Chapter 10

Microparticle Vaccines Against *Toxoplasma gondii*

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Abstract

Significant information indicates that future investigations on *Toxoplasma* vaccine development have to include adjuvants for enhancing protective immunity against *Toxoplasma gondii*. Especially, safe and effective adjuvants capable of fulfilling Th1-dependent cell-mediated immunity appear to be more likely to be allowed to use for anti *Toxoplasma* vaccine development. Recently, biodegradable and biocompatible polymers, such as poly (lactide-co-glycolide) (PLG) polymers, have been utilized as safe and efficacious adjuvants to encapsulate antigens for producing long-term release microparticle-based vaccines. PLG microencapsulation allows the sustained release of antigens and facilitates antigen uptake via antigen-presenting cells (APCs) to favorably generate Th1 cell-mediated immunity, which is required for the prevention of *T. gondii* infection. In our recent work, recombinant surface antigens (rSAGs), including rSAG1, rSAG2, and rSAG1/2, have been, respectively, encapsulated with the PLG polymer for production of PLG-encapsulated rSAG1 (PLG-rSAG1), PLG-encapsulated rSAG2 (PLG-rSAG2), or PLG-encapsulated rSAG1/2 (PLG-rSAG1/2) microparticles. This chapter describes adjuvant effect of PLG microparticles, controlled release of PLG microparticles, PLG microparticles-immune system interaction, *Toxoplasma* SAG-loaded PLG microparticles, protective immunity by *Toxoplasma* SAG-loaded PLG microparticles, and future prospects. PLG microparticle vaccines would be advantageous for their application in the development of long-lasting vaccines against *T. gondii* for future use in humans and animals.

Keywords: adjuvants, poly(lactide-co-glycolide) (PLG), antigen-presenting cells (APCs), PLG-rSAG1 microparticles, PLG-rSAG2 microparticles, PLG-rSAG1/2 microparticles

1. Introduction

Toxoplasma gondii is an intracellular protozoan parasite that uses felines as final hosts and various endothermic animals, including humans, as intermediate hosts [1]. Toxoplasmosis is of major clinical and veterinary importance. The infection in domestic animals such as sheep

or pigs usually generates adverse economic impact due to the induction of severe abortion and neonatal loss [2]. In addition, toxoplasmosis in pregnant women may result in severe congenital fetal disorders, including hydrocephaly, blindness, and mental retardation [3]. Toxoplasmosis in immunocompromised individuals such as AIDS patients often develops lethal toxoplasmic encephalitis as an important opportunistic infection [4]. Although prophylactic anti-*Toxoplasma* vaccines have been studied for a long time, only one commercial attenuated vaccine (Toxovax) has been licensed for use in sheep [5]. Most inactivated and recombinant vaccines developed in the past have produced only little to moderate protective efficacy against infections with a lethal challenge dose of the virulent strain of *T. gondii* [6].

Numerous earlier studies have demonstrated that cell-mediated immunity is considered as the 'appropriate' immune response in the prevention of *T. gondii* infection [7]. Therefore, potent adjuvants that can improve cell-mediated immunity have to include in *Toxoplasma* vaccine development in the future. In addition, gamma interferon (IFN-γ), one of Th1-type cytokines, mainly produced by $CD4^+$ Th1 cells is able to subsequently stimulate $CD8^+$ Tc cells to convert into cytotoxic effector cells for preventing acute and chronic *T. gondii* infection [6–8]. Thus, effective protection against *T. gondii* infection is critically dependent on the IFN-γ-associated Th1 cell-mediated immunity. Therefore, effective vaccines formulated with potent adjuvants that are promised to induce an IFN-γ-associated Th1 cell-mediated immune response seem more likely to be approved for use.

Most modern vaccines based on subunits of pathogens, such as purified proteins, are likely to be less immunogenic than traditional vaccine antigens and are often unable to initiate a strong immune response [9]. These subunit vaccines require effective adjuvants to aid them to elicit strong protective immune responses [10, 11]. Therefore, one of the current important issues in vaccinology is the urgent need for the development of new or improved adjuvants to enhance the immunogenicity or effectiveness of vaccines [9, 10, 12]. Different adjuvants capable of improving immunity and protection have been described in numerous studies [9, 10, 12]. However, the safety concern of an adjuvant is still a crucial issue to limit adjuvant development [13].

