# 5 Lipospheres for Vaccine Delivery

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## 5.1 INTRODUCTION

The tremendous advances of genetic engineering, and the ability to obtain many synthetic recombinant protein antigens derived from parasites, viruses, and bacteria, have revolutionized the development of new generation vaccines. Although the new, small, synthetic antigens offer advantages in the selection of antigenic epitopes and safety, a general drawback of small antigens is poor immunogenicity. Unfortunately, the body's immune system does not respond strongly to small peptides. In particular, macrophages do not readily ingest and process the small antigens, resulting in low antibody titers and the need for repeated immunizations. This lack of immunogenicity has created an acute need to identify pharmaceutically acceptable delivery systems for these new antigens.

One approach to enhancing the bioavailability and effectiveness of peptide-based vaccines is the use of microparticles as vaccine carriers. Several reports describing the

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improvement of immune response achieved by the association of antigens with lipid carriers such as liposomes [1,2] or microparticles like polymeric biodegradable microcapsules [3,4] have been published. The ability of these delivery systems to enhance immunogenicity was related to the physicochemical characteristics of the particles.

Significant attention has been given to developing formulations taken orally that induce immunization [5]. An appropriate microparticulate carrier may provide protection of the encapsulated peptide vaccine from enzymatic and environmental degradation, reduction of nonspecific interactions with food proteins, and facilitation of uptake by the gut-associated lymphoid tissue (GALT). Improved uptake of vaccine-loaded particles by the GALT can result in enhanced absorption across the intestinal epithelium and in avoidance of first-pass metabolism by the liver. In addition, the GALT has been shown to function in a manner that is analogous to lymph nodes, sampling antigenic and particulate material entering the gastrointestinal tract and mounting an immune response [6].

Particle size is a key factor, and it appears that particles of certain compositions in the size range of 50 to 3000 nm are capable of uptake and translocation [7,8]. Uptake increases with decreasing particle size. Surface hydrophobicity has a direct correlation with the immune response. Hydrophilic surfaces have little effect, as shown by confocal microscopy studies using polystyrene nanoparticles. Polystyrene was effectively targeted to the M-cell surface of the Peyer's patches, whereas absorbing poloxamers on these polystyrene particles resulted in a complete loss of the gastrointestinal absorption [9]. Further uptake can be induced by adding targeting ligands, such as monoclonal antibodies with specificity for M cells, onto the particle surface.

Lipospheres are fat-based encapsulation particulate systems developed for parenteral drug delivery [10–12] that also have been used successfully as carriers of vaccines and adjuvant [13,14]. Lipospheres have been used for topical applications, including with insect repellents and moisturizers with extended action. Lipospheres consist of water-dispersible solid microparticles composed of a solid hydrophobic fat core stabilized by one layer of phospholipid molecules embedded in their surface. Manufacture of liposphere–vaccine formulations is accomplished by gently melting neutral fat in the presence of phospholipid and dispersing the mixture in an aqueous solution containing the antigen by vigorous shaking. Upon cooling of this mixture, a phospholipid-stabilized solid hydrophobic fat core containing the antigen forms spontaneously.

Although the lipospheres seem to fit very well in vaccine formulations provided by injection or by oral intake, apparently not much has been published in this field since our last review [12]. This chapter is an update of the that review, with an emphasis on the possible use of lipospheres for oral immunization.

## 5.2 PREPARATION OF LIPOSPHERES

### 5.2.1 Formulations

In contrast to certain oil emulsions (including Incomplete Freund's Adjuvant), the liposphere approach uses pharmaceutically acceptable biodegradable constituents.

The internal hydrophobic core of lipospheres is composed of fats and biodegradable polymers, mainly triglycerides and lactide-based polymers, whereas the surface activity of liposphere particles is provided by the surrounding lecithin layer, composed of phospholipid molecules.

The neutral fats used in the preparation of the hydrophobic core of the several liposphere-vaccine formulations described here included tricaprin and tristearin, stearic acid, and ethyl stearate. The phospholipids used to form the surrounding layer of lipospheres were egg phosphatidylcholine and dimyristoyl phosphatidylglycerol. Polymeric biodegradable lipospheres were prepared from low molecular weight polylactide (PLA) and polycaprolactone-diol (PCL).

