

Poly-DL-lactide-co-glycolide microspheres as a controlled release antigen delivery system

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Abstract

Successful vaccine application means maximum protection with minimal number of administrations. A rational development of vaccines involves studies of the nature of the antigen as well as of the adjuvant to be used to improve the immune responses. This has provided the impetus for studies to design the degradable devices and for different approaches to antigen delivery by different routes of administration. The development of controlled release systems based on polymeric devices that permit a sustained or pulsed release of encapsulated antigens has attracted much interest. Polymeric delivery systems consist of polymers that release their content continuously in a controlled manner over a period of time. The development of a biocompatible delivery system for parenteral administration offers several advantages in terms of immunoadjuvanticity over other compounds. It was found that, in contrast to other carriers, microspheres are more stable, thus permitting administration by the oral or parenteral route. In the present study, we describe the main characteristics and potentialities of this new immunoadjuvant for oral and parenteral administration.

Key words

- Vaccine
- Adjuvant
- Microspheres
- Microencapsulation
- Biomaterial

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Introduction

The investigation of formulations for the controlled release in vaccine delivery is a top priority because of their potential for reducing the number of administrations required to induce protection (1-4). A rational development of vaccines involves studies of the nature of the antigen as well as the adjuvant used to improve the immune responses. With the progress in the development of new antigens resulting from biotechnology studies, the search for new adjuvants has become the main research subject for many groups who

study their role and safety for therapeutical use (5-8).

In order to improve the immune responses to highly purified and, therefore, weak antigens, a wide variety of experimental immunoadjuvants have been developed but they are a long way from rendering such vaccines strong or efficient enough for their use in humans (9). The adjuvant effect may be attained by three basic mechanisms: i) a depot formation at the site of administration; ii) acting as a vehicle delivery system for targeting the antigen to immunocompetent cells, and iii) acting as immunostimulants.

The ideal and safe adjuvant should not generate local or systemic reactions after administration. It should elicit an early, high and long-lasting immunoresponse and should be stable and chemically defined to permit reproducible manufacturing (5,8). A good adjuvant needs to be able to give signals to the immunological system so that lymphocytes and phagocytes migrate to the vaccinated area (10).

However, after many decades of research for adjuvants, only aluminum and liposomes (lipid vesicles) have been approved for use in humans (9). Although many advantages may be considered for alum as adjuvant, after years of safe history, the use of aluminum-based vaccines has declined considerably due to variations in the production of alum-precipitated vaccines (8). Other limitations are their ineffectiveness for certain antigens and their inability to elicit cell-mediated immune responses, particularly cytotoxic T-cell responses, which limit their application against intracellular parasites and viral infections. Also, aluminum adjuvants cannot be frozen or lyophilized (8).

Other adjuvants have been developed and a few have been evaluated in clinical trials, but most of them were never accepted for routine vaccines due to their adverse side effects. The use of liposomes as immunoadjuvants is also controversial. However, encapsulated antigens have the potential advantage of providing a higher antigen-carrying capacity. Both humoral and cell-mediated immune responses have been elicited by these systems. With the continuous hard effort of the past two decades, a considerable number of issues may yet be overcome such as shelf life and targeting specific cells.

Controlled delivery systems

The need for new adjuvants has led to the development of controlled release systems based on polymeric devices, which could permit a sustained or pulsed release of en-

capsulated or entrapped antigens (11-13). This has provided the impetus for active studies to design biodegradable materials and approaches for delivery by different routes of administration (14-17). Benefits of controlled release systems are delivery to a specific site, protection of the antigen from degradation, better patient compliance and more efficient antigen dosing, which may eliminate the need for boosters.

Polymeric delivery systems consist of polymers that release their content in a controlled manner continuously over time. The development of a biocompatible delivery system for parenteral administration offers several advantages in terms of immunoadjuvanticity over other compounds. In contrast to other carriers, polymeric microspheres are stable enough to permit their administration by the topical, oral or parenteral route. This is particularly advantageous when a mucosal immune response is needed. Furthermore, the association of antigens with microspheres has been able to elicit both cellular and humoral immune response (18). The stability of this formulation under storage conditions is related to hydrolytic degradation. However, the formulations can be freeze-dried and stored as a dry powder, which can be easily reconstituted immediately before use. The main goal of polymeric microencapsulation is to coat the antigenic material with a biodegradable polymer which will protect and control the antigen delivery. The possibility of obtaining free-flowing particles with well-defined diameters from 1 to 100 micrometers offers many advantages for its manufacture and interaction with immunocompetent cells.