Adjuvants are special substances used in combination with an antigen to generate a more robust immune response than the antigen alone [11, 14, 15]. This broad definition encompasses a very wide range of materials for adjuvant development. Actually, adjuvants can be broadly separated into two classes, vaccine delivery systems and immunostimulatory agents, based on their main mechanisms of action [11]. Vaccine delivery systems are generally particulate, such as emulsions [16], micro/nano particles [17], iscoms, and liposomes [18], and their main function is to deliver antigens into antigen-presenting cells (APCs) [11]. In contrast, immunostimulatory agents are predominantly derived from pathogens and often represent pathogen-associated molecular patterns (PAMP), such as lipopolysaccharides (LPS) [19], monophosphoryl Lipid A (MPL-A) [20], and CpG oligodeoxynucleotides (CpG-ODN) [21], which activate cells of the innate immune system to induce the following acquired immune response [11].

This chapter will focus predominantly on microparticle adjuvant, especially poly(lactide-co-glycolide) (PLG) polymer, to be used in tachyzoite surface antigen (SAG)-based subunit vaccines against *T. gondii*, the etiological agent of toxoplasmosis. In our recent work, recombinant SAGs

(rSAGs), including rSAG1, rSAG2, and rSAG1/2, have been, respectively, encapsulated with the PLG polymer for production of PLG-encapsulated rSAG1 (PLG-rSAG1) [22], PLG-encapsulated rSAG2 (PLG-rSAG2) [23], or PLG-encapsulated rSAG1/2 (PLG-rSAG1/2) microparticles [24]. This chapter describes adjuvant effect of PLG microparticles, controlled release of PLG microparticles, PLG microparticles-immune system interaction, *Toxoplasma* SAG-loaded PLG microparticles, protective immunity by *Toxoplasma* SAG-loaded PLG microparticles, and future prospects. The capability of these PLG microparticle vaccines to control the stable release of antigenic rSAG1 and effectively induce and extend protective immunity would be advantageous for their application in the development of long-lasting vaccines against *T. gondii* for future use in humans and animals.

2. Adjuvants effects of PLG microparticles

Microparticles, one of the vaccine delivery systems, derived from different biodegradable and biocompatible polymers, including poly(lactide-co-glycolide), alginate, starch, and other carbohydrate polymers, can be designed as safe carriers for proteins or drugs to perform the main function of delivery systems [25]. Particularly, PLG polymers approved by the US Food and Drug Administration (FDA) have been extensively used as sutures [26] and drug carriers [27] for many years. Different forms of PLG polymers can be generated according to the ratio of lactide to glycolide used for the polymerization [28]. In the recent 10 years, PLG polymers have further become safe and potent adjuvants or delivery systems to encapsulate vaccine antigens for the development of controlled release microparticle vaccines [29]. The PLG microparticles are biodegradable through hydrolysis to break down into the biocompatible metabolites, lactic and glycolic acids, which produce little inflammatory activity and are excreted from the body via natural metabolic pathways [28]. PLG polymers provide a number of practical advantages in acting as vaccine adjuvants or delivery systems following PLG encapsulation. The PLG microencapsulation protects antigens from unfavorable degradation [30], allows the sustained and extended release of antigens for a long period [31], and enhances antigen uptake by APCs, such as macrophages and dendritic cells, in specific lymphoid regions [32]. These adjuvant effects strengthen antigen immunogenicity to favorably generate strong specific immunity, especially cell-mediated immunity [29], which is urgently required for eliminating intracellular pathogens, such as *T. gondii*.

3. Microparticles-immune system interaction

Earlier significant studies have shown that potent cell-mediated immunity induced by PLG microparticles following vaccination is likely to be due to the uptake of PLG microparticles into APCs and the effective delivery of microparticle-containing APCs to specific lymphoid compartments [25, 32, 33]. The size of PLG microparticles used for animal vaccination is a crucial parameter in facilitating the uptake of APCs [32]. Particles smaller than 10 μm in diameter are appropriate for direct uptake by APCs, such as macrophages and dendritic cells [25, 32].