Liposphere formulations are prepared by solvent or melt processes. In the melt method, the active agent is dissolved or dispersed in the melted solid carrier (i.e., tristearin or polycaprolactone) and a hot buffer solution is added at once, along with the phospholipid powder. The hot mixture is homogenized for about 2 to 5 min, using a homogenizer or ultrasound probe, after which a uniform emulsion is obtained. The milky formulation is then rapidly cooled down to about 20°C by immersing the formulation flask in a dry ice–acetone bath, while homogenization is continued to yield a uniform dispersion of lipospheres.

Alternatively, lipospheres might be prepared by a solvent technique. In this case, the active agent, the solid carrier, and the phospholipid are dissolved in an organic solvent such as acetone, ethyl acetate, ethanol, or dichloromethane. The solvent is then evaporated and the resulting solid mixed with warm buffer solution, and mixing is continued until a homogeneous dispersion of lipospheres is obtained.

In a typical preparation, the active agent (200 mg), trilaurin (400 mg), and propylparaben (5 mg) are added to a 50-mL round-bottom glass flask. The flask is heated at 45°C to melt the triglyceride-active agent mixture, and hot 0.1 M phosphate buffer solution (pH 7.4, 45°C, 9.3 g) is added, along with egg phosphatidylcholine (100 mg). The mixture is homogenized for 2 min until a uniform milk-like formulation is obtained. The hot formulation is rapidly cooled to below 20°C by immersing the flask in a dry ice–acetone bath with continued mixing to yield a white, thin dispersion. If needed, the pH of the formulation is adjusted to 7.4 with a 1N HCl solution. The formulation may contain antioxidants such as tocopherol and preservatives such as parabens. Submicron-sized lipospheres are prepared by passing (four times) the liposphere formulation by extrusion through a submicron series of filters at a temperature 5°C above the melting point of the liposphere core composition. Particle size may be reduced to about 200 nm.

Polymeric biodegradable lipospheres can also be prepared by solvent or melt processes. The difference between polymeric lipospheres and the standard liposphere formulations is the composition of the internal core of the particles. Standard lipospheres, such as those previously described, consist of a solid hydrophobic fat core composed of neutral fats like tristearin, whereas in the polymeric lipospheres, biodegradable polymers such as polylactide or polycaprolactone were substituted for the triglycerides. Both types of lipospheres are thought to be stabilized by one layer of phospholipid molecules embedded in their surface. As lipospheres loaded with short peptides, luteinizing hormone-releasing hormone (LHRH) analogs have been recently reported [15]. In this study, peptides were incorporated into PLA lipospheres

by dissolving the peptide, the polymer, and the egg phospholipid in a 1:9 mixture of N-methylpyrrolidone (NMP) and dichloromethane. The solution was then evaporated to almost dryness, and buffer solution was added at 37°C and homogenized to form a uniform microdispersion of a particle size below 10 μm. The final formulation may contain traces of NMP in the aqueous medium; NMP is considered safe for injection. LHRH was constantly released from this formulation for more than 30 d. It is expected that vaccine peptides of similar molecular weight can be fabricated into a similar formulation and can provide extended action.

Sterile liposphere formulations are prepared by sterile filtration of the dispersion in the hot stage during preparation through a 0.2-μm filter at a temperature 5°C above the melting point of the liposphere core composition. Heat sterilization using a standard autoclave cycle decomposed the formulation. Gamma-irradiation sterilization of liposphere formulations did not affect their physical properties. Liposphere formulations of 1:4:2 and 2:4:2 bupivacaine:tristearin:phospholipid (w/w% ratio) were irradiated with a dose of 2.33 Mrad, and the samples were analyzed for particle size, bupivacaine content, *in vitro* release characteristics, and *in vivo* activity. The irradiated formulations had similar particle size, bupivacaine content, release rate, and anesthetic effectiveness in the rat paw analgesia model to those of a bupivacaine HCl solution (Marcaine). However, a more careful analysis of the formulation ingredients should be performed because phospholipids may degrade during irradiation [16].

Nanosized lipospheres have been prepared by homogenization by using a serial filter of reduced pore size, as described above. However, this method is limited to vaccines that are either soluble in the carrier systems or that are presented in a nanosize particle size. An alternative method for the preparation of nanosized lipospheres of particle size below 100 nm was recently developed using a dispersible concentrated oil system [17]. In this system, the drug, triglyceride, phospholipid, and other additives are dissolved in a mixture of surfactants (Tween and Span), and an organic solvent that is miscible with all components (propylene glycol, low–molecular weight polyethylene glycol, NMP, Cremophor, and polyethylene glycol–conjugated a-tocopherol). This clear, anhydrous solution spontaneously forms nanoparticles when gently mixed in buffer solution. Cationic or anionic nanolipospheres can be obtained when adding a cationic or anionic lipid, such as stearyl amine, phosphatidilethanolamine, stearic acid, or phosphatidic acid, to the solution. This concept has been applied for various water-insoluble drugs and peptides [17]. Solid lipid nanospheres, which are essentially nanosized lipospheres, have also been suggested for peptide and drug delivery, when phospholipid is used [18].