Poly-lactide-co-glycolide microspheres

Among the polymeric systems developed for pharmaceutical proposals, poly-lactide-co-glycolide (PLGA) microspheres have been widely explored in several immuno-

logical studies as a controlled delivery system of peptides, native and synthetic proteins and lately, nucleic acids (13-27). PLGA microspheres are composed of a spherical-shaped polymeric matrix ranging in diameter from 1 to 250 μm (Figure 1). Many factors are important to formulate these systems: i) ability to release the entrapped substance in a controlled way, which is influenced by polymer molecular weight, monomer ratio and morphology (28-30); ii) particle size, which is important in terms of interaction with phagocytic cells (12,13,31); iii) safety, which is related to the *in vivo* polymer degradation (32,33), and iv) stability, both in storage and in the biological fluids (34,35).

The antigen is physically entrapped into microspheres inside an injectable solid polymeric matrix. The combination of diffusion through pores and of polymer matrix biodegradation allows the control of antigen release rates. The biodegradation rate of the polymer depends on its molecular weight and it is well established that shorter chains are degraded faster (30,36). After their terminal chain hydrolysis, the resulting degraded products are monomers of lactide and glycolide, which are innocuous to the body. These degraded products are eliminated by the Krebs cycle as carbon dioxide and in the urine (37). During biodegradation, the encapsulated antigen is released. Since these polymers are chemically defined, the antigen release is uniform and reproducible. This release can vary from hours to months depending on the polymer combinations.

Microsphere diameter plays an important role in the interactions with phagocytic cells. Particles smaller than 10 μm may be phagocytosed faster by macrophages, which are recruited to the site of administration after subcutaneous injection, and particles larger than 10 μm act as a depot releasing the antigens in a second step (13,16). We also have to consider the fate of these particles across the digestive tract to be taken up by

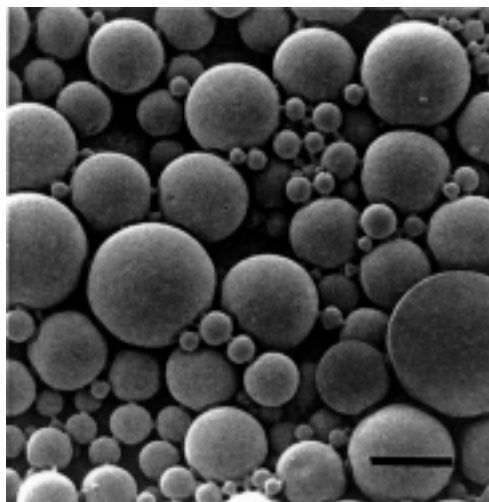


Figure 1 - Scanning electronic photomicrograph of PLGA microspheres obtained by the multiple emulsion W/O/W method. Bar = 5 μm .

the M cells of the Peyer's patches, when administered orally, since this could play a role by facilitating the antigen presentation to the immunocompetent cells and consequently the elicitation of an effective mucosal immune response. Enhancement of the stability of macromolecules may also be attained since they are protected from enzymes or other factors that could compromise the stability of the antigens.

Another important factor in favor of this new immunoadjuvant is related to its safety. These polymers have a long history as a safe material used in the composition of sutures and implants. This fact provided the basis for injectable delivery systems now used by 300,000 patients annually for treating advanced prostate cancer, endometriosis or precocious puberty (38).

Polymer selection is the most important step in the manufacture of microspheres because it critically influences their rate of biodegradation and hence devices with short- or long-lasting action can be constructed. Nowadays, several biocompatible polymers are commercially available. By changing the homopolymer ratio, different physicochemical compositions can influence its degradability and permeability, resulting in degradation times ranging from weeks up to several months depending on the shape, size and porosity of the device (Table 1). These pa-

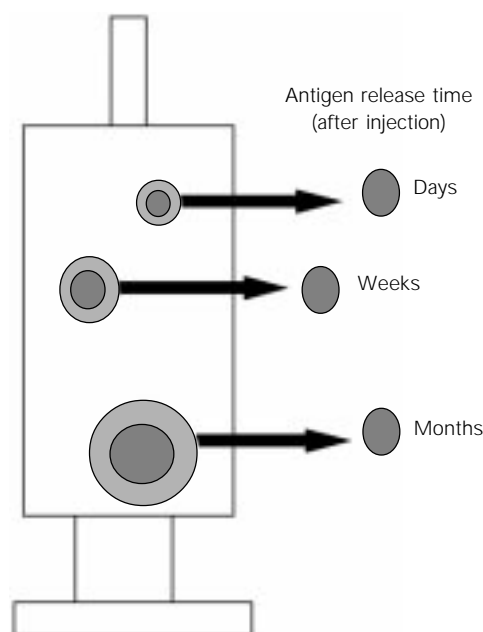
Table 1 – Physicochemical characteristics and biodegradation time of poly-lactide and co-polymers.