The proper size range thus can stimulate APCs to facilitate the microparticle uptake. Following the uptake of microparticles, the APCs containing microparticles then migrate to other lymphoid compartments [25], such as the spleen and mesenteric lymph nodes, where they effectively present antigenic epitopes to T lymphocytes, especially Th1 and Tc, thereby inducing strong specific cell-mediated immunity [32, 33]. In other words, facilitation of uptake and delivery of PLG microparticles by APCs can lead to more effective antigen processing and presentation to T lymphocytes capable of inducting cell-mediated immune responses [25, 32–34]. Significant earlier studies have further demonstrated that the APCs containing microparticles can travel to specialized mucosal lymphoid compartments, including mucosal-associated lymphoid tissues (MALTs), the inductive sites for stimulating potent immunity following intranasal or oral vaccination [35, 36]. Thus, PLG-encapsulated antigens can be designed as effective mucosal vaccines that have potential to stimulate mucosal systems, such as intestinal and vaginal tracks via intranasal or oral administration [36].

4. Controlled release of PLG microparticles

The capability of PLG microparticles to regulate the extended release rate of PLG-encapsulated antigens can lead to long-term immunity in microparticle-vaccinated animals [29, 37]. Various studies have demonstrated that PLG microparticles may perform pulsed and/or slow release of encapsulated antigens to promote effective immune responses [38]. The sustained and extended antigen release appears to substantially enhance and prolong antigen-specific immunity for achieving long-term protection [29, 31]. The antigen release from PLG microparticles is controlled by the degradation rate of PLG copolymer, which is largely due to the ratio of lactide to glycolide of PLG polymer, the molecular weight, and hydrophilicity of PLG polymer as well as the characteristics of PLG microparticles such as the morphology, size, and encapsulation efficiency [38]. The sustained antigen release of antigen-loaded PLG microparticles have been applied in the development of various potent microparticle vaccines [29, 31]. In addition, antigen-loaded PLG microparticles capable of sustaining release of an antigen also show potential for being designed as a single-dose vaccine without the need for booster doses [37, 39]. However, as some sophisticated events, including enhancement of protein load in PLG microparticles as well as optimization and stabilization of protein release are involved in the design of a single-dose vaccine [40], the feasibility needs to be assessed in future studies.

5. Encapsulation methods

The microparticles based on biodegradable PLG polymers can be prepared by number of methods, such as spray drying, double emulsion, and phase separation-coacervation [30]. However, the most widely used technique for preparation of protein-loaded microparticles is the double emulsion method [30].

The protein is encapsulated in 50:50 poly(lactide-co-glycolide) microparticles using the double emulsion method as described previously [41, 42], with minor modifications [22–24]. In the process, PLG polymer is first dissolved in an organic solvent. The organic solvent dichloromethane is mainly used to dissolve PLG polymer. Protein in aqueous solvent is then emulsified with nonmiscible organic solution of PLG polymer by high speed homogenization or sonication to produce a water/oil emulsion. The resulting emulsion is further transferred to a solution of polyvinyl alcohol, which is used as a stabilizer. Again homogenization or intensive stirring is necessary to generate a double emulsion of water/oil/water. The water/oil/water emulsion is then stirred for 18 h at room temperature (RT) and pressurized to promote solvent evaporation and microparticle formation in a laboratory fume hood. Solvent extraction can also be undertaken yielding microparticles containing protein. The microparticles are collected by centrifugation and washed with distilled water to remove nonentrapped protein.

Based on previous studies, proteins used for PLG encapsulation can be scaled down by using the water/oil/water double emulsion method [30]. In addition, this method also results in high microparticle yields and encapsulation efficiencies [30]. However, there is still a potential concern of antigen denaturation due to organic solvent exposure during the encapsulation process [41, 42], although numerous proteins have been successfully entrapped in PLG microparticles without loss of structural integrity, immunogenicity, or bioactivity [25, 30]. Especially, antigenicity retention following the process of double emulsion method is a critical event to subsequently initiate effective immunity by vaccinating antigen-loaded microparticles [30].

