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Enhanced Antigen Delivery Systems Using Biodegradable PLGA Microspheres for Single Step Immunization

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To demonstrate their possibilities as an enhanced vaccine delivery system, protein-loaded Poly lactide glycolide copolymer (PLGA) microspheres were prepared with different physical characteristics. Ethyl acetate (EA) solvent extraction process was employed to prepare microspheres and the effects of process parameters on drug release properties were evaluated. The biodegradability of microspheres was also evaluated by the pH change and GPC (Gel permeation chromatography). Primary IgG antibody responses in BALB/c mice were compared with protein saline solutions as negative controls and adsorbed alum suspensions as positive controls after single subcutaneous injection for *in vivo* studies. The microspheres showed a erosion with a highly porous structure and did not keep their spherical shape at 45 days and this result could be confirmed by GPC. *In vitro* release of proteinous drug showed initial burst effect in all batches of microspheres, followed by gradual release over the next 4 weeks. PLGA microspheres were degraded until 45 days and the secondary structure of OVA was not affected by the preparation method. Enzyme-linked immunosorbent assays demonstrated that the single subcutaneous administrations of OVA-loaded PLGA microspheres induced enhanced serum IgG antibody response in comparison to negative and positive controls. These results demonstrated that microspheres providing the controlled release of antigens might be useful in advanced vaccine formulations for the parenteral carrier system.

Key Words: PLGA, Ovalbumin, Microsphere, Solvent extraction, Immune response

INTRODUCTION

Vaccination against smallpox, polio, DTP (diphtheria, tetanus, pertussis), measles, and other pathogens has reduced mortality and contributed significantly to today's life expectancy. Despite these successes, vaccine development has significant problems because vaccine has traditionally involved with the use of live attenuated organisms. At the time of vaccine administration, the subject is often an infant or child, with no personal perception of immediate benefit. Because of such social issues, vaccines must be perceived as 'completely' safe, easy to administer, little pain upon delivery, and be effective against the pathogens of the region (Powell, 1996).

Recombinant, subunit, synthetic, and purified antigens could overcome many of these difficulties. But these peptide antigens are unfortunately poorly immunogenic and still require the presence of an immunomodulator to induce immunity (Newman et al., 1998). Moreover, they have multiple and high-dose injections required to achieve desirable therapy (Blanco et al., 1998). On this account, immunological adjuvants are commonly needed in animal studies include surfactants, cytokines and components of bacterial cell walls (McGee et al., 1994; Zahirul et al., 1994).

Aluminum hydroxide (alum) is the only widely used adjuvant for humans at present. The use of alum-type adjuvant for immunization, however, has also several disadvantages. First, Alum-type adjuvant has been reported to induce inflammation and to stimulate local production of granulomas. Second, although alum is efficient at increasing humoral immunity, cell-mediated immunity appears to be

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only slightly affected. Moreover, conventional alum-type vaccines require multiple recall injections at appropriately timed intervals in order to achieve long-lasting and optimal immune responses. Therefore, development of more efficient and safe adjuvant/vaccine delivery systems requiring single administration to obtain high and long-lasting immune responses is of primary importance (Men et al., 1995).

One promising approach to the development of new vaccines involves the encapsulation of antigens into microspheres prepared from PLGA polymers (McGee et al., 1994). The reason that these synthetic polyesters are approved by the FDA for human use, e.g. Zoladex™ (Zeneca), Decapeptyl™ (Ipsen Biotech) which are licensed for use in humans in Europe and the USA and have a 25 year history of safety (O'Hagen et al., 1991). Injected microspheres also exhibit good biocompatibility and induce only a minimal inflammatory response (Aguado, 1993; Claudio et al., 1996; Men et al., 1995; Sah et al., 1994; Singh et al., 1998). Microspheres have been chosen instead of alum-adsorbed protein suspensions and we present details of a multiple emulsion method for the preparation of PLGA microspheres with entrapped model protein; ovalbumin (OVA).

The purpose of the present study was to develop microparticulate systems for prolonged release vaccine delivery by microencapsulation techniques from our previously described systems (Cho and Kim, 2006) *in vitro* and *in vivo*. The microspheres prepared by our previous technique were evaluated by the immune response induced after single subcutaneous injection and antibody titers in immunized BALB/c mice were also determined using indirect ELISA method.