*Biodegradation time depends on the formulation, porosity, surface area and polymer molecular weight. Adapted from Ref. 35.

| Polymer | Crystallinity | Glass transition | Biodegradation time (months)* |
|---------------------------------------|---------------|------------------|-------------------------------|
| Poly (L-lactide) | Crystalline | 45-60°C | 18-24 |
| Poly (D,L-lactide) | Amorphous | 50-65°C | 12-16 |
| 50:50 Poly (D,L-lactide-co-glycolide) | Amorphous | 40-55°C | 2 |
| 85:15 Poly (D,L-lactide-co-glycolide) | Amorphous | 45-60°C | 5 |

rameters can be easily monitored using different polymer and copolymer compositions and controlling the variables during the manufacture process. Since the amorphous polymers are more permeable, poly-DL-lactide acid (DL-PLA), which is less amorphous than PLGA copolymers, has a slower degradation rate, usually over several months. The combination of particles with different diameters and polymer composition in the same formulation has permitted to establish the concept of the single-dose vaccine (1), which can be programmed to mimic the reinforcement dosages, inducing protection with a minimal number of administrations (Figure 2). Finally, PLA and its copolymers have

Figure 2 - The single-dose vaccination model based on PLGA microspheres. Adapted from Ref. 1.



proved to be biocompatible and produce little or no local or systemic toxicity (39,40).

PLGA subcutaneously administrated acts by means of three mechanisms: i) a depot formation at the site of administration; ii) targeting antigens to antigen-presenting cells, since it has been well described that macrophages are recruited to the site of the injection, or adherence to the Peyer's patches, which could facilitate the antigen presentation (41,42), and iii) protection of the antigen by storage, with a slow release of the remaining antigens that could eliminate the need for a booster (43,47).

Microsphere preparation

PLGA microspheres can be obtained by two main methods: simple emulsification (O/W) or multiple water-in-oil-in-water (W/O/W) emulsion followed by solvent elimination. The choice of method depends on the physical and chemical characteristics of the antigen, permitting the matrix entrapment of both lipophilic and hydrophilic molecules.

In the first case, an emulsion is formed by dissolving (or dispersing) the antigen in an organic solvent immiscible in water (methylene chloride, chloroform or ethyl acetate) containing the polymer, under strong mechanical agitation. This emulsion may be stabilized by a surfactant added to the aqueous phase. The solvent is eliminated by evaporation at room temperature, followed by washing and freeze-drying.

The multiple emulsion method involves water-in-oil-in-water emulsification (16,31). The inner aqueous phase containing the hydrophilic substances is obtained after emulsification with the immiscible organic solvent-containing polymer under strong mechanical agitation. This first emulsion is stabilized by the addition of an aqueous solution containing a surfactant (e.g., poly vinyl alcohol) and is further homogenized to produce a W/O/W double emulsion. This double emulsion is gently stirred with a homog-

enizer at room temperature for solvent evaporation. The microspheres are collected by centrifugation, washed with water and freeze-dried. The composition of the aqueous phase as well as the surfactant applied plays an important role in the pattern of antigen release since porosity and water permeability will be dependent on these factors. A mixture of hydrophilic and lipophilic molecules can also be successfully entrapped by using the W/O/W method (48).

Both of these methods allow high levels of antigen entrapment. The particles are characterized by determining the antigen entrapment rate, their average diameter, porosity and *in vitro* antigen release kinetics in appropriate medium. The maintenance of immunogenicity after antigen contact with organic solvents is also an important aspect to consider. Many studies have indicated that the antigenic properties and the structure of the proteins can be preserved during entrapment (26,44,49-53). Another important aspect is related to the possibility of scaling up the process under aseptic conditions, allowing the preparation of a pyrogen-free and sterile product (54).