6. Preparation of *Toxoplasma* SAG-loaded microparticles

Development efforts of subunit vaccines against *T. gondii* in our laboratory have been focused mainly on the major immunodominant SAGs of tachyzoites [22–24, 43, 44], the rapidly multiplying stage during the acute phase infection. Furthermore, SAG1 and SAG2 proteins have been identified as two major tachyzoite SAGs in the previous study [45]. These two proteins are involved in the process of host cell invasion [46] and can induce anti *Toxoplasma* immune responses [6]. Therefore, both SAG1 and SAG2 can be considered as potential candidate antigens for *Toxoplasma* vaccine development. SAG1 gene, SAG2 gene, and a hybrid gene consisting of SAG1 and SAG2 sequences had been, respectively, cloned in our previous work to produce recombinant SAG1 (rSAG1) protein [22, 43], recombinant SAG2 (rSAG2) protein [23, 43], and a recombinant chimeric protein, rSAG1/2 [24, 43]. Further animal studies in mice demonstrated that rSAG1, rSAG2, or rSAG1/2 emulsified with an oil adjuvant, Vet L-10, induced partial protection against a lethal subcutaneous challenge of *T. gondii* tachyzoites [43]. If alternative effective adjuvants, such as the PLG polymer are used to make these recombinant proteins more immunogenic, more protective immunity against *T. gondii* may be achieved in animals.

In our recent work, rSAG1, rSAG2, or rSAG1/2 was then, respectively, encapsulated with the PLG polymer by using the double emulsion method for production of PLG-encapsulated rSAG1 (PLG-rSAG1) [22], PLG-encapsulated rSAG2 (PLG-rSAG2) [23], or PLG-encapsulated

rSAG1/2 (PLG-rSAG1/2) microparticles [24]. Some microparticle characteristics, such as size (diameter), microparticle morphology, protein entrapment, and *in vitro* release were analyzed after PLG encapsulation (**Tables 1** and **2**). The morphological studies based on scanning electron microscopy showed that these microparticles are uniform population of spherical particles with a smooth surface. In addition, particle sizes of all three PLG microparticles in diameter were smaller than 10 μm (**Table 1**). Thus, the three PLG microparticles have an appropriate feature for direct uptake by APCs, such as macrophages and dendritic cells.

More importantly, the release of rSAG1, rSAG2, or rSAG1/2 from PLG microparticles was also analyzed (**Table 2**). We found that the *in vitro* cumulative release of rSAG1, rSAG2, and rSAG1/2 from PLG microparticles suspended in phosphate buffered saline (PBS) could be, respectively, sustained for 35, 33, and 56 days with three distinct phases consisting of an initial burst release, a very slow release, and a final rapid release (**Table 2**). Actually, based on previous critical investigations, such three-phase fluctuation in antigen release from PLG microparticles is due to the initial rapid diffusion of coated antigen on the PLG microparticle surface, the very slow and gradual diffusion of encapsulated antigen, and the final rapid diffusion of antigen because of the PLG microparticle degradation [47–49]. Furthermore, in the triphasic antigen release profile, both initial and final rapid release of entrapped antigen, respectively, look like priming and boosting doses usually employed in a conventional immunization procedure [49]. Thus, vaccination with a single dose of PLG-rSAG microparticles that are able to fulfill the triphasic rSAG release may be thought of as treating with two doses of rSAG protein. However, further improvements such as enhancement of protein load in PLG microparticles, as well as, optimization and stabilization of protein release are needed to evaluate the feasibility [40]. On the other hand, Western blotting assay with use of mouse monoclonal antibodies specific to tachyzoite SAGs demonstrated that released rSAG proteins from PLG microparticles still retained the original SAG antigenicity during the release from PLG microparticles [22–24]. These data indicate that both the encapsulation procedure and release from microparticles in our previous work are not detrimental to the antigenicity of

Microparticle	Mean particle size (μm)	Entrapment efficiency (%)	Reference
PLG-rSAG1	4.25–6.58	69–81	[22]
PLG-rSAG2	2.14–3.63	74–80	[23]
PLG-rSAG1/2	1.27–1.65	72–83	[24]

Table 1. Particle size and entrapment efficiency of PLG-rSAG microparticles.

