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Application of Liposomes for Construction of Vaccines

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1. Introduction

Vaccinology as a scientific field is undergoing a dramatic development. Never before such sophisticated techniques and in-depth knowledge of immunological processes have been at hand to exploit fully the potential of protecting from as well as curing diseases through vaccination. In spite of great successes like eradication of smallpox in the 1970s and poliomyelitis elimination from all but six countries in the world (two important milestones in the 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, new contacts between human and animals in endemic areas, poverty, and war conflicts in the third world, and many other factors contribute to the more frequent occurrence and rapid dissemination of new diseases. Three diseases that most heavily afflict global health are AIDS, tuberculosis, and malaria. As an example of new viral pathogens we can mention Ebola virus, SARS-coronavirus, or new strains of influenza virus (Wack & Rappuoli, 2005).

Among re-emerging diseases of the past few years, diphteria and cholera should be mentioned. Moreover, multi drug-resistant bacteria frequently occur as a result of overdosing on antibiotics. One of the most important future challenges will be to respond promptly to emerging diseases such as those mentioned above. Rapid sequencing of the genome of the pathogen implicated the speed of the development of diagnostic tools as well as the identification and expression of recombinant targets for vaccines and therapeutic agents development (Stadler et al., 2003). Immunotherapy of cancer represents a special field, where anticancer vaccines could be powerful weapons/tools for long-term effective treatment.

The progress in the vaccine development is closely related with the progress in immunology and molecular biology. A new term "Reverse vaccinology" was proposed by Rappuoli (Rappuoli, 2000) to specify a complex genome-based approach in the vaccine development. Unlike the conventional approach that requires a laborious process of a selection of individual components important for the induction of protective immune response, reverse vaccinology offers a possibility to use genomic information derived from *in silico* analysis of the sequenced organisms. This approach can significantly reduce the time necessary to identify the antigens for the development of a candidate vaccine and enables a systematic identification of all potential antigens of pathogens including those which are difficult or

currently impossible to culture. Of course, this approach is limited to the identification of protein or glycoprotein antigens, omitting such important vaccine components as polysaccharides and glycolipids. Nevertheless, reverse vaccinology can enable scientists to systemically classify the potential protective antigens, thereby helping to improve the existing vaccines and to develop efficient preparations against virtually any pathogen that has had its genome sequence determined.

As regards the process of activation of the immune system to produce an adaptive immune response, it is generally observed that the antigen by itself may not be adequate as a stimulating agent. Many potential antigens have no apparent immunizing activity at all when tested alone. In general, seamy side of pure recombinant protein antigens and synthetic peptide antigens is their poor immunogenicity. Therefore, potent adjuvants are required for highly purified antigen-based vaccines to be effective.

2. Adjuvants

2.1 Toll-like receptors and pathogen-associated molecular patterns

The word *adjuvant* is derived from the Latin root of *adjuvare*, which means to help. Thus, an adjuvant can be defined as any product which increases or modulates the specific humoral or cellular immune response against an antigen. 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 with the interaction of pathogen-associated molecular patterns (PAMPs) on the pathogen side with pattern-recognizing 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 and other PRRs and the co-receptors and intracellular adaptor signalling proteins with which they are associated. PAMPs and their derivatives are utilized 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 recognized by them, various PAMP agonists were tested as adjuvans. Examples of TLR-PAMP specific interaction include bacterial or viral unmethylated immunostimulating CpG oligonucleotides interacting with TLR9, 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. Further liposomal or lipid-based particle formulations of both CpG and MPLA are under development and testing.

2.2 Muramyl dipeptide and other muropeptides

Very specific group of PAMPs is 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 moity and one amino acid (Traub et al., 2006). One of the prominent muropeptides is muramyl dipeptide (MDP), which is known since the 1970s.

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, namely NOD2 and Cryopyrin (inflammasome-NALP-3 complex) (Agostini et al., 2004; Girardin and Philpott, 2004; McDonald et al., 2005). These two receptors recognize MDP/MDP analogues minimal recognition motifs for bacterial cell wall peptidoglycans (Girardin & Philpott, 2004; McDonald et al., 2005). NOD2 is also expressed in specialised epithelial cells, Paneth cells, localised in crypts of Lieberkün, which are producers of antimicrobial peptides having direct antimicrobial activity together with signalling functions within the immune system. Induction of an innate immune response against *Cryptosporidium parvum* infection by the liposomal preparation of lipophilic norAbuGMDP was demonstrated by us in newborn goats (Turanek et al., 2005) and this data is supportive of the present view of the role of MDP recognition in inducing both specific and innate immune responses.