Considerations about PLGA microsphere biocompatibility

Polymeric microspheres have attracted much attention because of their biocompatible characteristics. The phagocytosis of biodegradable and nonbiodegradable particles has been reported to depend on their size, surface charge and hydrophobicity (55,56). After subcutaneous administration, PLGA microspheres ranging in diameter from 1 to 10 μm are readily phagocytosed by macrophages recruited to the site of injection, thereby providing an intracellular delivery of the antigen. This mechanism may enhance antibody responses and consequently decrease the required antigen dose. On the other hand, particles larger than 10 μm in diameter would remain as a depot at the site

of injection providing a sustained release of antigen. Improving their hydrophobicity can increase phagocytosis of the particles, whereas microsphere preparations with different compositions of PLA and PLGA do not alter the extent of phagocytosis. However, precoating microspheres with opsonins can enhance phagocytosis (55). The intracellular fate of these particles is dependent on their composition. The rate of microsphere degradation inside cells could be controlled by changing the molecular weight and the monomer composition. PLGA microspheres are readily degraded releasing the entrapped molecules. Small particles of 200 nm in diameter are readily phagocytosed by Kupffer cells and reach the lysosomal compartment in liver tissues after intravenous administration, as demonstrated by transmission electron microscopy (56).

The relationship between the maximal dose of polymer and its implications in the intracellular residential time is not yet clear. In some reports of immunization studies, the entrapped protein:polymer ratio is very low, requiring administration of a huge amount of microspheres to attain the desirable antigen dose. It is well documented that the antigen and adjuvant doses interfere with the immune responses. Studies conducted with subcutaneous implants indicate that the physicochemical characteristics of PLA and PLGA polymers play a decisive role in the *in vivo* degradation time and also in the local inflammatory reaction (32,33,39). These results highlight an important aspect to be considered in the development of microspheres as carriers, i.e., the dose of polymer. We focused our studies on the clarification of the local responses after subcutaneous administration of microspheres, using an air pouch model in rats. We observed that 6 h after the administration of a single dose of 125 mg or a higher dose of 625 mg of polymer per kg rat body weight, 14.3×10^3 and 31.5×10^3 cells were recruited to the air pouch, respectively. Seven days after injection

tion, the number of cells decreased conspicuously without a significant difference between the treated groups and the control group treated with saline only. Histological analysis allowed us to identify an organic reactivity in the injected area characteristic of macrophage infiltration. Using soluble and particulate *Leishmania* antigens as a model, we found that the mononuclear infiltrate obtained at the injection site was larger with antigen-loaded microspheres as compared to unloaded ones. No granulocytic cell infiltration was observed. Higher doses (250 and 625 mg/kg) provoked the formation of a fibrovascular capsule surrounding the microspheres. In contrast, after administration of lower doses this reaction was not observed and PLGA microspheres were found to be completely degraded one month after injection. We selected the maximal polymer dose of 150 mg/kg to design our experimental protocols.

Systemic immunization

The diameter of the particles plays an important role in the induction of protection by parenteral immunization. In fact, when ovalbumin was adsorbed to or entrapped in PLGA microspheres of different diameters, drastic differences were observed in the antibody responses (12,13). Two weeks after immunization with a single dose by the subcutaneous route, the serum IgG antibody response to ovalbumin was significantly greater than the response to soluble ovalbumin. This response was more significant for particles of 1.5 μm than for particles larger than 73 μm . Interestingly, the level of antibodies remained high even one year after injection (16). Uchida et al. (31) reported similar results showing the superior efficacy of microspheres over alum. Vaccine formulations prepared by simple mixing of blank microspheres (without antigens) and ovalbumin exhibited low primary immune responses, which were only elevated by boosting (45).

Three batches of PLA and PLGA microspheres were used to investigate the single dose vaccine delivery by incorporating tetanus toxoid (18). Its comparison with aluminum hydroxide as immunoadjuvant provided data to support the superior efficacy of PLGA microspheres to elicit T cell and antibody responses after subcutaneous administration. These results were confirmed by Walker et al. (57). The cellular and antibody responses of mice to tetanus toxoid were compared after subcutaneous administration of the toxoid alone or incorporated into aluminum hydroxide and PLGA microspheres. The safety of a similar vaccine was demonstrated in rats (40).

The entrapment of a synthetic peptide in PLGA microspheres was evaluated by Partidos et al. (23). Intraperitoneal immunization with particles smaller than 10 μm containing a chimeric peptide constructed by synthesis of a potential B cell site induced anti-peptide antibody responses after a single dose. This single intraperitoneal administration protocol was also applied to induce protection against *Bordetella pertussis* respiratory infection in mice (26). Although, PLGA microspheres containing pertussis fimbriae elicited lower levels of serum antibody than those produced by alhydrogel-containing antigen, both formulations protected against intranasal infection by *B. pertussis*.