### 5.2.2 Antigens

The feasibility of developing a human malaria sporozoite vaccine was demonstrated in a clinical trial by using irradiated sporozoites as antigens. Protection against sporozoite infection apparently can be achieved by inducing a high titer of anti-sporozoite antibodies. It is currently presumed that it is only during the brief period (a few minutes or hours) when the sporozoite resides in the blood that antibodies

can gain access to the sporozoite and prevent continuation of the malaria infection. Therefore, a major goal of a sporozoite immunization scheme is to maintain a high titer of antibodies at the time of transfer of the organism from the mosquito to the host. A major challenge is to induce a high antisporozoite antibody titer that is also long-lived, hopefully with a protective duration of several months or more.

The major sporozoite antigen that is responsible for inducing protective immunity is a protein, the circumsporozoite (CS) protein, that covers the outer surface of the sporozoite. A region containing repeating tetrapeptides in the middle of the CS protein is thought also to be capable of inducing protective immunity. It is widely believed that high titers of antibodies to the CS protein can interrupt the life cycle of the sporozoite stage and provide protection against infection.

The two malaria antigens used in this study, R32NS1 and R32LR, were supplied under a Cooperative Research and Development Agreement by SmithKline Beecham Pharmaceuticals (King of Prussia, Pennsylvania). R32NS1 is a fusion protein with the following amino acid sequence: $[MDP(NANP)_{15}NVDP(NANP)_{15}NVDP]NS1_{81}$. The R32 refers to the 32 repeats of the tetrapeptide NANP interspersed with two tetrapeptide NVDP repeats from the immunodominant repeat region of the CS protein of the human malaria parasite (*Plasmodium falciparum*), and $NS1_{81}$ refers to 81 amino acids from the nonstructural protein of influenza virus. $NS1_{81}$ is added because it is thought to include human T helper cell epitopes and to function as a carrier protein [19]. In the case of R32LR, R32 is linked to the first two amino acids, leucine and arginine (LR), from a nonsense reading of the tetracycline gene of the vector [20]. The R32LR was used as capture antigen in the enzyme-linked immunosorbent assay (ELISA) because it contains the same repeating units as the R32NS1 antigen used for immunization [21].

The liposphere platform formulation for vaccines and adjuvants can be applied for a range of vaccination agents, for either oral or injectable administration. Bacterial toxins such as the cholera toxin and its nontoxic receptor binding B subunit have been formulated for oral intake and showed strong stimulation with mucosal secretory immunoglobin A and plasma immunoglobin G (IgG) antitoxin responses that last for months in the intestine. However, this adjuvant may induce toxicity and nonspecific stimulation of the immune system [5,22]. DNA plasmids that code for antigenic proteins have been recently considered for immunization. The antigen is synthesized *in vivo* directly from the protein coding sequences. An advantage of this approach is that the vector is unlikely to become toxic. However, one should consider the need for an effective transfection agent that will allow safe and efficient presentation of the plasmid into the nucleus of a selected cell or tissue. The complex of the anionic DNA with a cationic lipid or polymeric carriers might be sufficient for both delivery and transfection, with no need for liposphere carrier.

### 5.2.3 Adjuvants

It is widely believed that optimal methods for immunization against certain synthetic antigens may require the use of adjuvants, and this belief has stimulated a considerable amount of research aimed at developing new or improved adjuvants. The

most widely used adjuvants consist of aluminum compounds, particularly aluminum hydroxide (alum), which is used in diphtheria and tetanus toxoid vaccines [23].

A variety of lipid adjuvants and protein mediators have also been shown to influence the immune response to antigens encapsulated in liposomes. The most widely used examples of such adjuvants for practical immunization procedures are endotoxin (including lipid A and lipopolysaccharide) and numerous types of lipophilic derivatives of muramyl dipeptide.