Here GMDP abbreviates N-acetylglucosaminylmuramyl dipeptide. Another recently reported sensor of MDP is Cryopyrin (also known as CIAS1 and NALP3), which is a member of the NOD-LRR family (Agostini et al., 2004). 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 analogues were not pyrogenic even at a high concentration, much higher than the concentrations used for vaccination. We supposed that the modification introduced into the structure of MDP to get norAbuMDP analogues had not changed their affinity to NOD2 but had substantially decreased the affinity to cryopyrin. This hypothesis is in accordance with our data on pyrogenicity and is being currently tested in appropriate *in vitro* models. In addition, murabutide, another nonpyrogenic derivative of MDP was shown not to be able to induce detectable level of IL-1ß in sera of treated volunteers (Darcissac et al., 2001).

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. Although the immuno-stimulatory effects of MDPs have been described for over three decades, the process of molecular recognition and binding of MDP/MDP analogues to NOD2 and cryopyrin receptors remains unclear. 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. The biological effects of muramyl peptides have been described for over three decades. The mechanism underlying their internalization of MDP to the cytosol, where it is sensed by NOD2 and cryopyrin, remains unclear. 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. In case of liposomal formulation of various MDP analogues, the relevant intracellular pharmacokinetics, molecular recognition and binding affinity towards NOD2 and Cryopyrin remain to be determined. Such differences found for various MDP analogues are responsible for their various biological activities (e.g., pyrogenicity, ability to induce the innate immune response etc.) and, therefore, could be utilised for a precise tuning of the intensity and type of immune

response. 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 (Fig. 1) was approved for the treatment of osteosarcoma.

2.2.1 Mifamurtide (MTP-PE)

In pyrogenicity test in rabbits, pyrogenic activity of Mifamurtide i.v. was comparable to that of MDP. In several studies with cancer patients refractory to standard therapy, infused with liposomal Mifamurtide at a dose range of 0.01 – 1.8 mg/m²/dose, dose-dependent fever (in common about 70% of patients) and rigor (about 50% of patients) were the most prominent from a number of acute systemic toxicities (Creaven et al., 1990).
Mifamurtide was also assayed as an additional immunomodulator in an MF59-adjuvanted influenza virus vaccine (Keitel et al., 1993) and HIV-1 vaccine (Keefer et al., 1996); systemic symptoms including fever, chill, and nausea made these vaccines unsuitable for clinical use. Today, the main interest lies in clinical trials for liposomal Mifamurtide as a component of three-drug chemotherapy of osteosarcoma (Anderson P.M, 2006; Anderson et al., 2010).

2.2.2 Romurtide (Muroctasine)

In healthy volunteers, s.c. administration of Romurtide (Fig. 1) at a dose of 200 μg induced - besides local pain and redness - an approximately 1° C increase in body temperature with great individual variability in the course of pyrogenicity curves; normalization occurred within 48 hours (Ichihara et al., 1988). Fever accompanied by chill and headache was also the most common adverse reaction in cancer patients treated with Romurtide at a dose range of 100 – 400 μg/dose s.c. for the restoraion of haemopoiesis after chemotherapy and/or radiotherapy (Tsubura et al., 1988) (Azuma & Seya, 2001; Tsubura et al., 1988).

2.3 nor-Muramyl glycopeptides

We found that a combination of structural modifications both in the saccharide and peptide moiety of MDP and GMDP molecules leads to significant suppression or elimination of pyrogenicity and potentiation of immune-stimulatory activity (Fig. 2). The substitution of muramic acid with normuramic acid and L-alanine with L-2-aminobutyric acid has lead, in the case of the norAbu-MDP molecule, to a decrease of pyrogenicity and, at the same time, to the potentiation of immunoadjuvant activity. If the same structural change is carried out in GMDP molecule, a non-pyrogenic and highly immunoadjuvant analog, norAbu-GMDP is obtained. Furthermore, it has been demonstrated that by the introduction of bulky lipophilic residues into the molecules of these analogs, immunomodulatory activity can be effectively profiled, while the favourable pharmacological parameters of the parent structures are retained. These facts motivated our aims to design and prepare the new groups of lipophilic analogs of norAbu-MDP and norAbu-GMDP, which differ in the character and topology of the lipophilic residue. We primarily aimed to modify their immunopharmacologic parameters. norAbu-MDP-Lys(L18), i.e. MT05, belongs to them. In accordance with our premise, all the new compounds were nonpyrogenic (rabbit test), and the character and topology of the lipophilic residue had a significant effect on their immunologic parameters. As an example, the structural differences between norAbu-MDP-Lys(18) (MT05) and Romurtide, which