MATERIALS AND METHODS

1. Chemicals

PLGA; molecular weight 40,000, lactide: glycolide ratio = 50:50, Poly vinyl Alcohol (PVA; molecular weight 13,000 ~23,000), OVA (chicken egg, grade V) was supplied by Sigma-Aldrich, USA. Sodium dodecyl sulfate (SDS) from Bio-rad, USA. Tetramethyl benzidine substrate solution (TMB), Goat Anti-Mouse IgG (H+L), Horseradish peroxidase (HRP), N,N',N'-tetramethylethylenediamine (TEMED), Micro-BCA and isoelectric marker were supplied by Pierce, USA. BALB/c mice was supplied by Hanlim, Co. Korea. All materials were used as received.

2. Preparation of PLGA microspheres

The procedure and each concentration of microspheres were determined by the method from our previous report (Cho and Kim, 2006). Brifely, A solution of OVA in phosphate buffer containing PVA solution was emulsified with polymer in EA. The resulting water in oil emulsion was then emulsified with a PVA solution to produce a W/O/W emulsion. After W/O/W emulsion was produced, the solvent was rapidly extracted in an aqueous isopropyl alcohol solution. Microspheres were then collected by centrifugation, washing and freeze-dried. The final product was stored in the same condition as described previously. Antigen and polymer ratio was fixed at 1:5 and the viscosity of the external aqueous phase was determined by dissolving PVA at 5.0% (W/V).

3. Measurement of average molecular weight

The PLGA molecular weight during the degradation in the aqueous phase were determined by gel permeation chromatography (GPC) (Cohen et al., 1991; Park, 1994). For the determination of molecular weight for the polymer, the following conditions were adopted: column (Waters PLGel column, USA); column temperature: 35℃; mobile phase: tetrahydrofuran; flow rate: 1 ml/min; detection: differential refractmeter (Waters Model 410, USA). At the appropriate intervals, Microspheres were dissolved in tetrahydrofuran after centrifugation and lyophilisation and injected into the GPC equipment. Weight average molecular weights were calculated from the GPC curve using a series of polystyrene standards and were expressed as the weight average molecular weight.

4. *In vitro* release and pH change of PLGA microspheres

Accurately weighed microspheres (40 mg) were suspended in 4.0 ml PBS and retained in a orbital shaking incubator (Jeio-tech, SI900R, Korea) at 37℃ with successive shaking (250 rpm) (Alonso et al., 1993; Singh et al., 1995). At predetermined time intervals, pH of the suspensions was measured and the microsphere were centrifuged (12,000 rpm, 10 min) and replaced with 2 ml of fresh PBS. Protein concentration in the release medium was analyzed using micro-BCA assay. Release profiles were calculated in terms of cumulative release (%) with incubation time. In addition,

the pH change of solution released from microsphere was monitored at appropriate time.

5. Biodegradability of microspheres

For determination of *in vitro* degradation, the microspheres suspended in pH 7.4 phosphate buffered saline (PBS) solution. The microspheres were collected at fixed time points (15, 30, 45 days). The samples were dried and the surface appearance of the PLGA microspheres was analyzed by scanning electron microscopy (SEM) (JEOL 35CF, USA). For the surface analysis, freeze-dried microspheres were mounter onto metal slubs using double sided adhesive tape, dried under vacuum and coated with gold-palladium.

6. Immunization protocols

Female BALB/C mice, aged up to 6~8 weeks and weighing about 25 g, were used and maintained on a normal mouse diet throughout the study. To make the positive control preparation, OVA (4.4 mg) was added to 5 ml of and alum suspension (8.4 mg/ml) and incubated at room temperature for 24 hr orbital rotation. The alum samples were isolated from the incubation medium by centrifuging and washed once by resuspension in 5 ml of water. The washed samples were analyzed for protein content by a micro BCA assay and the amount of OVA adsorbed to the alum substrate was determined by subtraction (Coombes et al., 1996). Then, mice were injected with OVA in 0.25 ml physiological saline subcutaneous sites. Three groups of eight mice were immunized with 300 g OVA in the following formulations (Uchida et al., 1994).