The magnitude of the immune response induced by peptides is comparable or superior to that induced by peptide emulsified in complete Freund's adjuvant (58). *In vitro*, peptides (carrying an immunodominant T-helper cell epitope delineated from the rabies virus nucleoprotein) incorporated into PLGA microspheres induced the proliferation of a peptide-specific T cell line. After subcutaneous immunization of mice, the immune response induction was related to the nature of the polymer.

Other encouraging trials have been conducted providing data to confirm the superior efficacy of this system over other adju-

vants in a birth control vaccine (25), HIV-1 vaccine (52,54), rabies virus nucleoprotein (58), malaria antigen (59) and type A botulism (60).

Mucosal immunization

New approaches to vaccine development have become possible after a common mucosal defense system was recognized where an antigen interacting with localized lymphoid tissue could stimulate IgA precursor cells that could migrate to other mucosal surfaces (12,13,61). The mucosal immune system displays a unique ability to respond to an array of immunogens presented by the respiratory and oral routes. Oral vaccines are often more desirable than other routes because of easy administration to large populations and a reduced number of side effects (62). Since PLGA microspheres are readily absorbed by the Peyer's patches, they have considerable potential as a vehicle for oral immunization. They are taken up by modified epithelial cells (M cells) and transported to the lymphoid tissue where they encounter antigen-presenting cells including macrophages, dendritic cells and B cells. So, the use of microspheres has two advantages: i) protection from low pH and from proteases (since the antigen and/or adjuvant are entrapped in the polymer matrix, they are protected from both gastric and gastrointestinal proteolysis), and ii) targeting to IgA inductive sites.

It was found that peroral immunizations with a toxoid vaccine of staphylococcal enterotoxin B encapsulated in 1-10 μm PLGA microspheres stimulated circulating IgM, IgG and IgA antitoxin antibodies in mice. A disseminated mucosal IgA response was also observed. Systemic immunization was effectively primed for a mucosal IgA response when a booster was administered by the oral or intratracheal route, indicating that under these experimental conditions the systemic immunization did not induce tolerance to or

down-regulation of a subsequent mucosal antibody response. Nonencapsulated controls did not elicit any significant response. Microspheres less than 5 μm in diameter were effectively taken up by Peyer's patches and carried by macrophages to the mesenteric lymph nodes and spleen. On the other hand, microspheres in the range of 5-10 μm remained in the Peyer's patches throughout the 35 days of the experiment. Microspheres larger than 10 μm in diameter were not adsorbed at any point in the gastrointestinal tract (13).

The entrapment of a branched peptide immunogen representing a portion of the principal neutralizing determinant of HIV-1 has been developed for oral and combined oral and subcutaneous immunization against HIV. Under aseptic conditions a pyrogen-free and sterile preparation was obtained. The system showed high levels of both serum IgG and neutralizing antibodies against HIV in guinea pigs in both protocols. The authors proposed the formulation for clinical trials (52,54). Recently, it was demonstrated that immunization with a recombinant HIV envelope protein entrapped in PLGA microspheres induced consistent HIV-specific CD4⁺ and CD8⁺ T-cell responses in mice. Cytotoxic T lymphocyte (CTL) responses were detected after a single systemic immunization with gp120 entrapped in microparticles; when given by the intranasal route these microparticles induced HIV-specific CD8⁺ CTL and secretory IgA. The induction of Th1 cells was observed after generation of CD4⁺ T cells that secreted moderate to high levels of interferon- γ following immunization with gp120 entrapped in microparticles (63).

Entrapped plasmid DNA was recently proposed for oral administration (27). Entrapped DNA expressing the insect protein luciferase under the transcriptional control of the human cytomegalovirus promoter was administered by the oral and intraperitoneal routes. Entrapment increased the DNA sta-

bility and stimulated the secretion of IgA, IgG and IgM after oral administration. On the other hand, only the IgM and IgG responses were significant after intraperitoneal administration.

Concluding remarks

PLGA microspheres with an incorporated antigen represent a good antigen delivery system for both cellular and humoral responses. Further efforts are needed to elaborate an effective controlled release system

for antigens. The promising use of PLA and PLGA microspheres has been confirmed by experimental and clinical trials. The question whether pulsed or continuous antigen release provides the better immune responses remains to be clarified. The easy manufacture of microspheres and the possibility of administration by different routes offer the additional advantage of their use as a pharmaceutically acceptable adjuvant for vaccines. These findings may have major implications for the design of novel vaccine delivery strategies.

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