influenced the profile of the effects and lead to the elimination of pyrogenicity, are depicted in Fig. 3. (Ledvina M., Turánek J., Miller A.D., Hipler K.: Compound (Adjuvants): PCT appl., WO 2009/11582 A2, 2009.)

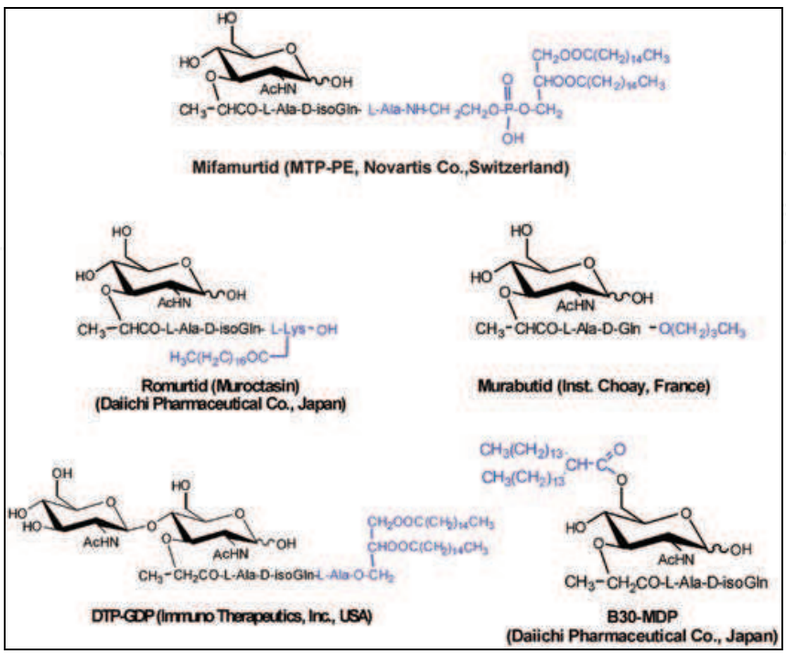


Fig. 1. MDP derivatives developed by various pharmaceutical companies as adjuvants and immunotherapeutics.

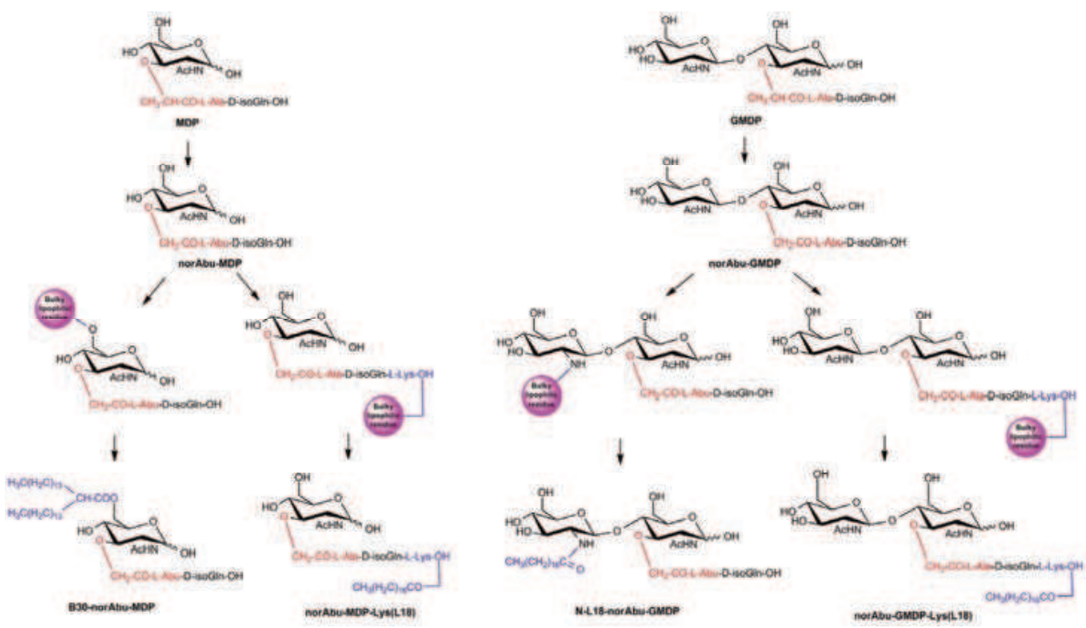
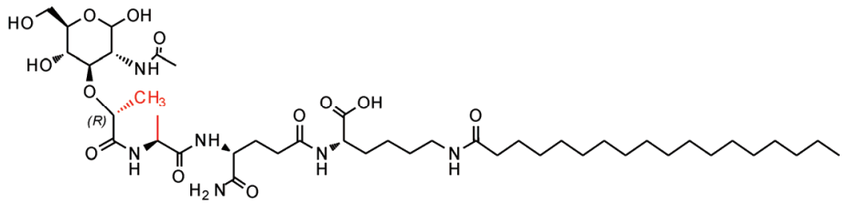
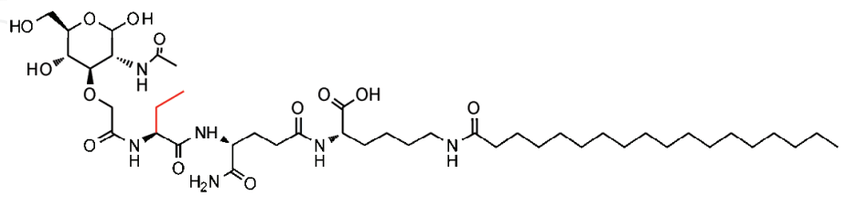