Lipid A is the portion of Gram-negative bacterial lipopolysaccharide. In addition to containing nearly all of the endotoxic activity of lipopolysaccharide, lipid A is responsible for numerous other biological activities that are ordinarily associated with lipopolysaccharide [24]. Because of its potent endotoxic activities, lipid A by itself has had limited applicability as an adjuvant for use in human vaccines. Lipid A isolated from Salmonella Minnesota R595 (obtained from List Biological Laboratories, Campbell, California) was used as adjuvant in some of the liposphere-vaccine formulations. Alum has also been used as an additional adjuvant in some of the liposphere-R32NS1 formulations.

All liposphere formulations prepared remained stable during the 3-month period of the study, and no phase separation or appearance of aggregates were observed. The difference between polymeric lipospheres and the standard liposphere formulations is the composition of the internal core of the particles. Standard lipospheres, such as those previously described, consist of a solid hydrophobic fat core composed of neutral fats like tristearin, whereas, in the polymeric lipospheres, biodegradable polymers such as polylactide or polycaprolactone were substituted for the triglycerides. Both types of lipospheres are thought to be stabilized by one layer of phospholipid molecules embedded in their surface.

## 5.3 IMMUNOGENICITY OF LIPOSPHERES

### 5.3.1 Effect of Liposphere Fat Composition

The effect of the type of fat used in the preparation of liposphere on their immune response to encapsulated antigen was tested. Mice were immunized twice at weeks 0 and 4 with lipospheres containing R32NS1 malaria antigen. For all liposphere formulations, the first immunization at week 0 caused a very small immune response. However, after the boost injection, a very marked increase of mean IgG antibody levels was observed. For most of the six liposphere–vaccine formulations tested, the immune response obtained remained at very high levels of IgG antibody titers, even after the 12-week period of the experiment. The most immunogenic liposphere formulation was the one made of ethyl stearate, where lipospheres made of stearic acid showed the lowest IgG ELISA titers. The complete order of immunogenic activity of the six liposphere formulations tested was: ethyl stearate > olive oil > tristearin > tricaprin > corn oil > stearic acid. No correlation between liposphere particle size or chemical characteristics and immunogenicity was found. It is worth noting that the IgG antibody ELISA titers obtained on immunizing rabbits with LS (R32NS1) were superior to those obtained following similar immunizations with

the free antigen absorbed to alum, which showed no antibody activity at the same antigen concentrations. It was previously shown that this antigen was also poorly immunogenic in humans when injected alone as an aqueous solution or when adsorbed on alum [14,19].

### 5.3.2 Influence of Phospholipid Composition

Incorporation of a negatively charged phospholipid, dimyristoyl phosphatidylglycerol, in the liposphere lipid phase caused a significant increase in the antibody response to the encapsulated R32NS1 antigen [13]. Enhancement of immunogenicity by inclusion of charged lipids has also been observed with certain antigens in liposomes. Negatively charged liposomes produced a better immune response to diphtheria toxoid than positively charged liposomes [25]. However, when liposomes were prepared with other antigens, positively charged liposomes worked equally as well as those bearing negative charge [26,27]. Further studies are needed to determine whether negative charges in lipospheres have a general ability to enhance immunogenicity or whether, as with liposomes, charge effects are dependent on individual antigen composition.

### 5.3.3 Influence of Fat/Phospholipid Molar Ratio

An interesting correlation was observed between the liposphere fat to phospholipid (F/PL) molar ratio, particle size, and immunogenicity. Low F/PL ratios (0.75) were found to induce the formation of lipospheres of small particle size (70% less than 10 μm in diameter), and this apparently resulted in increased antibody titers [13]. Among the ratios tested, a maximal level of IgG antibody production was obtained at a F/PL ratio of 0.75, whereas at larger ratios, decreased antibody production was observed. Although the reason for this phenomenon is unknown, a possible explanation may be the occurrence of better antigen orientation and epitope exposures in the small lipospheres because of higher surface curvature. It may be relevant to note that small liposomes have also been reported to generate higher antibody titers against encapsulated antigen than large liposomes [28].

### 5.3.4 Effect of Particle Size Distribution

Two populations of particles usually coexist in liposphere formulations: one in the size range of 1 to 10 μm in diameter (population A), and a second population with a diameter between 10 and 80 μm (population B). As mentioned in the previous section, the particle size distribution of lipospheres depends on the F/PL molar ratio, and the immune response to liposphere-encapsulated R32NS1 was also dependent on the F/PL ratio. The average size of the particles increases with increasing F/PL molar ratio. Under conditions in which the F/PL ratio is high (2.5), the large-particle population is predominant (approximately 80% of the particles had an average size of 73 μm), whereas for F/PL ratios of 0.75, most of the lipospheres have a diameter of less than 10 μm [13].

