Functionalised Nanoliposomes for Construction of Recombinant Vaccines: Lyme Disease as an Example

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Abstract

Liposomes (phospholipid bilayer vesicles) represent an almost ideal carrier system for the preparation of synthetic vaccines due to their biodegradability and capacity to protect and transport molecules of different physico-chemical properties (including size, hydrophilicity, hydrophobicity, and charge). Liposomal carriers can be applied by invasive (e.g. *i.m.*, *s.c.*, *i.d.*) as well as non-invasive (transdermal and mucosal) routes. In the last 15 years, liposome vaccine technology has matured and several vaccines containing liposome-based adjuvants have been approved for human and veterinary use or have reached late stages of clinical evaluation.

Given the intensifying interest in liposome-based vaccines, it is important to understand precisely how liposomes interact with the immune system and how they stimulate immunity. It has become clear that the physicochemical properties of liposomal vaccines – method of antigen attachment, lipid composition, bilayer fluidity, particle charge, and other properties – exert strong effects on the resulting immune response. In this chapter

we will discuss some aspects of liposomal vaccines including the effect of novel and emerging immunomodulator incorporation. The application of metallochelating nanoliposomes for development of recombinant vaccine against Lyme disease will be presented as a suitable example.

36.1 Introduction

Vaccinology as a scientific field is undergoing dramatic development. Sophisticated techniques and rapidly growing in-depth knowledge of immunological mechanisms are at hand to exploit fully the potential for protecting from, as well as curing of diseases through vaccination. In spite of great successes like eradication of smallpox in the 1970s and in a lesser extent poliomyelitis (two important milestones in medical history), new challenges have arisen to be faced. Rapidly changing ecosystems and human behaviour, an ever-increasing density of human and farmed animal populations, a high degree of mobility resulting in rapid spreading of pathogens in infected people and animals, poverty and war conflicts in the third world, and many other factors contribute to the more frequent occurrence and rapid dissemination of new as well as some old infectious disease.

The three infections that most heavily afflict global health are AIDS, tuberculosis, and malaria. As an example of new viral pathogens, Ebola virus, SARS coronavirus, or new strains of influenza virus can be mentioned [1]. Rapid sequencing of the genome of pathogens leads to development of sensitive molecular diagnostic tools and augments identification and expression of recombinant antigenic targets for future vaccines [2]. Special field represents immunotherapy of cancer, where anticancer vaccines could be a powerful weapon for long-term effective treatment.

The progress in vaccine development is tightly connected not only with new findings in immunology but also in molecular biology and biotechnology. The new term "reverse vaccinology" was proposed by Rappuoli to describe a complex genome-based approach toward vaccine design [3]. In comparison with conventional approaches which require a laborious process of attenuation or inactivation of pathogens, or selection of individual components important for induction of immune response, reverse vaccinology offers the possibility of using genomic information derived from in silico analyses for direct design and production of protective antigen using recombinant technology.

This approach can significantly reduce the time necessary for the identification of antigens for development of candidate vaccine and enables systematic identification of all potential antigens even from pathogens which are difficult or currently impossible to culture. Of course, this approach is limited to identification of protein or glycoprotein antigens, omitting such important vaccine components such as polysaccharides and glycolipids. The principal question for reverse vaccinology consists in identification of protective antigen, which presents the main hurdle of this approach. Nevertheless, once the protective antigen is identified it enables scientists to systemically classify such antigens, and develop efficient preparations virtually against any pathogen that has had its genome sequence determined.

Subunit vaccines offer superior safety profiles and can be manufactured with minimal risk of contamination [4, 5]. When coupled with appropriate adjuvants, they can also focus the immune response on protective or highly conserved antigenic determinants that may not elicit a potent response during natural infection or after vaccination with an inactivated or attenuated pathogen [6, 7].

A common observation from the process of elicitation of the adaptive immune response is that the antigen by itself is not a stimulating agent. In other words, administration of absolutely pure recombinant protein antigens and synthetic peptide antigens generally does not induce specific immune response. Therefore there is a need for potent co-stimulation by co-administration of appropriate adjuvants, biocompatible carrier systems and application devices for vaccines consisting of highly purified antigens. These particulate systems are supposed to mediate efficient delivery to antigen-presenting cells and may induce inflammation through activation of innate immunity [8–10].

36.2 Lyme Diseases

Lyme disease is the most frequent zoonosis both in Europe and the United States. Disease may progress into a chronic form and cause damage of the nervous or cardiovascular system, joints, skin, or eyes. Infected patients can be affected by prolong work disability or even permanent invalidity. Prevention and treatment of disease thus becomes a long-term priority for medical research.

36.3 History

Some symptoms typical for Lyme disease have been known since the beginning of the twentieth century – acrodermatitis chronica atrophicans, erythema migrans, lymphocytoma, and meningo-polyradiculoneuritis [11]. Nevertheless, the aetiological agent *Borrelia burgdorferi* was described quite recently on the basis of endemic juvenile arthritis accompanied with erythema migrans [12, 13].

Later it was shown that *B. burgdorferi* sensu lato is a complex of several sibling species. Currently, 12 species are distinguished and new variants are identified continuously, so the number of *Borrelia* species is probably not final. At least three species are known to be pathogenic for human – *B. burgdorferi sensu stricto, B. afzelii* and *B. garinii*. The number of annually reported Lyme disease cases continually increases in many geographical areas. This may be due to the actual spread of the disease or alternatively, to improved diagnostic methods [14].

36.4 Aetiological Agents

The genus *Borrelia* Swellengrebel 1907 is a member of the family Spirochaetaceae together with genera *Leptospira* and *Treponema*. In the USA, the only one causative agent – *B. burgdorferi* s.s. – was described. In Europe, two species – *B. afzelii* and *B. garinii* – are the most common.

Borreliae are typical for their spiral-shaped cells, 10–30 μm in length and 0.2–0.3 μm in diameter. This shape is caused by periplasmatic flagella responsible for the high motility in viscous environment like connective tissue. *Borrelia* lacks the rigid cell wall and the surface is composed mainly of lipoproteins with strictly controlled expression pattern, essential for adaptation for external conditions [15].