Microparticle	Release protein	Release period	Release profile	Reference
PLG-rSAG1	87.8% rSAG1	35 days	Three phases	[22]
PLG-rSAG2	88.3% rSAG2	33 days	Three phases	[23]
PLG-rSAG1/2	88.5% rSAG1/2	56 days	Three phases	[24]

Table 2. Release of rSAG from PLG microparticles.

rSAG. Thus, rSAG proteins have been successfully encapsulated with PLG polymers by the double emulsion method and the resulting PLG-rSAG microparticles not only properly preserved the SAG's antigenicity, but also sustained the controlled, stable release of the antigenic rSAG proteins from PLG microparticles. Based on these data, therefore, released rSAG proteins from PLG microparticles have the potential to induce anti SAG immune responses.

7. Protective immunity by *Toxoplasma* SAG-loaded microparticles

Although different adjuvants capable of improving immune responses and protection against *T. gondii* have been studied [6, 50], the biodegradable and biocompatible PLG polymers are so far seldom used as potent adjuvants for *Toxoplasma* vaccine development. Stanley and his coauthors first employed the double emulsion method to produce PLG-encapsulated microparticle vaccine against *T. gondii* [51]. In the same study, the PLG microparticle vaccine containing a tachyzoite extract plus a mucosal adjuvant, cholera toxin, failed to provide protection in sheep [51]. The unexpected protection in sheep indicates that more effort is therefore needed to improve not only the stability of encapsulated *Toxoplasma* antigens but also the immune responses and protection they induce in animals.

On the other hand, the adjuvant effects of the PLG encapsulation had been exercised to, respectively, prepare PLG-rSAG1, PLG-rSAG2, and PLG-rSAG1/2 microparticles in our three previous studies [22–24]. The ability of these PLG-rSAG microparticles to trigger protective immunity against *T. gondii* was subsequently evaluated in BALB/c mice by vaccination through the intraperitoneal route. Results showed that both PLG-rSAG1 and PLG-rSAG1/2 microparticles effectively induced not only significant long-lasting (10 weeks) specific humoral and cell-mediated immune responses, accompanied by secretion of a large amount of IFN-γ, but also high protection (80% for PLG-rSAG1 microparticles and 83% for PLG-rSAG1/2 microparticles) against *T. gondii* tachyzoite infection [22, 24]. However, PLG-rSAG2 microparticles could induce sustained (10 weeks) lymphocyte proliferation and IFN-γ production [23]. Furthermore, after a lethal subcutaneous challenge of 1×10^4 *T. gondii* tachyzoites (RH strain), PLG-rSAG2 microparticles also improved anti *Toxoplasma* protection (87%) [23], which is higher, though not statistically significant, than either 80% of PLG-rSAG1 microparticles [22] or 83% of PLG-rSAG1/2 microparticles [24].

As *T. gondii* is an obligate intracellular parasite, protective immunity to *T. gondii* is largely mediated by Th1 cell-mediated immunity [6, 7]. Previous studies have shown that induction of both lymphocyte proliferation and IFN-γ production (one of Th1-type cytokines) positively correlates with protective Th1 cell-mediated immunity against *T. gondii* [43, 44, 51]. In addition, IFN-γ has been demonstrated to be a critical mediator that has to be secreted for as long as possible in order to maintain anti *Toxoplasma* immunity [52, 53]. We found that sustained lymphocyte proliferation and significant IFN-γ production readily detected in mice immunized with PLG-rSAG microparticles in our previous studies [22–24]. These findings indicate that immunization with PLG-rSAG microparticles really elicits the IFN-γ-associated Th1 cell-mediated immunity, which is the expected response that we aimed to induce in mice.