### 5.3.5 Effect of Route of Administration

To examine the influence of different routes of administration of lipospheres on their immunogenicity, rabbits were immunized orally or parenterally (by subcutaneous, intraperitoneal, intramuscular, and intravenous routes) with lipospheres made of tristearin and lecithin (1:1 molar ratio) and containing the malaria antigen. The immune response obtained was followed with time for a period of 12 weeks postimmunization.

No antibody activity was found after oral immunization in any of the individual rabbits immunized with liposphere R32NS1–vaccine formulation. However, rabbit immunization by all parenteral routes tested resulted in enhanced immunogenicity, with increased antibody IgG levels over the entire postimmunization period. The individual rabbit immune response shows that immunization by subcutaneous injection was the most effective vaccination route among all parenteral routes of administration tested.

## 5.4 LIPOSPHERES AS CARRIERS OF ADJUVANTS

### 5.4.1 Adjuvant Activity of Lipid A

The adjuvant activity of lipid A on the immunogenicity of lipospheres was investigated. Lipid A was included in the lipid phase of lipospheres because it has been used effectively by many laboratories to enhance humoral immunity to a wide range of antigens because of its adjuvant properties [2]. The adjuvant activity of liposomal lipid A has been recently investigated [2], and it has been found that liposomes can serve as a vehicle that allows expression of the adjuvant activity of lipid A and simultaneously can reduce certain unwanted side effects of lipid A. It has been established that incorporation of lipid A into liposomes greatly reduces many of the toxic effects normally associated with endotoxin as pyrogenicity and neutropenia with no significant reduction of its adjuvant activity [2].

A successful human trial of alum-adsorbed liposomes containing monophosphoryl lipid A recently demonstrated that a formulation consisting of a combination of oil/water and adsorbent adjuvants can have considerable safety and efficacy and may be useful in the development of a potential vaccine against *Plasmodium falciparum* [29].

A comparison was made between 100-μg injection of R32NS1 malaria antigen incorporated in lipospheres lacking lipid A and R32NS1 entrapped in lipospheres containing lipid A, with both formulations administered in the absence of alum. Incorporation of lipid A in lipospheres significantly increased the immune response to R32NS1 malaria antigen, resulting in double IgG levels compared with R32NS1 lipospheres lacking the lipid A. The adjuvant effect of lipid A incorporated in lipospheres was observed even after 1600-fold dilution of the rabbit sera.

The adjuvant effect of different doses of lipid A in lipospheres was also examined by immunizing rabbits with lipospheres containing R32NS1 and prepared at different final concentrations of lipid A. The ELISA titers of the individual rabbit groups immunized, as determined by dilution of serum obtained at 6 weeks after primary immunization, have shown a gradual increase in IgG antibody titer with increasing

lipid A dose. The strongest antibody activity was obtained with lipospheres containing 150 μg of lipid A/rabbit. At a higher lipid A dose (200 μg/rabbit), a decrease in ELISA units was observed.

### 5.4.2 Effect of Alum

The effect of alum as adjuvant was also tested in the liposphere–vaccine formulation. In the presence of lipid A, enhanced immune response is obtained even in the absence of alum. This observation is very important because there is increasing concern about the toxic side effects of alum in the long term. Research has suggested a link between aluminum and diseases of the brain, including Alzheimer's disease.

## 5.5 POLYMERIC BIODEGRADABLE LIPOSPHERE VACCINES

Over the last decade, the use of polymeric materials for the administration of pharmaceuticals and as biomedical devices has increased dramatically. The most important biomedical applications of biodegradable polymers are in the form of implants and devices for surgical dressings and are in the area of controlled drug delivery systems. Several articles have been published describing the adjuvant effect achieved by the association of antigens with biodegradable polymeric microparticulate delivery systems, showing controlled release of several immunogens [30–34].

The improvement of the efficiency of essential vaccines by the combination of new immunological adjuvants and advanced delivery systems based on controlled release technology is actually one of the major priorities of the World Health Organization Program for Vaccine Development, as announced by the World Health Organization (WHO) [35]. The general objective is to improve vaccine immunogenicity and to simplify delivery trough conversion of multiple-dose vaccines to single-dose vaccines, with an emphasis on controlled release systems to induce a protective immune response as soon as possible after first immunization with delayed boost of immunity. The preparation and use of polymeric biodegradable lipospheres as a potential vehicle for the controlled release of vaccines was studied. The recombinant R32NS1 malaria antigen was incorporated in biodegradable polymeric lipospheres in the absence or presence of lipid A as an adjuvant.