36.5 Epidemiology, Clinical Disease, and Treatment

The disease has a vector character. Most of the time, it is transmitted to humans by infected ticks of the genus *Ixodes*, but *Borrelia* has also been found in the midgut of the mosquitoes and other blood sucking insect. The role of haematophagous insect in disease transmission is still unclear [16, 17]. Reservoir competence has been described in the broad spectrum of wildlife animals, e.g. rodents (genera *Apodemus, Clethrionomys, Microtus, Rattus*, etc.), squirrels (*Sciurus sp.*), hares (*Lepus sp.*), and birds (genera *Turdus, Phasianus, Carduelis and Fringilla*, etc.). Human is a terminal host, incapable of further spreading of infection [18].

Lyme disease can have three stages – early localised infection, early disseminated infection, and late persistent infection, but not all stages develop in every infected individual. It appears that a significant portion of infections has asymptomatic course without clinical symptoms, but with elevated levels of anti-*Borrelia*-specific antibodies. The first stage is characterized by non-specific symptoms including fever, chills, headache, lethargy, and/or muscle and joints pain, about 70 % is accompanied by erythema migrans at the site of bacterial entry. If the infection is not eliminated by the host immune system or antibiotics treatment, it may disseminate and affect central nervous system (meningitis, radiculopathy, seventh cranial nerve palsy) or cardiovascular system (atrioventricular heart block, myopericarditis). The late persistent infection may develop months to years after the transmission and commonly affects the nervous system, skin, joints, or less frequently heart, eyes, or other organs [15].

Treatment of Lyme disease is based on the application of antibiotics. For adults administration of doxycycline is recommended. For children

mainly beta-lactam antibiotics are prescribed. For the treatment of advanced forms, cephalosporins or penicillin G are recommended. Long-term usage of antibiotics in persistent forms of Lyme disease has only limited effect [19].

The first effort to design vaccine against Lyme disease followed shortly after the discovery of *Borrelia* as the aetiological agent causing this disease. First experiments demonstrated the immunogenicity of bacterial whole cell lysates. Later, specific proteins were identified to be recognised by the host immune system [20].

36.6 Antigens and Immune Response

Expression of surface antigens by *Borrelia* is highly variable. In the tick host, *Borrelia* expresses outer surface proteins A (OspA) and B (OspB). Genes coding both proteins are located within one operon and expression of these proteins is dependent on housekeeping sigma factor RpoD (σ^{70}). Presumed function of OspA and OspB is the adhesion to tick gut epithelium. When tick starts blood feeding on the vertebrate host, both proteins are downregulated. At the same time the expression of proteins dependent on alternative sigma factor RpoS (σ^{38}) is induced, e.g. (OspC) and OspF and decorin-binding proteins DbpA and DbpB. These proteins are required for *Borrelia* transmission to vertebrate and for initial stages of vertebrate infection. In later stages, OspC is downregulated and the expression of VlsE protein (Vmp-like sequence, expressed) is induced. It enables *Borrelia* escape from specific humoral response due to high VlsE variability.

Early humoral response to *Borrelia* is characterised by production of IgM antibodies specific to OspC, flagellins (p39 and p41), and BmpA (p39). Later IgG antibodies against p39, p41, p83/100, DbpA and VlsE arise. In some cases, antibodies against OspA and OspB can be detected in later stages of the disease [15].

Subunit vaccines of first generation were based on OspA antigen, which is highly expressed during cultivation of Lyme disease spirochetes in vitro. Preclinical trials with vaccines based on OspA antigen demonstrated protection in animal models. OspA antigen was produced either as a recombinant protein or lipoprotein, or it was expressed on the surface of *Escherichia coli*, *Salmonella typhimurium*, or *Mycobacterium bovis*.

Two OspA-based vaccine candidates were developed and tested. The ImuLyme vaccine (Pasteur Merieux-Connaught) based on purified recombinant OspA antigen expressed in *E. coli* and LYMErix (GlaxoSmithKline) containing purified recombinant OspA from *B. burgdorferi* s.s. [21]. In both vaccines protein was adsorbed on aluminium hydroxide. In clinical trials involving more than 10,000 people, LYMErix vaccine was found to confer protective immunity to *Borrelia* in 76 % of adults and 100 % of children with only mild or moderate and transient adverse effects [20]. Based on these results, LYMErix was approved by the Food and Drug Administration (FDA) on December 21, 1998.

Subsequently, hundreds of vaccinee reported the development of autoimmune side effects. Supported by some patient advocacy groups, a number of class-action lawsuits were filed against GlaxoSmithKline, alleging the vaccine had caused these health problems. These claims were investigated by the FDA and the US Centers for Disease Control (CDC), who found no connection between the vaccine and the autoimmune complaints [22, 23]. Despite the lack of evidence, sales plummeted and LYMErix was withdrawn from the US market by GlaxoSmithKline in February 2002 in the setting of negative media coverage and fears of vaccine side effects.

There are a number of reasons for the slow development of a Lyme disease vaccine, including reluctance by pharmaceutical companies to get burned in a similar way to LYMErix manufacturers. Others argue that it is simply more profitable for drug manufacturers to 'treat' the symptoms of Lyme palliatively, giving anti-inflammatory medications, pain relievers, and other medications where antibiotics fail to address a patient's ailments. The continued availability of a Lyme disease vaccine for dogs has made many people question the motives of drug companies and the psychological component of many symptoms often blamed on Lyme disease.

36.7 Licensed Veterinary Lyme Disease Vaccines

Unlike humane medicine, several licensed veterinary Lyme disease vaccines are available for companion pets. Three are formulations of *B. burgdorferi* bacterin (Merilym, Merial, Germany; Galaxy Lyme, Schering Plough, USA; LymeVax, Fort Dodge, USA). A European vaccine based on combination of bacterins from *B. garinii* and *B. afzelii* is produced for dogs (Biocan, Bioveta, Czech Republic) [24]. There are also available veterinary subunit vaccines based on recombinant OspC antigen (ProLyme, Intervet, USA; Recombitek Lyme, Merial, USA). All of the veterinary vaccines are applied in two-dose immunisation scheme with boosters recommended yearly. Safety data are limited and minor cross-reactivity between heterologous *Borrelia* species has been reported [25]. Thus, the poor cross-protective coverage is a problem of currently available vaccines.