Based on previous studies, PLG microparticles appear to favorably facilitate a size-dependent interaction with APCs, such as macrophages and dendritic cells [32–34]. The particles, like PLG-rSAG microparticles prepared in our previous studies (**Table 1**), smaller than 10 μm in diameter are directly taken by APCs [32]. Based on our previous results, the proper size range thus could stimulate peritoneal macrophages to facilitate the uptake of these PLG-rSAG microparticles administered in the mouse peritoneal cavity [22–24]. Therefore, the microparticle-containing macrophages in the peritoneal cavity then traveled to other lymphoid compartments, including the spleen, and effectively presented SAG epitopes to Th1 and Tc; thereby, inducing strong SAG-specific Th1 cell-mediated immunity to protect mice from the tachyzoite challenge. Our previous studies [22–24] and those recorded by others [32–34] have shown that facilitation of uptake and delivery of PLG-rSAG microparticles by macrophages can lead to more effective antigen processing and presentation to T lymphocytes capable of inducting cell-mediated immunity. Thus, the high survival rates in mice have demonstrated that PLG-rSAG microparticles effectively elicit protective Th1 cell-mediated immunity to remove tachyzoite-infected cells for limiting parasite dissemination during the experimental tachyzoite challenge [7].

In addition to Th1-dependent cell-mediated immunity, in our previous studies, high titers of anti *Toxoplasma* IgG in mouse sera elicited by PLG-rSAG microparticles have indicated that systemic humoral immunity mediated by Th2 may participate in the prevention of *T. gondii* infection [54, 55]. However, further measurements by the dye test are still needed to assay these antibodies to elucidate their functional lytic activities. Therefore, peritoneal vaccination of mice with PLG-rSAG microparticles may generate mixed Th1/Th2 immunity against *T. gondii*.

8. Conclusions and future prospects

PLG polymers are the primary candidates for the development of microparticle vaccines [25]. The rSAG proteins (rSAG1, rSAG2, and rSAG1/2) prepared in our laboratory have been successfully encapsulated with PLG polymers to generate PLG-rSAG microparticles capable of sustaining long-term stable release of antigenic rSAG proteins. Moreover, following peritoneal immunization in mice, PLG-rSAG microparticles induce not only long-term (10 weeks) SAG-specific humoral and cell-mediated immune responses, but also high protection against a lethal challenge of *T. gondii* tachyzoites [22–24]. Our studies provide a valuable basis for developing long-lasting vaccines against *T. gondii* for future use in humans and animals. Our experimental data indicate that the encapsulation procedure we used for production of PLG-rSAG microparticles is feasible at the laboratory level. However, this procedure have never been used to try mass production of PLG-rSAG microparticles. More effort is therefore needed to evaluate the optimized encapsulation conditions used to fulfill the need for mass production [40].

The PLG-rSAG microparticles we prepared previously could allow the sustained and extended release of rSAG proteins over a long period. Such long-term release of rSAG proteins could

repeatedly stimulate the immune effector cells to maintain enhanced immunity following immunization with PLG-rSAG microparticles [29, 37]. However, the triphasic rSAG release detected in the cumulative release assay we carried out in the previous studies was done *in vitro* in PBS and; therefore, may not completely reflect *in vivo* release in mice [22–24]. Further studies are therefore needed to confirm the critical effect of triphasic rSAG release on *in vivo* anti *Toxoplasma* immune responses.

One adjuvant effect acted by PLG microparticle vaccines is to facilitate antigen uptake via APCs [25, 32, 33]. Different APCs populated in various administrating routes are able to perform the uptake of antigen-loaded PLG microparticles and then process and present the epitopes of PLG-encapsulated antigen on the major histocompatibility (MHC) molecules [32]. Therefore, different routes of delivery of antigen-loaded PLG microparticles give rise to different vaccine efficacy in animals [32]. The mouse protective immunity induced by intraperitoneally administered PLG-rSAG microparticles protected mice from a lethal subcutaneous challenge of *T. gondii* tachyzoites. However, such intraperitoneal administration of the microparticle vaccine appears to be inappropriate for use in large animals such as sheep or swine. In order to corroborate the conclusions drawn from the mouse model, more studies are needed to evaluate the proper route for administration of PLG-rSAG microparticles in target animals. In addition, due to the natural infection initiated by ingesting oocysts released in cat feces or consuming meat from infected animals containing the long-lived tissue cysts, future experiments will also be necessary to assess whether mucosal administration (oral or nasal route) of PLG-rSAG microparticles protects these animals from an oral challenge of oocysts or tissue cysts of *T. gondii*.

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