The immunogenicity of polymeric lipospheres composed of PLA or PCL was tested in rabbits after intramuscular injection of the formulations [14]. High levels of specific IgG antibodies were observed in the sera of the immunized rabbits up to 12 weeks after primary immunization, using a solid-phase ELISA. PCL lipospheres containing the malaria antigen were able to induce sustained antibody activity after one single injection in the absence of immunomodulators. PCL lipospheres showed superior immunogenicity compared to PLA lipospheres, with the difference being attributed to the different biodegradation rates of the polymers.

The important factors in PLA biodegradation are the molecular weight and polydispersity, as well as the crystallinity and morphology of the polymers [36]. Others factors that may affect PLA degradation include chemical and configurational structure, fabrication conditions, site of implantation, and degradation conditions.

Biodegradation of the aliphatic polyesters occurs by bulk erosion. The lactide/glycolide polymer chains are cleaved by random nonenzymatic hydrolysis to the monomeric lactic and glycolic acids and are eliminated from the body through the Krebs cycle, primarily as carbon dioxide and in urine.

On the basis of the differences in the biodegradation profiles of PLA and PCL, it can be assumed that the higher degradation rate of PLA results in faster release of a R32NS1 malaria antigen from the lipospheres, causing the observed temporary increase in antibody activity followed by a gradual time-dependent decrease in IgG ELISA titers. In contrast, PCL, which is known to biodegrade at a slower rate, is probably released from the lipospheres in a more sustained way over a longer period of time, resulting in prolonged immunogenicity.

The adjuvant effect of lipid A on the immunogenicity of polymeric lipospheres was also tested [14]. Incorporation of lipid A in PCL lipospheres had no effect on the IgG ELISA titers. However, in the case of PLA lipospheres, lipid A significantly increased the immune response to R32NS1 malaria antigen, resulting in IgG levels similar to those obtained with PCL lipospheres. The adjuvant effect of lipid A incorporated in PLA lipospheres was observed even after 1600-fold dilution of the rabbit sera [14].

Most vaccines require two or three primary immunizations, followed by a booster for optimum immune response. If one injection of the immunization schedule is missed, it leads to manifold loss of effective antibody titers. According to WHO statistics, more than 30% of the patients do not return for the next injection at each period of the immunization schedule. The effect of noncompliance is most severe in third world countries, where more than a million children die each year from vaccine-preventable diseases.

The ideal method for substantial improvement of current vaccines is to develop formulations that would provide time-released doses of immunogens that could replace the need for multiple visits and booster shots. Controlled release vaccines would be particularly advantageous in the third world, where a repeated immunization of the vaccine by health-care personnel is difficult to achieve [37].

The data described here showed sustained high levels of IgG antibody production following one single immunization of rabbits immunized with biodegradable lipospheres containing malaria antigen. These results are very promising, with the expectation that biodegradable polymeric lipospheres might be very useful in the conversion of multiple-dose vaccines to single-dose vaccinations, avoiding the need for repeated immunizations.

## 5.6 CONCLUSIONS

The results presented in this chapter demonstrate that enhanced immunogenic efficacy can be achieved by using liposphere-based formulations, indicating the potential usefulness of lipospheres in the formulation of human and veterinary vaccines. The liposphere approach employs the fat–lipid environment to achieve several goals: to serve as a carrier to protect the antigen, to serve as a "depot," and to provide a surface interphase necessary for adjuvant activity. The ability to provide different surface

properties to the lipospheres, in addition to reducing the particle size to below 100 nm, makes lipospheres attractive for oral immunization.

It is reasonable to presume that the immunogenic and adjuvant activity of lipospheres may be the result of a combination of factors. These factors may include a focused and enhanced delivery of the antigen to an antigen-presenting cell (macrophage) and protection of the antigen from metabolic destruction at other sites in the body that do no participate in the immune response.

The binding of the antigen to a surface, or the presentation of a special type of surface for antigen adsorption, appears to be critical for the biological activities of many agents that are reported to have adjuvant activities [38]. The data obtained with the liposphere-encapsulated antigen in this study confirm the proposed relationship that exists between physicochemical properties of surface-active systems and their ability to serve as adjuvants. It has been proposed that the ability of surfactants to act as adjuvants is dependent on their capability to concentrate adjuvants and immunogens on hydrophobic surfaces, where they are more effectively presented to cells of the immune system [39].