Second generation of OspA subunit vaccines is based on recombinant OspA with genetically removed potentially cross-reactive T-cell epitopes, formerly suspected for the induction of autoimmunity [26]. Development of broadly cross-protective rOspA vaccine must address issues pertaining to sequence variation, because at least seven serotypes of OspA do exist. Multivalent-chimeric OspA proteins have been developed by molecular cloning incorporating the protective epitopes from several OspA serotypes [27]. Nevertheless, OspA antigen-based vaccines require repeated vaccination to keep high titre of specific antibodies in vaccinee serum to prevent transfer of bacteria from tick into human host.

36.8 Ferritin

New interesting antigen for vaccine development is tick ferritin 2, which is responsible for maintaining iron homeostasis. Iron is an essential but potentially toxic element; therefore, during feeding, ticks must deal with the challenge of an enormous iron supply in the blood meal. Ferritins, the iron storage proteins, play a pivotal role in this process. It was shown that vaccination with recombinant FER2 significantly reduces tick infestations in vaccinated rabbits infected with *Ixodes ricinus* and in cattle infected with *Rhipicephalus microplus* and *Rhipicephalus annulatus*. These results support the inclusion of FER2 as a promising candidate antigen for development of new anti-tick vaccines [28]. In spite of the fact that ferritin 2 is not the antigen derived from *Borrelia*, the vaccination with this antigen prevents long-term feeding of tick and transmission of *Borrelia* as well as other tick-transmitted pathogens into host. In this aspect ferritin 2 could be superior to OspA and is of interest to include it in development of combined multiantigen vaccine.

36.9 OspC Antigen

As mentioned above, several new antigens were discovered and studied as possible targets for new vaccines. We focus on OspC antigen, which received considerable attention in the effort to develop a broadly protective Lyme disease vaccine. OspC is an essential virulence factor that is critical for the establishment of early infection in mammals [29] and the sequence of the protein is not undergoing mutation during infection [30]. OspC is a 22 kDa immunodominant lipoprotein that is anchored in the spirochete outer membrane by an *N*-terminal tripalmitoyl-*S*-glyceryl-cysteine [31].

The factor complicating the exploitation of OspC as a vaccine candidate is the existence of OspC variation. Molecular phylogenetic analyses have revealed that OspC sequences form at least 38 distinct OspC types. This OspC variation arises primarily through genetic exchange and recombination and not by hypermutation with concomitant immune selection [32]. Owing to this sequence variation the protective range of single OspC-based vaccine is narrow. A potential approach to solve the high variability of the OspC is to prepare (a) polyvalent vaccine containing several OspC variants [33]; (b) generation of recombinant chimeric protein that consists of

protective epitopes from those OspC types associated with disease [34]; and (c) combination of two or more different recombinant immunogens like OspC, DbpA, and fibronectin-binding protein (BBK32) [35].

From the biotechnological point of view the full-length OspC is difficult to express in a high yield and purity as a recombinant protein [36]. The removal of the lipidisation signal substantially increases both the yield (28 mg/1 of the bacterial culture) and purity (93 %) of the recombinant OspC protein [37]. On the other side delipidised rOspC exerted very low immunogenicity [38, 39] and strong adjuvanticity of vaccine formulation is necessary to induce specific antibody response especially in IgG2 subclass (in experimental mice) important for effective complement activation and opsonisation.

In effort to adhere to all above requirements for OspC vaccine, we developed experimental vaccine based on functionalised metallochelating nanoliposomes and synthetic non-pyrogenic adjuvants derived from muramyl dipeptide. This formulation induced strong immune response in experimental mice, which was superior to aluminium hydroxide formulation. Owing to the versatility and safety as an adjuvant, liposomes thus represent promising platform for developing the clinically acceptable vaccine against Lyme disease. Next part of the chapter is focused on liposomal-based vaccines.

36.10 Liposomes as Biocompatible and Versatile Carriers for Construction of Recombinant Vaccines

Of the numerous particulate delivery systems that have been developed until now, phospholipid bilayer vesicles (liposomes) are among the most promising. Gregoriadis and Allison first reported the use of liposomes as immunological adjuvants in 1974 [40, 41]. Since that time, liposomes and related vesicular carriers have been established as robust systems for induction of humoral and cell-mediated immunity to a broad spectrum of infectious diseases and cancers [42].

Liposomes represent the oldest nanoparticle systems described for applications in biological studies as model membranes and in medicine. Over 44 years, liposomes have been shown to be suitable drug delivery systems for applications ranging from cosmetics and dermatology to medical applications such as therapy of infections, anticancer therapy, and veterinary vaccination [43].

Liposome-based vaccines have been around for approximately 30 years, and numerous liposome variants have been developed, some with evident immune-stimulatory properties and attractive safety profiles, resulting in registration of several products on the market and progressing of others to advanced stage of clinical testing. Epaxal® (Crucell, hepatitis A, formalin-inactivated hepatitis A virus adsorbed to virosomes) and Inflexal® V (Crucell, influenza, virosomes – reconstituted influenza viral membranes) are two examples of marketed liposome-based vaccines for human application. Inflexal® V is licensed in 43 countries with over 60 million doses applied [44, 45].

Furthermore, at least eight liposome-based adjuvant systems are currently approved for human use or undergoing clinical evaluation [46].

