

RING-OPENING COPOLYMERIZATION OF DL-LACTIDE AND POLY(ETHYLENE GLYCOL) INITIATED BY LANTHANUM ACETATE AND APPLICATION OF THE COPOLYMER AS MATRIX OF MICROSPHERES CONTAINING *VIBRIO CHOLERA* ANTIGENS*

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Abstract Poly-dl-lactide-poly(ethylene glycol) (PELA) triblock copolymers were synthesized with lanthanum acetate as the initiator. PELA microspheres with entrapped *Vibrio Cholera* antigen and outer membrane protein (OMP) were prepared by a double emulsion W/O/W based on solvent extraction methods. The obtained microspheres showed smooth and spherical surface and their size varied between 0.5 and 5.0 μm , which are suitable for oral targeting delivery system. The distribution tests in rabbits and mice through scanning electronic micrography and fluorescence microscope indicated that microspheres have successfully reached the immunization-related tissues, such as the liver, spleen and intestinal peyer's patches, following oral administration. The PELA microspheres were also evaluated as an efficient antigen delivery system by enhancing a higher protective ratio against live *Vibrios Cholera*.

Keywords Poly-dl-lactide-poly(ethylene glycol), *Vibrio Cholera* antigen, Lanthanum acetate, Microsphere, Protective ratio

INTRODUCTION

In recent years, there are various attempts to demonstrate new immunization strategies to induce higher level and larger duration of immune responses following parenteral^[1] and/or oral^[2] administration. Antigens for oral immunization, which is thought as an economical, safe and convenient way, are required to avoid degradation in the intestine and enhance absorption into the intestinal peyer's patches. The potential applicability of biodegradable microspheres as oral antigen delivery system is based not only on sustained antigen release and their ability to protect the antigen from acidic and proteolytic degradation in the intestine, but also on the fact that microspheres with a diameter under 5 μm could be phagocytized and transported by macrophages into the draining lymph nodes from the intestinal lumen^[3]. Polylactide (PLA) and poly(lactide-co-glycolide) (PLGA) are widely known as biodegradable and biocompatible polymer, which have

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been used in many biomedical fields including as matrix of antigen delivery system^[4]. The second component polyethylene glycol (PEG) is introduced into PLA to form triblock polymer PLA-*b*-PEG-*b*-PLA, which is expected to increase the affinity of matrix polymer and water-soluble antigens and to decrease the amount of emulsifier used in microspheres preparation.

Poly-dl-lactide-poly(ethylene glycol) (PELA) block copolymer was studied by a number of investigators. Deng and Zhu *et al.* performed the pioneering investigations on the synthesis of PELA by using dl-lactide and PEG as the starting materials with various initiators^[5-7]. PELA were also prepared by using the transesterification method from PDLLA and PEG^[8] and by using macroinitiators^[9], such as potassium poly(ethylene glycol)ate and sodium poly(ethylene glycol)ate. But more investigations were carried out for PELA, which was synthesized from dl-lactide and PEG with such initiators as stannous 2-ethyl hexanoate and stannous chloride. The potential problem in the synthesis is related to the toxicity of this kinds of initiators in clinical uses. To enhance the biocompatibility of polymers, in recent years, much interest has been placed on exploring new non-toxic initiators with high performance. Rare earth compounds meet these requirements due to their rich resource in China, no significant adverse effects to humans and extraordinarily active for ring-opening polymerization of lactones^[10-11]. Up to now, the widely used rare earth catalysts are rare earth alkoxides, which have been proved to be good initiators for living ring-opening polymerization^[12], and alkyl rare earth compounds^[13]. But these catalysts are limited and cannot be widely used because of their complex preparation procedure, harsh process conditions and high sensitivity to water and oxygen gas.

In the present study, triblock copolymer PELA was synthesized with lanthanum acetate as the initiator, which was more stable and easier to prepare. PELA was characterized by ¹³C-NMR and ¹H-NMR, and factors that influence the yield and molecular weight were also investigated. The PELA microspheres containing *Vibrio Cholera* antigen and outer membrane protein (OMP) were prepared by a double emulsion W/O/W based on solvent extraction methods. The microspheres obtained were administered into New Zealand rabbits and their distribution in the liver, spleen and intestinal peyer's patches was inspected one month post-administration. The protective ratio against *Vibrio Cholera* was tested on *Balb/c* mice by counting the survival ratio after the introduction of live *Vibrio Cholera* throughout abdominal cavity.