(1) OVA free solution
(2) OVA adsorbed on alum
(3) OVA-loaded 50:50 PLGA microparticles prepared by multiple emulsion solvent extraction

Blood samples were collected from the tail veins of the mice at 2 week intervals for 12 weeks after immnunization. Thereafter, blood samples were collected from the tail veins at 4 week intervals until the termination of the study. In the mice, one of the lower veins on the underside of the tail was nicked at appropriate time intervals so that blood samples could be taken. The microcentrifuge tube containing blood was incubated at 37℃ for 1 h and was flicked several times to dislodge the blood clot. After storing the tube at 4℃ for overnight, it was centrifuged to collect serum. The serum was frozen at -20℃ until assayed for the levels of anti-OVA antibody (Harlow et al., 1988; O'Hagen et al., 1991; Sah et al., 1996).

7. Measurement of IgG by ELISA

Sensitive enzyme immunoassays were developed to monitor serum anti-OVA antibody titers in mice. These assays were indirect ELISA method and Antibody (IgG) responses in immunized animals are monitored using a modified microplate ELISA (Igartua et al., 1998). Briefly, 96-well ELISA microtiter plates (Nunc-Immuno Plate U96 Polysorp. Nunc) were coated with 50 μl/well of OVA 15 g/ml dissolved in Tris (hydroxymethyl)-aminomethane buffer saline (TBS) solution (pH 7.4) and incubated overnight at 4℃. The plates were thoroughly washed two times in 0.05% v/v Tween-20 solution TBS and followed by sterile saline and allowed to dry and blocked for 2 h at 37℃ with 200 μl of 1% bovine serum albumin (BSA) and the sera obtained from mice were diluted 1:1 in TBS and 50 μl of the diluted samples was loaded into each well of the microtiter plates. After incubation at 37℃ for 2 h, they were washed as same as before. Then each well was dispensed with 50 μl goat anti-mouse Ig horse radish peroxidase (HRP) conjugate (diluted to 1:2000 in 1% BSA). The microtiter plates were incubated at room temperature for 2 h followed by extensive washings with TBS. Then tetra methyl benzidine (TMB) as a substrate solution was added to each well and incubated again at room temperature for 15 min. The 50 μl of stopping solution (2 M H_2SO_4) was added and incubated again for 15 min. Results were expressed at optical density at 450 nm for each serum sample with a microplate reader. The antibody titers are shown as absorbance for all time points on sera.

RESULTS

1. Characteristics of PLGA polymers

To investigate the mechanism of protein release from the mcirspheres, we followed the evolution of the polymer Mw during the in vitro release process. From the GPC traces, the total amount of water solubilized degradation product released can be determined by measuring the peak area at different incubation periods. Since the amount of degradation products in the aqueous medium per unit time can be regarded as a polymer degradation rate. Fig. 1 shows the

plot of GPC peak area vs. various incubation time. Residual molecular weight of the polymer evaluated by GPC showed that the erosion of microparticles occurred until 45 days. Until 30 days, slow degradation was investigated but fast degradation was showed after 30 days. So, we carried out the *in vitro* release test by 45 days.

2. *In vitro* release of ovalbumin

Microspheres showed an initial burst release to 40% and gradual release over the next 6 weeks (Fig. 2). This release study demonstrates that the drug release rate may be controlled by selecting the method of preparation and adjusting the particle size. Typically, the protein is released from PLGA microspheres in three phases: an initial burst, diffusion controlled release, and erosion controlled release (Cleland et al., 1996; Cohen et al., 1991). The initial burst phase is a rapid release (within a few days) of protein at or near the surface of the microspheres. The diffusion controlled release phase consists of protein diffusion through pores or channels in the microspheres and erosion controlled release is produced by polymer erosion (hydrolysis). Although the microspheres represent the typical release pattern of a bioerodible matrix system, there is a lack of additional release in the erosion state, which might be due to low antigen loading or interaction of the PLGA polymer with ovalbumin. These phenomena are documented very well in numerous publications. For example, Lai et al. (1993) reported 70% of isoprenaline release within a few hours. Uchida et al. (1994) also reported that PLGA 50/50 microspheres with a small diameter (1.2 μm) showed an 80% burst release within one day, although additional release was very low as time passed.