36.11 Chemical, Physicochemical, and Structural Parameters Affecting Activity of Liposome-Based Vaccine

Liposomes represent almost ideal carrier/delivery systems for the components of synthetic vaccines due to their biodegradability and their ability to retain/incorporate a variety of essential vaccine components simultaneously, even components possessing quite different physicochemical properties (different size, hydrophobicity, charge, etc.). Different synthetic vaccine components can be encapsulated within the aqueous cavities of liposomes (if hydrophilic) or associated with liposome bilayers (if at least partially hydrophobic in character). Furthermore, essential components can be attached to either internal or external outer leaflet membrane by electrostatic, covalent, or metallochelation interactions. Biochemically the
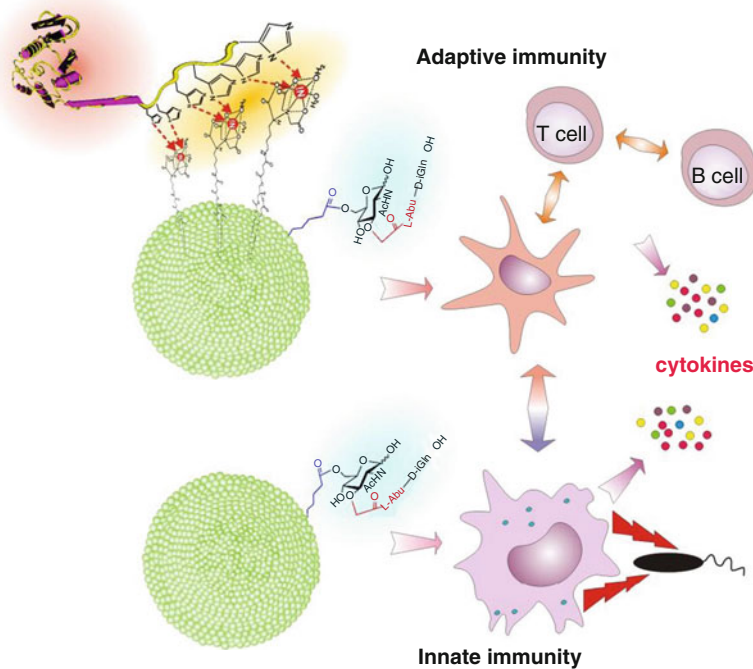
Fig. 36.1 Interaction of liposome-based immunomodulators and proteoliposomal vaccines with immune cells. Functionalised nanoliposomes facilitate co-delivery of antigen to immune cells (dendritic cells, macrophages and B-cells). Highly oriented protein antigen on the liposomal surface is able to interact with membrane-bound immunoglobulins on B-cells followed by internalisation of whole complex liposomal antigen. Synthetic lipophilic derivative of muramyl dipeptide (norAbuMDP) provides the danger signal via intracellular receptors like NOD2 and NALP-3 to induce innate and adaptive immune responses. Moreover, glycopeptide part of the molecule exposed on the liposomal surface forms molecular pattern recognised by immune cells and enhancing internalisation of liposomes (Liposomes and immune cells are not in scale)

most diverse vaccine components are adjuvants required for efficacious activation of innate immunity cells including antigen-presenting cells (APC) (e.g. monophosphoryl lipid A, CpG oligonucleotides, muramyl dipeptide, and analogues). In addition, adjuvants can be combined with antigens such as soluble or membrane proteins to provoke strong specific immune response. Finally, the liposomes may present carrier for targeting of antigens and adjuvants to antigen-presenting cells [47]. The laboratory and industrial procedures for the liposome preparation have been established, and liposomes have been approved by the US Food and Drug Administration for biomedical applications. The potential for the participation of liposome-based recombinant vaccines in the human and veterinary vaccine market is very promising [48].

The beneficial effect of liposomal carriers consists in their ability to ensure (1) protection and stabilisation of the antigen, including reconstitution of its native conformation, (2) enhanced uptake by APC by passive or active targeting and (3) enhanced or controlled antigen processing after uptake.

Interaction of liposomal vaccine and liposomal immunomodulators with immune cells and cooperation between innate and adaptive branches of immune system is schematically represented in Fig. 36.1.

Liposome-based nanoparticle formulations used for vaccination can be broadly grouped into several classes:

Conventional liposomes composed of neutral lipids and cholesterol have been the most widely studied due to their greatest versatility – desired

formulation parameters can be achieved through modification of the lipid composition or vesicle preparation method. These liposomes were used for steric entrapment of protein antigens in early stage of liposome-based vaccine research.

These classical liposomes are relatively ineffective at enhancing the immunogenicity of antigens because the phospholipids do not act as adjuvants. Adjuvant effect of these liposomes is based on multimeric presentation of antigen, and their adjuvant effect can be optimised by variation of lipid composition, co-incorporation of various adjuvant molecules (e.g. MLP-A, MDP, CpG oligonucleotides, trehalose dibehenate), and attachment of antigen.

Transferosomes are ultradeformable liposomes with enhanced skin penetration properties. They are composed of phosphatidylcholine and cholate (9:2 M ratio). Presence of cholate endowed these vesicles with high elasticity enables them to squeeze through pores in the stratum corneum. As a result of high elasticity of the bilayer, vesicles of the size of 200–300 nm can pass the highly compact stratum corneum and deliver their content into deep parts of the skin. Transferosomes are utilised as carrier system for topical application of vaccines [49]. Cationic transferosomes were also tested for noninvasive topical vaccination with pDNA vaccine against hepatitis B [50].

36.12 Cationic Liposomes

Surface adsorption on the cationic liposomes, via electrostatic interactions, is another useful method for attachment of antigens (primarily proteins) on liposomes. The recent work of Christensen, Perrie, and others with protein antigens adsorbed to cationic DDA/TDB (dimethyldioctadecylammonium/trehalose-6,6-dibehenate) liposomes has demonstrated that formulations with surface-adsorbed antigens can be highly stable and elicit robust antibody and cell-mediated immune responses in mice and ferrets [42, 51–53]. Incorporation of synthetic immunostimulatory molecules like lipophilic TDB into cationic liposomes is able to potentiate significantly their adjuvant effect [52]. It was also observed that certain cationic lipids, originally synthesised as transfection reagents, induce robust IFN-γ and TNF-α response in serum and antitumour immunity in murine models of pulmonary metastasis and fibrosarcoma [54]. DOTAP is an example of such a cationic lipid used for many years as component of liposomal gene delivery system exerted an enantiospecific adjuvant effect in dendritic cells [55].

Virosomes are a special class proteoliposomes prepared from reconstituted influenza virus membranes supplemented with PC [56]. The physicochemical features of virosomes are constrained by their well-defined composition and method of preparation, but these vesicles benefit greatly from the inherent delivery properties (efficient cell binding, internalisation, and cytosolic release) and immunogenicity of the influenza virus.