EXPERIMENTAL

Materials

DL-lactic acid was obtained from Chemical Factory of Hubei University. *Vibrio Cholera* antigen OMP (M_w : 41000) was provided by West China University of Medical Sciences. Rhodamine was from Sigma Company. PEG (M_w = 6000) and other reagents were commercially available.

Synthesis and Characterization of Copolymer PELA

The initiator lanthanum acetate was prepared and characterized as described previously^[14]. The triblock copolymer PELA was produced by ring-opening polymerization of lactide and PEG. Briefly, the pretreated lactide and PEG were transferred to a clean and dry ampoule bottle and freshly prepared initiator was added. The bottle was evacuated at 80°C, purged with nitrogen gas for three times, sealed after evacuating for 2 h and placed into an temperature-controlled oven. After the desired polymerization period, the crude product was dissolved in methylene chloride and precipitated in excess methanol. The precipitated polymer was dried in vacuum at 40°C. PELA was characterized by IR, ¹³C-NMR and ¹H-NMR. The average molecular weights of PELA were obtained from viscosity measurement and calculated according to following equation: $[\eta] = 1.04 \times 10^{-4} M_v^{-0.75}$ ^[15]. The intrinsic viscosity of PELA in tetrahydrofuran was measured at a Ubbelohde capillary viscometer in a water bath at a constant temperature of $37 \pm 0.2^\circ\text{C}$.

Preparation and Characterization of PELA Microspheres Containing OMP

PELA-10 (PEG content: 10%) microspheres containing OMP were prepared by using a water-in-oil-in-water emulsion based on solvent extraction process, as described previously^[16]. The surface morphology of microspheres was evaluated by scanning electronic micrography (SEM, Amray, USA), and their size was obtained by measuring the diameters of at least 500 particles shown in SEM. The level of residual methylene chloride within microspheres was determined by gas chromatography (Shanghai Analytical Instrument Factory), and compared with a set of standard samples with known amounts of methylene chloride. The OMP entrapment was measured by dissolving a sample of microspheres in methylene chloride, extracting OMP with double distilled water and determining the amount of OMP in the extracted solution by an ultraviolet spectrophotometer (Shimadzu UV-120, Japan). Rhodamine (fluorogenic substance) labeled microspheres were prepared by adding rhodamine into the PELA microspheres suspension and stirring for several hours. The solid microspheres were collected by centrifugation and rinsed three times with distilled water.

Determination of the Distribution of Microspheres in Vivo

New Zealand rabbits were administered microspheres suspended in water using a feeding needle inserted into the stomach. One month later, the rabbits were killed and their representative tissues, such as the liver, spleen and intestinal peyer's patches were collected. The fracture sections of these tissues, prepared by freezing in liquid nitrogen and breaking, were inspected by SEM to determine the targeting distribution of microspheres in animals. Similarly, groups of *Balb/C* mice were administered with rhodamine (as a tracer) labeled microspheres. On the 3rd, 7th and 15th day, two of the mice were killed and such tissues as the liver, spleen and small intestine were collected. The tissues were frozen in liquid nitrogen and cut into series sections, which were viewed on a fluorescence microscope to qualify the dynamic distribution of microspheres in each tissue.

Test of Immunization Protective Ratio

Three groups of mice were immunized twice at an interval of one week. Two groups of mice were orally and subcutaneously administered PELA microspheres containing *Vibrios Cholera* antigen, the third group was orally immunized with free antigens. At the 10th day after the latest immunization, the immunized and unimmunized (as a control group) groups of mice were challenged through abdominal cavity with 0.5 mL suspension (5000 *Vibrios Cholera* per milliliter) of live *Vibrios Cholera* 18003 strain (EL-TOR, Inaba), the death/survive ratio was inspected for 72 h. The LD₅₀ was determined from unimmunized group of mice challenged with different amount of live *Vibrios Cholera*.