The liposphere delivery system as a fat-based adjuvant formulation may both provide the surface interface necessary for solubilization and proper orientation of the adjuvant-active material, and provide potential carriers for vaccines, which may allow better position for processing and presentation of the incorporated antigens, resulting in enhanced immunogenicity.

The feasibility of polymeric biodegradable lipospheres as carriers for the controlled release of a recombinant malaria antigen was also demonstrated. Polymeric lipospheres containing R32NS1 malaria antigen were able to induce very high levels of antibody activity after a single injection, in the absence of immunomodulators.

Polymeric lipospheres prepared with a copolymer mixture of PCL–PLA, as well as other biodegradable polymers, can also be prepared using the same procedure described here. An advantage of the copolymer lipospheres delivery system is the ability to control the time or rate at which the incorporated immunogen is released. In the case of vaccines, this allows for better scheduling of the antigen release in such a manner as to maximize the antibody response following a single administration, thus avoiding the need for repeated vaccinations.

## REFERENCES

[1] Alving, C.R., Liposomes as Carriers of Vaccines, in *Liposomes: From Biophysics to Therapeutics,* M.J. Ostro, ed., Marcel Dekker, New York, 1987, 195.

[2] Alving, C.R., Liposomes as Carriers of Antigens and Adjuvants, *Journal of Immunological Methods.* 140, 1, 1991.

[3] Eldridge, J.H. et al., Biodegradable Microspheres as a Vaccine Delivery System, *Molecular Immunology.* 28, 287, 1991.

[4] Cohen S. and H. Bernstein, *Microparticulate Systems for the Delivery of Proteins and Vaccines,* Marcel Dekker, New York, 1996.

[5] Hillery, A.M., Microparticulate Delivery Systems: Potential Drug/Vaccine Carriers via Mucosal Routes, *Pharmaceutical Science and Technology Today.* 1, 69, 1998.

[6] McGhee, J.R. et al., The Mucosal Immune System: from Fundamental Concepts to Vaccine Development, *Vaccine.* 10, 75, 1992.
[7] Hillery, A.M., P.U. Jani, A.T. Florence, Comparative, Quantitative Study of Lymphoid and Nonlymphoid Uptake of 60 nm Polystyrene Particles, *Journal of Drug Targeting.* 2, 151, 1994.
[8] Jani, P. et al., The Uptake and Translocation of Latex Nanospheres and Microspheres after Oral-Administration to Rats, *Journal of Pharmacy and Pharmacology.* 41, 809, 1989.
[9] Hillery, A.M. and A.T. Florence, The Effect of Adsorbed Poloxamer 188 and 407 Surfactants on the Intestinal Uptake of 60-nm Polystyrene Particles after Oral Administration in the Rat, *International Journal of Pharmaceutics.* 132, 123, 1996.
[10] Domb, A.J., L. Bergelson, S. Amselem, Lipospheres for Controlled Delivery of Substances, in *Microencapsulation, Methods and Industrial Applications,* Marcel Dekker, 1996, 377.
[11] Maniar, M. et al., Characterization of Lipospheres: Effect of Carrier and Phospholipid on the Loading of Drug into the Lipospheres, *Pharmaceutical Research.* 8, 1991.
[12] Amselem, S., Delivery Systems for Vaccines, in *Microparticulate Systems for Drug Delivery,* S. Cohen and H. Bernstein, eds., Marcel Dekker, New York, 1996, 149.
[13] Amselem, S., A.J. Domb, C.R. Alving, Lipospheres as Vaccine Carrier Systems: Effects of Size, Charge, and Phospholipid Composition, *Vaccine Research.* 1, 383, 1992.
[14] Amselem, S., C.R. Alving, A.J. Domb, Polymeric Biodegradable Lipospheres as Vaccine Delivery Systems, *Polymers for Advanced Technologies,* 3, 351, 1992.
[15] Rasiel, A. et al., Phospholipid Coated Poly(Lactic Acid) Microspheres for the Delivery of LHRH Analogues, *Polymers for Advanced Technologies.* 13, 127, 2002.
[16] Marathe, D. and K.P. Mishra, Radiation-Induced Changes in Permeability in Unilamellar Phospholipid Liposomes, *Radiation Research.* 157, 685, 2002.
[17] Bekerman, T., J. Golenser, A. Domb, Cyclosporin Nanoparticle Lipospheres for Oral Administration, *Journal of Pharmaceutical Sciences,* 93, 1264, 2004.
[18] Müller, R.H., K. Mäder, S. Gohla, Solid Lipid Nanoparticles (SLN) for Controlled Drug Delivery: A Review of the State of the Art, *European Journal of Pharmaceutics and Biopharmaceutics.* 50, 161, 2000.
[19] Hoffman, S.L. et al., Plasmodium-Falciparum: Sporozoite Boosting of Immunity Due to a T-Cell Epitope on a Sporozoite Vaccine, *Experimental Parasitology.* 64, 64, 1987.
[20] Verma, J.N. et al., Phagocytosis of Liposomes by Macrophages: Intracellular Fate of Liposomal Malaria Antigen, *Biochimica et Biophysica Acta.* 1066, 229, 1991.
[21] Edelman, R., Vaccine Adjuvants, *Reviews of Infectious Diseases.* 2, 370, 1980.
[22] Holmgren, J., N. Lycke, C. Czerkinsky, Cholera-Toxin and Cholera-B Subunit as Oral Mucosal Adjuvant and Antigen Vector Systems, *Vaccine*. 11, 1179, 1993.
[23] Baylor, N.W., W. Egan, P. Richman, Aluminum salts in vaccines: US perspective, *Vaccine*. 20, S18, 2002.
[24] Takada, H. and S. Kotani, Structural Requirements of Lipid-a for Endotoxicity and Other Biological Activities, *CRC Critical Reviews in Microbiology.* 16, 477, 1989.
[25] Allison, A.C. and G. Gregoria, Liposomes as Immunological Adjuvants, *Nature*. 252, 252, 1974.
[26] Heath, T.D., D.C. Edwards, B.E. Ryman, Adjuvant Properties of Liposomes, *Biochemical Society Transactions*. 4, 129, 1976.
[27] Vanrooijen, N. and R. Vannieuwmegen, Endotoxin Enhanced Adjuvant Effect of Liposomes, Particularly When Antigen and Endotoxin Are Incorporated within the Same Liposome, *Immunological Communications*. 9, 747, 1980.