Archaeosomes are liposomes prepared from special lipids isolated from archaebacteria, which are extremophiles, and their cell membranes contain lipids and proteins resistant to extreme temperatures, pH, and salt concentration. These lipids are less sensitive to oxidation and the membrane of archaeosomes is resistant to action of bile acid salts. Therefore they are suitable for enteral application. Immune responses induced by archaeosomes are comparable to those induced by complete Freund's adjuvants [57].

Functionalised liposomes in general represent various liposomal structures having their surface modified by various ligands (biotin, oligosaccharides, peptides), lipids with reactive head groups, pH or temperatures sensitive polymers, monoclonal antibodies or their fragments, lectins, etc. to tailor their functions with respect to selective interaction with targeted extra or intracellular structures (e.g. pathogenic microorganisms, tumour cells, immune cells, blood clothes). Targeting of antigen to antigen-presenting cells is of great importance for enhancement of efficacy of subunit recombinant vaccines. In this chapter we will introduce a new system for construction of liposomal-based vaccines. This system employs metallochelating lipids for binding of HIS-tagged recombinant antigens onto the surface of liposomes.

36.13 Binding of Antigen to Liposomes

One of the most critical parameters influencing the immunogenicity of liposomal vaccines is the method by which the antigen is physically or chemically associated with the formulation. The most common modes of association include covalent lipid conjugation (either pre- or post-vesicle formation), non-covalent surface attachment (via biotin, NTA-Ni-His, or antibody–epitope interactions), encapsulation, and surface adsorption (Fig. 36.2). Many of the early studies of liposomal peptide and protein antigenicity in mice compared encapsulated antigens to those conjugated to the surface of preformed liposomes. Early studies by Alving, Gregoriadis, Therien, and others confirmed that both methods are generally effective for inducing antibody and T-cell responses to associated protein antigens such as albumin and tetanus toxoid [58–60].
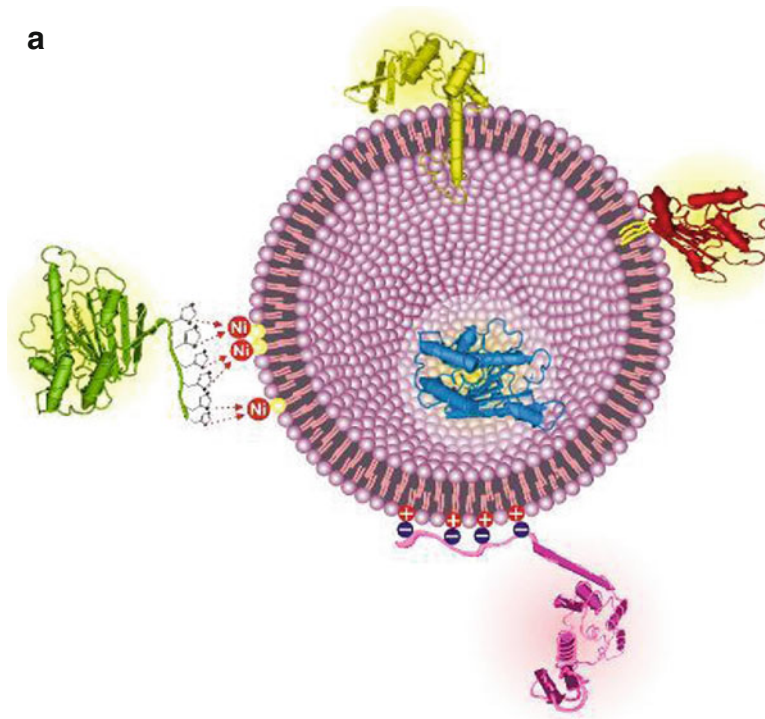