RESULTS AND DISCUSSION

Synthesis and Characterization of PELA

To inspect factors influencing the molecular weight and yield of PELA, different batches of polymerization were carried out and the results are shown in Table 1. From the data (Nos. 1, 2 and 3), it is suggested that higher yield is achieved with an increase in the amount of initiator used (i.e. a decrease in the weight ratio of monomer to initiator, M/I). The number of active centers increases with an increase in the amount of initiator, which leads to higher yield but lower molecular weight due to the excessive number of active centers. Both larger yield and higher molecular weight are obtained by extending the polymerization time, but the molecular weight does not increase any longer when it reaches a certain value, which reveals that polymerization maintains an equilibrium (Nos. 2 and 4). The PEG content has considerable effect on the molecular weight (Nos. 4, 5, 6, 7 and 8). A higher PEG content leads to a lower molecular weight of obtained copolymer, because the hydroxyl groups of PEG interrupt the propagation of polymer chain. The

decrease in the molecular weight also suggested that the obtained polymer should be copolymer of LA and PEG. It was determined from the structural analysis of oligomers prepared under the present initiator and polymerization conditions that the polymerization was agreement with cationic mechanism, which had been investigated in detail in a previous study^[14].

Table 1. Copolymerization of LA and PEG with lanthanum acetate^a

No.	<i>M/I</i> ^b	PEG (%)	<i>t</i> (h)	Yield(%)	[η] (dL/g)	<i>M_v</i> (×10 ⁴)
1	500	10.0	5	81	0.22	2.8
2	1000	10.0	5	79	0.24	3.1
3	1500	10.0	5	61	0.26	3.4
4	1000	10.0	10	84	0.27	3.6
5	1000	5.0	10	87	0.30	4.1
6	1000	20.0	10	76	0.16	1.8
7	1000	30.0	10	78	0.15	1.7
8	1000	50.0	10	68	0.13	1.4

a) Polymerization temperature: 180°C,
b) The weight ratio of monomer to initiator, weight of monomer: 2.0g

The spectral data of PELA-10 with 10% of PEG content are listed below. IR (in KBr): 1756 cm⁻¹ (C=O, straight strain); ¹³C-NMR (in CDCl₃): δ = 75.1 (CH₂, in the middle of the PEG chain), δ = 64.5 (CH₂, CH₂—CH₂—O—C(=O)—); ¹H-NMR (in CDCl₃): δ = 1.55 (CH₃), δ = 3.6 (CH₂), δ = 5.12 (CH). The spectral data confirmed the block polymerization of LA and PEG and the structure of triblock copolymer PELA.

Characterization of Microspheres Containing OMP

It has been reported that there is limited efficiency of microspheres to be phagocytized and transported by macrophages into the draining lymph nodes from the intestinal lumen following oral administration^[3], so the size and its distribution are critical for microspheres to reach the targeting sites efficiently. All batches of PELA microspheres had smooth spherical surface structure, devoid of pits and pores, as shown under SEM (Fig. 1), while the microspheres prepared from PDLLA homopolymer appeared to be pitted and some of them had collapsed according to a previous report^[16]. A mean microspheres diameter was less than 5 μm and a typical size distribution is shown in Fig. 2. The level of residual methylene chloride present in the microspheres was less than 20 ppm as measured by gas chromatography, which was safe to humans according to the USP XXIII Requirement. The amount of OMP entrapment varied between 2% ~ 4% (W/W) and OMP loading efficiency between 40% ~ 60% (W/W), depending on the preparative parameters.

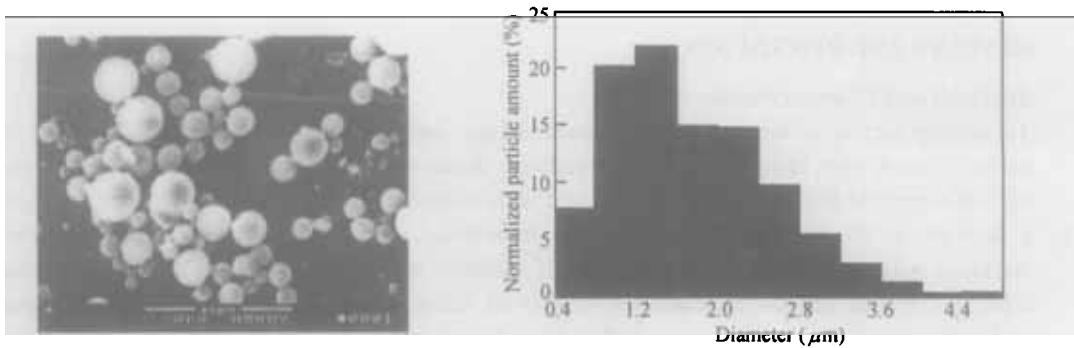


Fig. 1 The SEM of PELA microspheres containing OMP

Fig. 2 The size distribution of PELA microspheres containing OMP

Distribution of Microspheres in the Immunization-Related Tissues of Mice and Rabbits