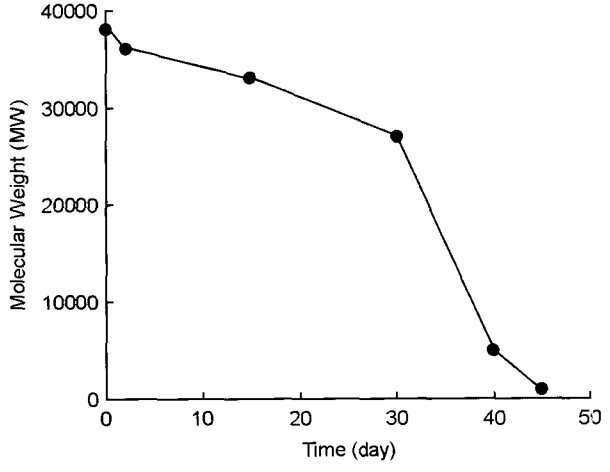

Fig. 1. Molecular weight change measured by Gel permeation chromatography (GPC). Column (Waters PLGel column, USA); column temperature: 35℃; mobile phase: tetrahydrofuran; flow rate: 1 ml/min; detection: differential refractmeter (Waters Model 410, USA).

3. pH change from PLGA microspheres

The dispersed PLGA microsphere suspensions indicated that there was no significant degradation due to the hydrolysis of the ester linkages of the polymer. There is little decrease of pH. An extensive degradation of the polymer should result in a decrease of the pH, because the random hydrolytic chain scission of polyester linkages generates free carboxylic acid end groups. A sufficient concentration

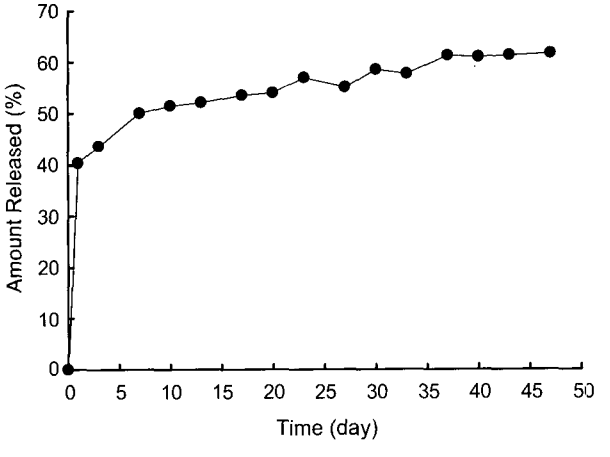

Fig. 2. Cumulative release profile of OVA from PLGA microspheres in 5% PVA concentration. Microspheres (40 mg) were suspended in PBS and retained in a orbital shaking incubator at 37℃ with successive shaking.

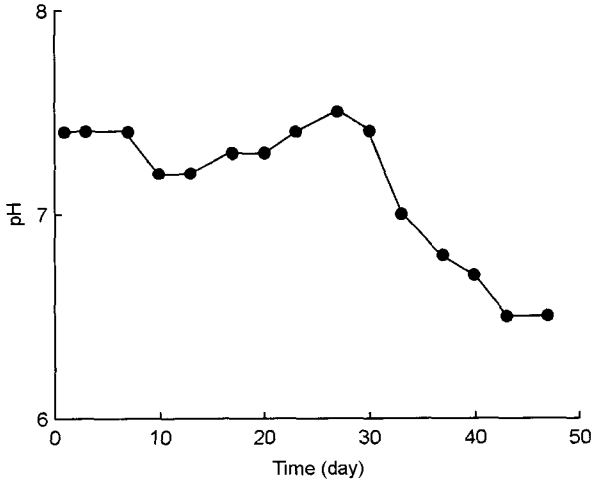

Fig. 3. pH change during *in vitro* release of OVA-loaded PLGA microspheres. the pH change of solution released from microsphere was monitored at appropriate time.