Fig. 36.2 (**a**) Association of protein antigen with liposome. Protein antigen can be associated with liposome by various ways. Membrane protein can be reconstituted in their natural conformation in liposome membrane (*yellow*); lipophilised protein (e.g. palmitoylated protein) can be anchored in lipid membrane by lipidic residue (*red*). Functionalised liposomes can be prepared to facilitate covalent or non-covalent binding of protein onto the surface of liposomes. Selective metallochelating bond is applicable for His-tagged recombinant proteins (*green*) and this non-covalent bond can be transformed to a covalent one via carbodiimide conjugation. All above-mentioned examples represent highly oriented binding of protein onto liposomal surface. Covalent coupling of protein onto liposomal surface functionalised by, e.g. reactive maleiimide phospholipid headgroup, leads to random orientation of bound protein. Electrostatic interaction represents another way for association of proteins with liposomes. This association is non-specific and relatively labile in biological milieu (*pink*). Molecule of soluble protein can be also sterically entrapped in water compartment of liposome (*blue*). This is of importance especially for peptide antigens and protein antigens intended for direct delivery into the cytoplasm of immune cells to mimic virus-like pathways for antigen processing. (**b**) Binding of protein onto liposomal surface. *A*. Oriented non-covalent binding of HIS-tagged GFP (green fluorescent protein) onto liposomal surface. *B*. Transformation of metallochelating bond into covalent amide bond via carbodiimide chemistry preserving oriented binding of GFP. Ni$+2$ ions are removed by EDTA as a metallochelation agent. *C*. Example of random orientation of GFP bound onto liposomal surface by application of carbodiimide chemistry without pre-orientation via metallochelating bond
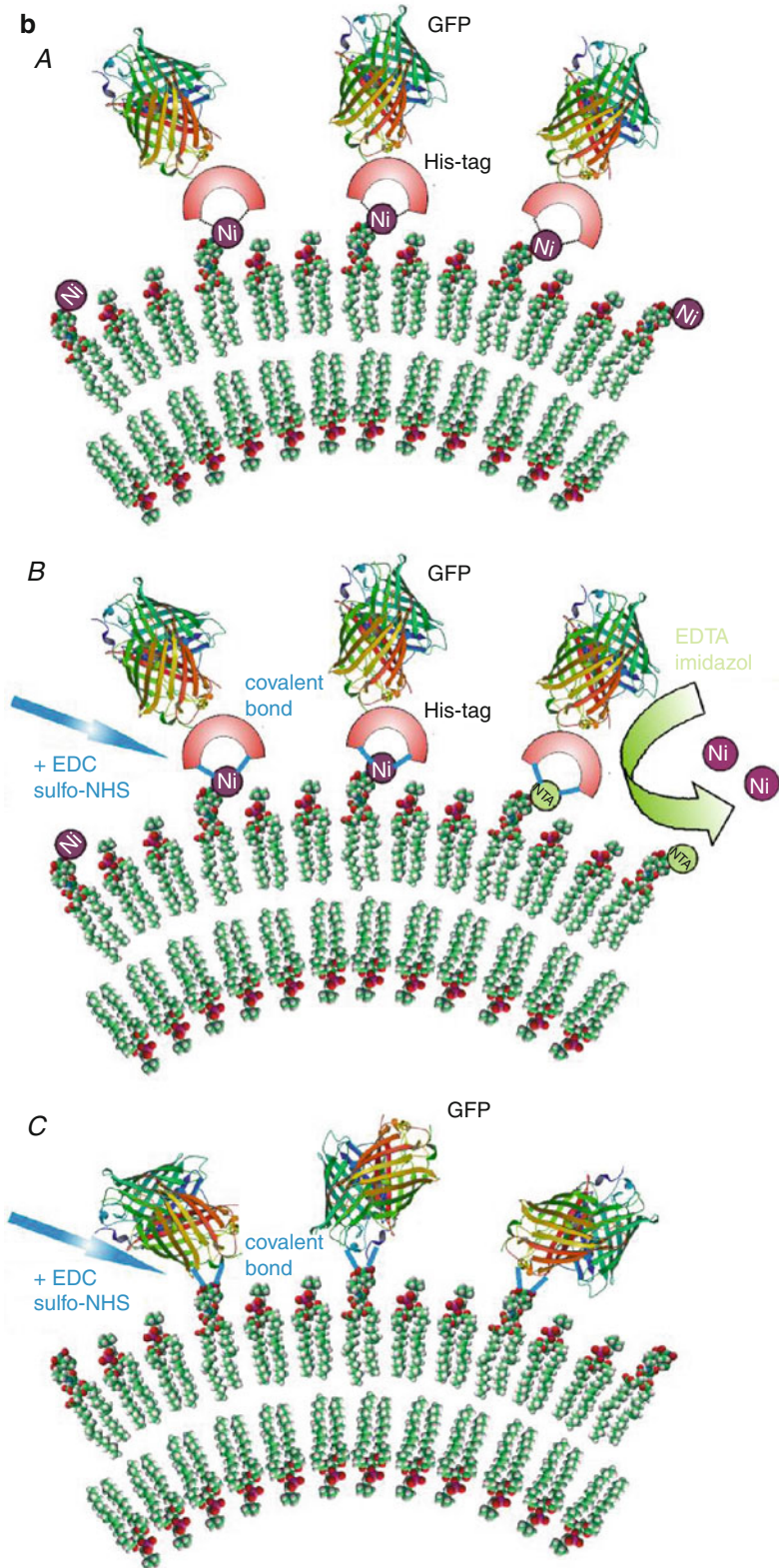
In some cases, covalent antigen conjugation results in superior antibody induction, which is not surprising because B-cell receptors can recognise intact antigen on the liposome surface [61]. As the size and complexity of the antigen decreases, the benefit of their surface conjugation for antibody induction becomes more pronounced, as found for the synthetic peptides for which surface conjugation provides immune responses superior to encapsulation [61, 62].