[28] Shek, P.N., B.Y.K. Yung, N.Z. Stanacev, Comparison between Multilamellar and Unilamellar Liposomes in Enhancing Antibody Formation, *Immunology.* 49, 37, 1983.
[29] Fries, L.F. et al., Liposomal Malaria Vaccine in Humans: A Safe and Potent Adjuvant Strategy, Proceedings of the National Academy of Sciences of the United States of America. 89, 358, 1992.
[30] Singh, M., A. Singh, G.P. Talwar, Controlled Delivery of Diphtheria Toxoid Using Biodegradable Poly(D, L-Lactide) Microcapsules, *Pharmaceutical Research*. 8, 958, 1991.
[31] Ohagan, D.T. et al., Biodegradable Microparticles as Controlled Release Antigen Delivery Systems, *Immunology.* 73, 239, 1991.
[32] Kreuter, J., Nanoparticle-Based Drug Delivery Systems, *Journal of Controlled Release*. 16, 169, 1991.
[33] Hunziker, I.P. et al., Perspectives: Towards a Peptide-Based Vaccine against Hepatitis C Virus, *Molecular Immunology.* 38, 475, 2001.
[34] Raghuvanshi, R.S. et al., Improved Immune Response from Biodegradable Polymer Particles Entrapping Tetanus Toxoid by Use of Different Immunization Protocol and Adjuvants, *International Journal of Pharmaceutics*. 245, 109, 2002.
[35] Classified announcements, *Nature.* 353, 1991.
[36] Moynihan, J.S. et al., A Novel Microencapsulated Peptide Vaccine against Hepatitis B, *Vaccine*. 19, 3292, 2001.
[37] Bloom, B.R., Vaccines for the Third World, *Nature*. 342, 115, 1989.
[38] Woodard, L.F., Surface Chemistry and Classification of Vaccine Adjuvants and Vehicles, in *Bacterial Vaccines,* A. Mizrahi, ed., Alan R. Liss, New York, 281, 1990.
[39] Hunter R., S.F., F. Kezdy, The Adjuvant Activity of Nonionic Block Polymer Surfactants: I. The Role of Hydrophile-Lipophile Balance, *Journal of Immunology.* 127, 1244, 1981.