The possibility of using PELA microspheres as an oral vaccine delivery system was tested by investigating the dynamic distribution of microspheres containing rhodamine in the immunization-related tissues, such as the intestinal peyer’s patches, liver and spleen of mice. Fluorescence microscopic observation of frozen-sectioned tissues revealed that microspheres assembled in the draining lymph nodes of the small intestine on the 3rd day after administration. On the 7th day the number of microspheres decreased in the lymph nodes but increased in the liver and spleen, while the total number of microspheres present in the three tissues fell significantly on the 15th day after administration. The obtained result is in agreement with Eldridge’s report, in which PLGA was performed as the polymer matrix^[3]. The data also showed that, following oral administration, microspheres with a size of lower than 5 μm were absorbed into the intestinal peyer’s patches, and then migrated into the spleen and liver, where antigen release would be expected to induce systemic and secretory antibody response.

Figure 3 shows the PELA microspheres locating in the intestinal peyer’s patches (a), spleen (b) and liver (c) of rabbits one month after oral administration, which indicated that microspheres containing OMP had successfully reached the targeting sites of immunization-related tissues.

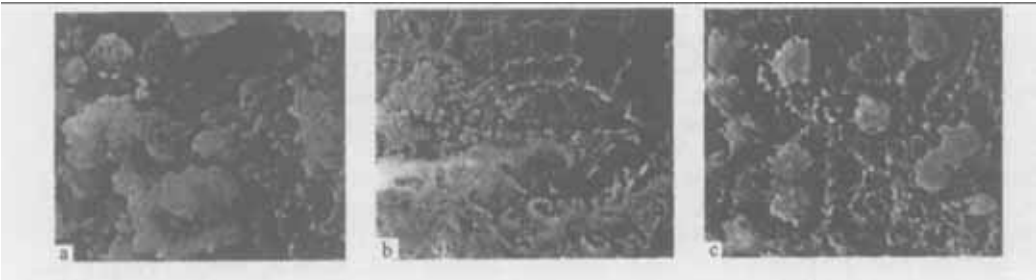


Fig. 3 The SEM of the fractured sections of the intestinal peyer’s patches (a), liver (b) and spleen (c) of rabbits 30 days after administration of PELA microspheres containing OMP

Protective Immune Response of Orally Immunized Microspheres

The LD₅₀ value was determined to be 833 *Vibrios Cholera* for each mouse. Immunized and unimmunized mice were administered 2500 live *Vibrios Cholera* each through the abdominal cavity and the results are shown in Table 2. As seen in Table 2, the control group of mice and mice orally administered free antigen produced nearly no protective immune response (0% and 10%, respectively) because the amount of live *Vibrios Cholera* was three times higher than LD₅₀. The protective ratio of mice orally immunized with PELA microspheres containing OMP was 70%, slightly lower than those of mice with the equal amount of microspheres by subcutaneous administration (80%), which is widely used in nowadays. But oral administration is known to be a convenient and more acceptable way of immunization as not only can it decrease the cross infection caused by injection, but it is also suitable for preventing widely-spread infectious diseases and is also even more preferred in developing countries where there are shortages of needles and

Table 2. Immunization protective test *in vivo*

Groups	Immunization (OMP μg/mouse)	Mice number	Death/Survive			Protection ratio (%)
			24 h	48 h	72 h	
Oral MS-OMP	100×2	10	2/8	3/7	3/7	70
S.C. MS-OMP	100×2	10	0/10	1/9	2/8	80
Oral Free OMP	100×2	10	4/6	6/4	9/1	10
Control		10	5/5	8/2	10/0	0

Challenged strain: EL-TOR biotype Inada 18003, challenged amount: 2500/mouse (LD₅₀ : 833/mouse), S.C.: subcutaneous, MS: PELA microspheres

trained personnel to administer the drug.

In conclusion, PELA was synthesized from lactide and PEG initiated by lanthanum acetate, a non-toxic, stable and active initiator. PELA microspheres containing the *Vibrios Cholera* antigen, OMP with smooth spherical surface and a certain size for oral administration were obtained. The PELA antigen microspheres were evaluated as an efficient antigen delivery system by oral administration.

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