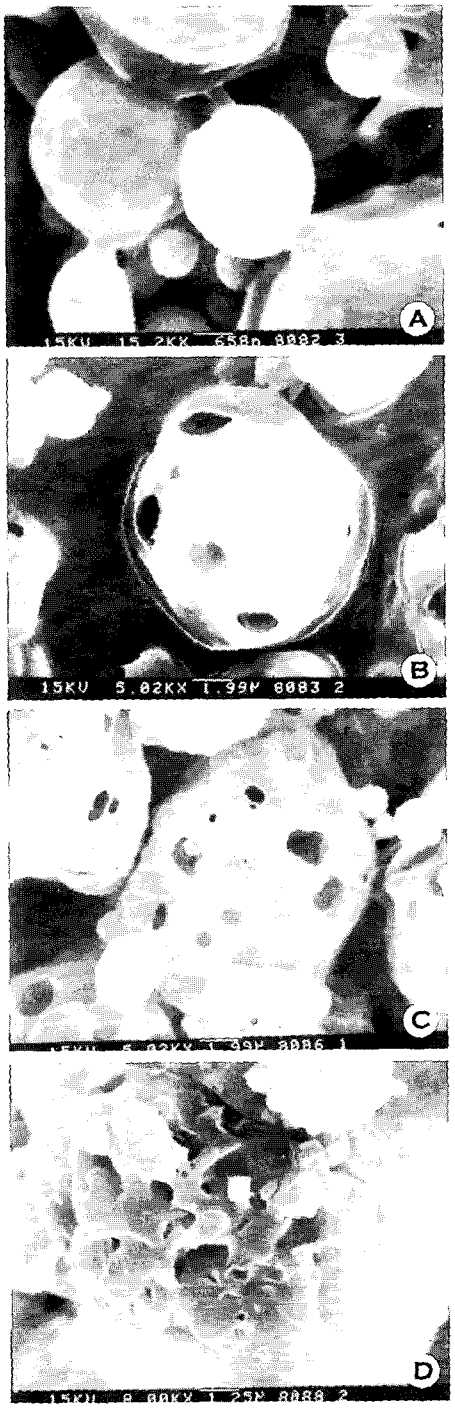

Fig. 4. Scanning electron microscopy (SEM) of PLGA microspheres at different degradation states. **(A)** Immediately after preparation; **(B)** After 15 days; **(C)** After 30 days; **(D)** After 45 days in releasing medium at 37℃.

of carboxylic acid end groups depletes base reserve of the phosphate buffer ion. Then, the microsphere suspensions no longer possess buffer capacity, and are subject to pH change (Sah et al., 1994) (Fig. 3).

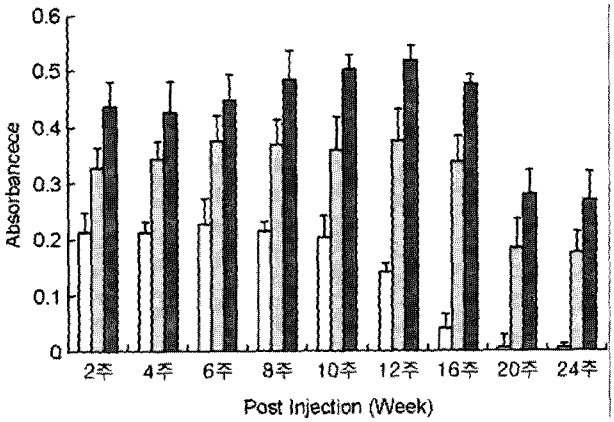

Fig. 5. Serum Ig G antibody titers of OVA. Mice was immunized with a single subcutaneous injection of 300 μg OVA. The levels of specific IgG anti-OVA antibody in sera was elicited by single subcutaneous injections for the full 24 weeks.
☐ OVA solution ☐ Alum solution ■ Microspheres

4. Biodegradability of PLGA microsphers

SEM examination showed different morphological characteristics at various time periods during *in vitro* degradation. The microspheres showed an intact outer surface immediately after preparation (Fig. 4A). After 15 days in the releasing medium, small pores were generated (Fig. 4B). This was interpreted as surface erosion in which the microspheres adsorbed the water when it was immersed in the releasing medium. The pore size was increased with time and, at 30 days (Fig. 4C), the microspheres showed a greater erosion with a highly porous structure although they maintained their spherical shape. At 45 days, there was a collapse in the structure and the microspheres did not keep their spherical shape (Fig. 4D).

5. Immunogenicity and adjuvant effect of PLGA microspheres

In vivo experiments using mice were carried out to investigate the immunogenicity of a model antigen OVA. Mice was immunized with a single subcutaneous injection of 300 μg OVA. Before the ELISA experiment, the sera obtained were diluted to 1:6400 in tris buffered saline (TBS). The levels of specific IgG anti-OVA antibody in serum after a single subcutaneous administrations of OVA-loaded PLGA microspheres were prolonged serum IgG antibody response levels than that elicited by administration of OVA adsorbed on alum or OVA saline solutions for the full 24 weeks following single subcutaneous injections (Fig.

