of the formation of passages is achieved and a higher release rate is observed. Besides, we know that the degradation of PLA will generate acid substances, which will acidify the degradation medium generally, whereas the acid condition is harmful to the DNA molecules. However, the PELA copolymer is able to support a more stable condition for DNA. By virtue of the hydrophilicity of PEG in PELA, the swelling of PELA is easier than that of PLA. The swelling leads to a rapid exchange of ions, which results in a neutral pH condition inside the microspheres. The neutral condition prevents aggregation and denaturation of the DNA molecules. Consequently, it is assumed that the release behavior of PELA microspheres may be in accordance with the swelling mechanism in addition to the diffusion and erosion mechanisms.

CONCLUSIONS

In the current studies, the mean size of λDNA-loaded PELA microspheres was about 3.5 μm, which was needed for them to be taken up by the phagocytes. After being encapsulated, λDNA was able to mainly retain its structural integrity. Furthermore, being encapsulated by PELA can prevent λDNA from being degraded by DNase. The *in vitro* degradation of the λDNA-loaded microspheres was faster than that of the blank PELA microspheres, and the release of λDNA from the PELA microspheres showed a biphasic form: an initial burst release followed by a sustained release. The degradation profile of the PELA microspheres is quite different from that of the PLA microspheres. The introduction of hydrophilic PEG domains in the polymer and the presence of DNA within the microspheres show a remarkable influence on the degradation and release profiles. Compared with the PLA microspheres, the PELA microspheres exhibited some advantages, such as swelling in the degradation medium, the generation of a more stable condition for DNA, and the minimization of the initial burst release. In the studies, a good agreement was obtained between the degradation profile and the release profile.

As a novel matrix polymer for a gene-delivery system, PELA has shown some advantages, such as the

existence of a hydrophilic group and a controllable release rate depending on controllable composition. However, it is clear that more detailed investigations are necessary to clarify the effect of the preparative parameters on the microsphere characteristics and the stability of DNA, the effect of the polymer matrix on the degradation of microspheres and the release of DNA, and the effect of the characteristics of different DNA molecules. Also, the gene transfection and gene expression *in vitro* and *in vivo* all need urgent further studies. There is a long way to go. But with the developing of experiments, we will find that the PELA microspheres can be used as a controlled release-delivery system for DNA successfully and that it is also a potential method in future studies of gene therapy.

The authors gratefully acknowledge the National Project 973 of China and the National Natural Science Foundation of China (Grant 20004009) for financial support.

References

1. Behr, J. P. Acc Chem Res 1993, 26, 274.
2. Mulligan, R. C. Science 1993, 260, 926.
3. Katayose, S.; Kataoka, K. Bioconjug Chem 1997, 8, 702.
4. Felgner, P. L. Adv Drug Deliv Rev 1990, 5, 163.
5. Mumper, R. J.; Wang, J.; Claspell, J. M.; Rolland, A. P. Proceed Int Symp Control Rel Bioact Mater 1995, 22, 178.
6. Haensler, J.; Szoka, F. C. Bioconj Chem 1993, 4, 372.
7. Uchida, T.; Goto, S. J Pharm Pharmacol 1994, 47, 556.
8. Cohen, S.; Yoshioka, T.; Lucarelli, M.; Hwang, L.; Langer, R. Pharm Res 1991, 8, 713.
9. Siegel, R.; Langer R. Pharm Res 1984, 1, 2.
10. Wang, D.; Robinson, D. R.; Kwon, G. S. Samuel, J. J Control Rel 1999, 57, 9.
11. Ando, S.; Putnam, D.; Pack, D. W.; Langer, R. J Pharm Sci 1999, 88, 126.
12. Kenley, R. A.; Lee, M. O.; Mahoney, T. R.; Sanders, L. M. Macromolecules 1987, 20, 2398.
13. Deng, X. M.; Xiong, C. D.; Cheng, L. M. J Polym Sci Polym Lett 1990, 28, 411.
14. Li, X. H.; Xiao, J.; Deng, X. M.; Li, X. Y.; Wang, H. L.; Zhang, W. B.; Jia, W. X.; Men, L.; Yang, Y.; Zheng, Z. X. J Appl Polym Sci 1997, 66, 583.
15. Deng, X. M.; Li, X. H.; Yuan, M. L.; Xiong, C. D.; Huang, Z. T.; Jia, W. X.; Zhang, Y. H. J Control Rel 1999, 58, 123–131.
16. Sisay, G.; Byung, W. H.; Patrick, P. D. AAPS Pharm Sci Tech 2000, 1, 1(article 28).
17. Eldridge, J. H.; Hammond, C. J.; Meulbroek, J. A. J Control Rel 1990, 11, 205.
18. Bitz, C.; Doelker, E. Int J Pharm 1996, 131, 171–181.
19. Holland, S. J.; Tighe, B. J.; Gould, P. L. J Control Rel 1986, 4, 155.