36.14 Metallochelating Bond and Metallochelating Liposomes

With respect to potential application of metallochelating liposomes for the construction of vaccines, the question of in vitro and especially in vivo stability is of great importance. This problem could be divided into two fields: first, the stability of the liposomes themselves and second, the effect of the components presented in biological fluids (e.g. proteins and ions) on the stability of the metallochelating bond. In the example of OspC proteoliposomes, the gel permeation chromatography data indicated a good in vitro stability of the OspC proteoliposomes during the chromatographic process, within which they experience a shear stress and dilution. Also after incubating OspC proteoliposomes in serum at 37 °C, we demonstrated the stability of the metallochelating bond linking the protein to the liposomal surface [37]. In fact, in vivo *fate* of liposomes after the intradermal application is different from the situation after intravenous injection. First, dilution of proteoliposomes after intradermal application is not so rapid and second, the ratio of tissue fluid proteins to proteoliposomes is more favourable to proteoliposomes owing to their relatively high concentration at the site of application. In the case of another route of application – intradermal – the flow rate of tissue fluid within intradermal extracellular matrix is even lower in comparison with muscle tissue or blood vessels. This fact is often overlooked. The stability of metallochelating bond probably depends also on the character of the particular protein. It was shown that Ni-NTA3–DTDA
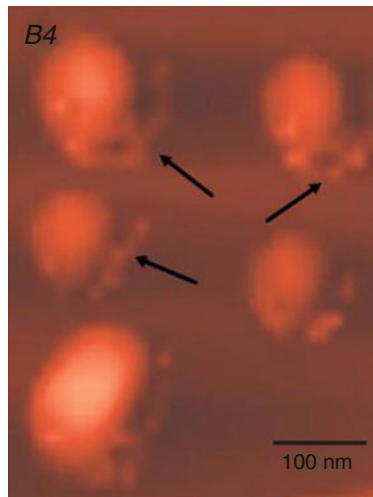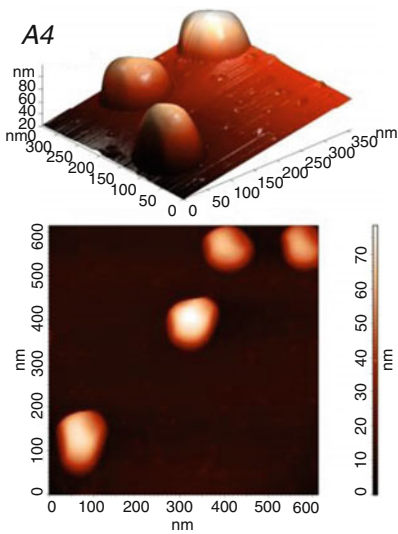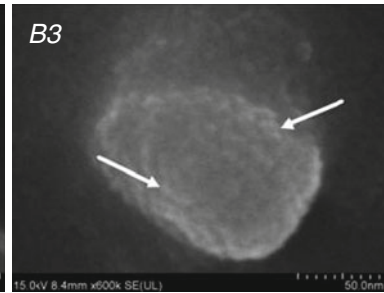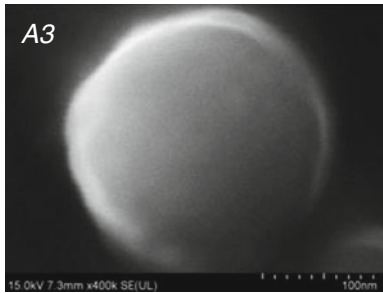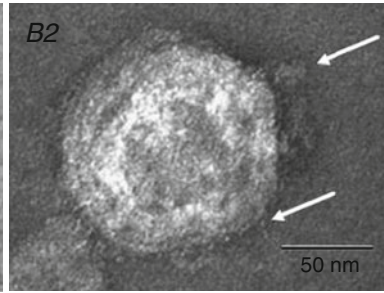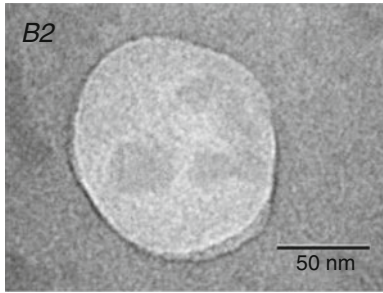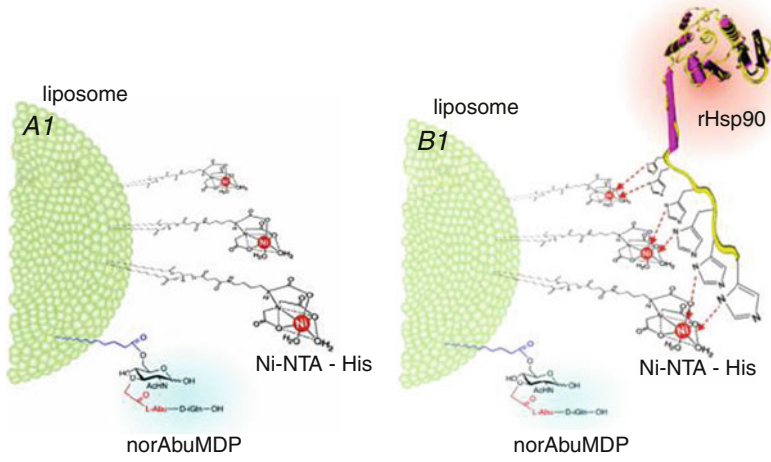
liposomes (containing three functional chelating lipid) with single-chain Fv fragments (anti CD11c) bound onto the liposomal surface were able to target dendritic cells in vitro as well as in vivo. The application of Ni-NTA3–DTDA probably endows the metallochelating bond with a higher in vivo stability [63], but this improved stability did not influence higher immunogenicity [64]. Generally, metal ions, physicochemical character of the metallochelating lipids, and their surface density belong to the factors that could be optimised to get a required in vivo stability and therefore strong immune response.

Binding of recombinant OspC (rOspC) onto metallochelating liposomes was confirmed by TEM, GPC, SDS PAGE and dynamic light scattering methods. Stability of the metallochelating bond in model biological fluids was studied by the incubation of rOspC liposomes with human serum. This study showed that after 1 h incubation at 37 °C, more than 60 % of rOspC was still associated with liposomes. Based on this data, therefore, the half-life of rOspC proteoliposomes in serum was estimated to be at least 1 h. Increase in stability of surface-exposed antigens on the liposomes can be achieved by chemical binding onto the outer liposomal surface that is appropriately functionalised. With respect to a potential application for the construction of vaccines, the question of in vitro and especially in vivo stability is of great importance.

There are only few references reporting the metallochelating bond implemented in the construction of supramolecular structures as vaccine carriers [65–69]. Various ways for association of protein antigen with liposome is depicted in Fig 36.2a. Real structures revealed by various microscopy methods are presented in Fig. 36.3.

36.15 Adjuvants for Liposomal-Based Vaccines

The interaction between the innate and the adaptive immune responses is paramount in generating an antigen-specific immune response. The initiation of innate immune responses begins

liposome
A1
Ni-NTA - His
norAbuMDP

liposome
B1
rHsp90
Ni-NTA - His
norAbuMDP

B2
50 nm

B2
50 nm

A3
15.0kV 7.3mm x400k SE(UL)   100nm

B3
15.0kV 8.4mm x500k SE(UL)   50.0nm

A4

B4
100 nm

with the interaction of pathogen-associated molecular patterns (PAMPs) on the pathogen side with pattern-recognising receptors (PRR) such as Toll-like receptors (TLRs) on the host cells involved in the innate immunity (e.g. dendritic cells). A major functional criterion commonly used for the evaluation of various new adjuvants involves their ability to stimulate the innate immunity cells. This would include engaging other PRRs and the co-receptors and intracellular adaptor signalling proteins with which they are associated. PAMPs and their derivatives are utilised by adjuvant developers to harness the power of innate immunity to channel the immune response in a desired direction.

Based on the identification of several TLRs and PAMPs recognised by them, various PAMP agonists were tested as adjuvants. Examples of TLR–PAMP-specific interaction include bacterial or viral unmethylated immunostimulating CpG oligonucleotides interacting with TLR9 and liposaccharide and its component monophosphoryl lipid A (MPLA) interacting with TRL4. These two types of adjuvants are in advanced stage of testing in clinical trials and some already licensed vaccines contain MPLA in liposomal form [70, 71].

New lipophilic adjuvant (see Fig. 36.4) suitable for construction of liposome-based vaccines is the mycobacterial cord factor trehalose-6,6-dimycolate (TDM), and its synthetic analogue trehalose-6,6-dibehenate (TDB) are potent adjuvants for Th1/Th17 vaccination that activate Syk-Card9 signalling in APCs [72].

The last group of potent synthetic or semisynthetic adjuvants is derived from muramyl dipeptide (MDP).