5). We concluded by *in vivo* result that the induction of a serum IgG antibody response of lasting duration following a single immunization with antigen entrapped in microspheres is encouraging. The results obtained in the present study serve to illustrate the potential of PLGA microspheres as enhanced antigen delivery systems and provide the adjuvant effect.

DISCUSSION

This study examine the effects of process parameters on particle characteristics such as *in vitro* release profile and polymer properties. We also inspect the effect of the microsphere preparative process on the immune responses of PLGA microspheres as a vaccine delivery carrier system. OVA was selected as the model antigen because it is poorly immunogenic and has been recommended as the standard model poor immunogen (McGee et al., 1994; Nihant et al., 1994). Also, we focused on the development of a microencapsulation process utilizing ethyl acetate as a dispersed organic solvent in stead of dichloromethane. The application of microencapsulation to vaccine technology revealed that polymeric delivery systems were capable of continuous antigen release and stimulation of a humoral immune response. They biodegrade through hydrolysis of ester linkages to yield the normal body constituents lactic acid and glycolic acid. The degradation rate of lactide copolymers is controlled by various factors including molecular weight, lactide: glycolide ratio and polymer crystallinity. They can be varied from several weeks to over a year, thus potentially allowing control over the time and rate of vaccine release (Yeh et al., 1995). A technique based on a water-oil-water (W/O/W) double emulsion method has been proposed as an alternative method for the encapsulation of hydrophilic drugs (Arshady, 1991; Cohen and Bernstein, 1996; Yeh et al., 1996; Sah, 1997; Newman et al., 1998). The advantages of the W/O/W process for antigen delivery are that proteins can be used for encapsulation as an aqueous solution, scaling-down is possible, and high yields and encapsulation efficiency are obtained. The main disadvantages are to be seen in the complexity of the process, the sensitivity to polymer properties, and the difficulties in modifying release profiles of drugs from these microsperes. When the antigen delivery system was developed, Two alternative release profile can be proposed: pulsed and continuous delivery systems. The first would mimic the conventional vaccination schedules, e. g., three to four dosed released over a year. Pulsed released formulations are being prepared that contain a mixture of beads of different sized and composition. They are expected to provide a strong optimal immune response (protective antibody) as soon as injection and a boost at a later point. (Aguado, 1993). The second is a continuous delivery of antigen over a similar period of time. It is currently unknown whether 'pulsed' or 'continuous' release of antigen is most effective for the induction of potent immune responses. In both studies. the microsphere formulations induced immune responses that were comparable to potent immunological adjuvants. Consequently, the question for which is the most appropriate release profile for antigens from microparticles must remain open and currently each laboratory has a different conclusion on the possible duration of protein delivery because of different microsphere formulations and various microencapsulation techniques (Sah et al., 1995). In accordance with recommendations for the testing of new adjuvants, we have assessed the primary immune responses to OVA entrapped in our delivery system, PLGA microspheres. Biodegradable PLGA microspheres have been successfully prepared by W/O/W emulsion solvent extraction method with EA. The results described in the present study showed that the fabrication method, including solvent selection and PVA concentration, could influence the drug release of protein from PLGA microspheres. Also, we concluded by *in vivo* result that the induction of a serum IgG antibody response of lasting duration following a single immunization with antigen entrapped in microparticles was encouraging. Shelf life and storage condition, and stability of the parenteral vaccine delivery under *in vivo* conditions are aspects requiring careful investigation, too. It is hoped that sing step vaccines would not require refrigeration. These claims, however, are at present purely speculative, and clearly, more information is needed for their substantiation (Zahiru et al., 1994; Cohen et al., 1996). The results obtained in the present study serve to illustrate the potential of PLGA microspheres as controlled-release antigen delivery systems and provide the adjuvanticity effect. In conclusion, PLGA microspheres are delivery systems capable of encapsulating a wide range of materials, formulation with various entrapped antigens prepared by several techniques. The controlled release of antigens using this polymer might be useful in prolonged vaccine formulations for the parenteral carrier system.

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