Muramyl dipeptide and other muropeptides are derived from very specific group of PAMPs represented by peptidoglycans (PGN). Both Gram-positive and Gram-negative bacteria contain PGN which consists of numerous glycan chains that are cross-linked by oligopeptides. These glycan chains are composed of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) with the amino acids coupled to the muramic acid. Muropeptides are breakdown products of PGN that bear at least the MurNAc moiety and one amino acid [73]. One of the prominent muropeptides is muramyl dipeptide (MDP), which is known since the 1970s. We will deal with this compound in more detail.

Recently, the molecular bases for MDP recognition and subsequent stimulation of the host immune system have been uncovered. Myeloid immune cells (monocytes, granulocytes, neutrophils, and also DCs) possess two types of intracellular receptor for MDP/MDP analogues, namely, NOD2 and cryopyrin (inflammasome-NALP-3 complex) [74–76]. These two receptors recognise MDP/MDP analogues minimal recognition motifs for bacterial cell wall peptidoglycans [77]. Another recently reported sensor of MDP is cryopyrin (also known as CIAS1 and NALP3), which is a member of the NOD–LRR family [74]. Cryopyrin is a part of the inflammasome complex that is responsible for processing caspase-1 to its active form. Caspase-1 cleaves the precursors of interleukin IL-1β and IL-18, thereby activating these proinflammatory cytokines and promoting their secretion. IL-1β is known to be a strong endogenous pyrogen induced by MDP. We showed that norAbu-MDP and that norAbu-GMDP analogues

Fig. 36.3 Structure of metallochelating liposomes and proteoliposomes revealed by TEM, SEM and AFM. Recombinant heat shock protein derived from *Candida albicans* was used as illustrative example. *A1*. Schematic presentation of a metallochelating liposome with metallochelating lipids and incorporated lipidized norAbuMDP molecules. *A2*. TEM photograph of a metallochelating liposome with metallochelating lipids and incorporated lipidized norAbuMDP molecules. *A3*. SEM photograph of a metallochelating liposome with metallochelating lipids and incorporated lipidized norAbuMDP molecules. *A4*. AFM photograph of a metallochelating liposome with metallochelating lipids and incorporated lipidized norAbuMDP molecules. *B1*. Schematic presentation of a metallochelating liposome (A1) with rHsp90 bound via metallochelating bond. *B2*. TEM photograph of a metallochelating liposome with rHsp90 bound via metallochelating bond. *B3*. SEM photograph of a metallochelating liposome with rHsp90 bound via metallochelating bond. *B4*. AFM photograph of a metallochelating liposome with rHsp90 bound via metallochelating bond
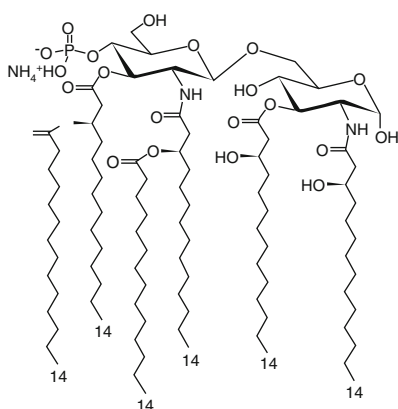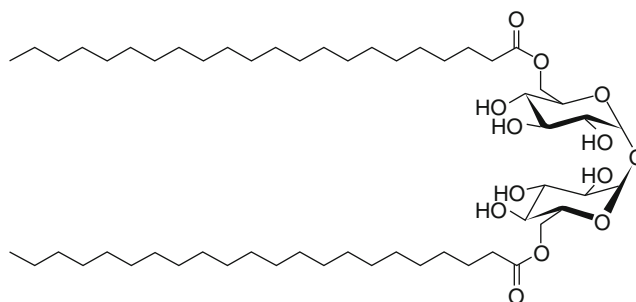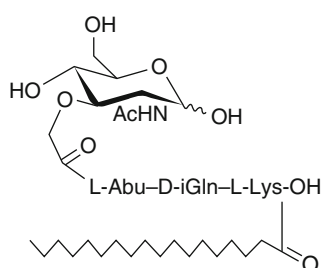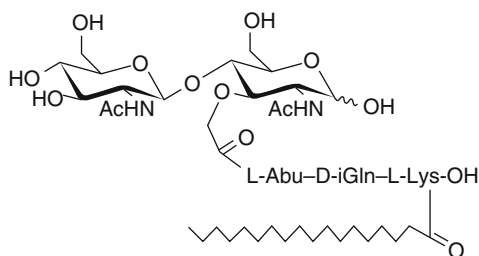
Monophosphoryl Lipid A (MPL A)
(TDB)

D-(+)-trehalose 6,6'-dibehenate

nor-AbuMDP
(lipophilised in peptide part)

nor-AbuGMDP
(lipophilised in peptide part)

L-Abu–D-iGln–L-Lys-OH

L-Abu–D-iGln–L-Lys-OH

Fig. 36.4 New lipophilic adjuvants for liposomal vaccines

were not pyrogenic even at a high concentration, much higher than the concentrations used for vaccination [15, 65, 66].

The expression of NOD2 in dendritic cells is of importance with respect to the application of MDP analogues as adjuvants. Nanoparticles like liposomes are able to provide a direct co-delivery of a danger signal (e.g., MDP) together with the recombinant antigen and therefore to induce an immune response instead of an immune tolerance. This is especially important for weak recombinant antigens or peptide antigens. Clearly, the recognition of MDP by DCs is crucial for the application of MDP analogues as adjuvants. Within the cell, MDP/MDP analogues trigger intracellular signalling cascades that culminate in the transcriptional activation of inflammatory mediators such as the nuclear transcription factor NF-κB. Liposomes probably play the role of efficient carriers for MDP and its analogues on the pathway from extracellular milieu into the cytosol, where they trigger intracellular signalling cascades that culminate in transcriptional activation of inflammatory mediators such as the nuclear transcription factor NF-κB pathway. Since the discovery and first synthesis of MDP, about one thousand various derivatives of MDP have been designed, synthesised, and tested to develop an appropriate drug for an immunotherapeutic application that would be free of the side effect exerted by MDP. The main side effects of MDP are pyrogenicity, rigor, headache, flue-like symptoms, hypertension, etc. Only several preparations reached the stage of clinical testing and only mifamurtide was approved for the treatment of osteosarcoma [77].

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