Fig. 2. Transformation of MDP into norAbu-MDP and GMDP into norAbuGMDP and formulae of their hydrophobised derivatives suitable for development of lipid-based adjuvants.



Romurtide (MDP-Lys(L18))



MT-05 (MDP-Lys(L18))

Fig. 3. Structural differences between Romurtide and MT05.

3. Liposomes

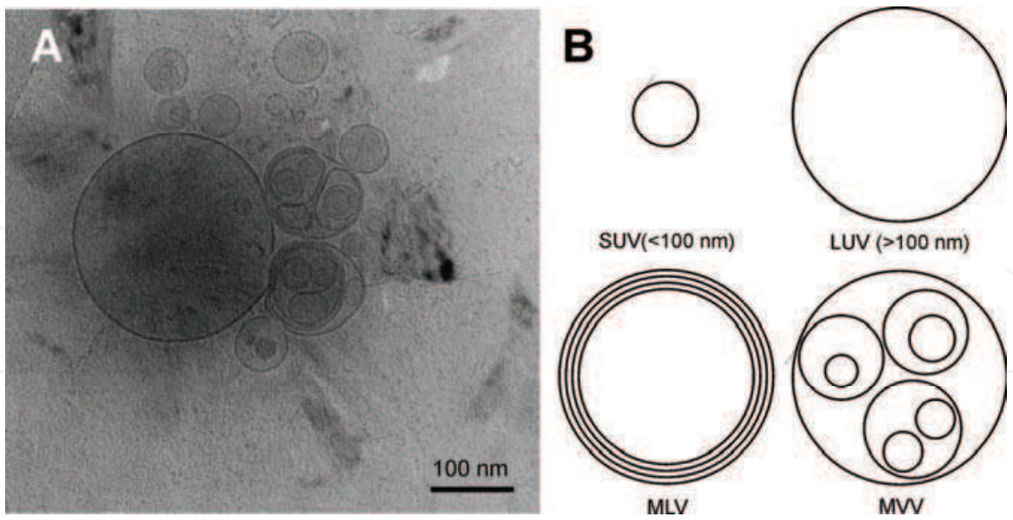
3.1 General characterisation of liposomes

Liposomes, membrane-like spherical structures consisting of one or more concentric lipid bilayers enclosing aqueous compartments were first formulated and described by Alex Bangham in 1965 (Bangham et al., 1965), and have become a useful tool and model in various areas of science. Liposomes represent the oldest and the most explored nano- and micro systems for biological studies on model membranes and for medical applications, especially for drug formulations, because they eliminate or suppress organ-specific toxic side-effects of various drugs (Allen, 1997). Through their 46-year history, liposomes have been approved as suitable delivery systems for applications ranging from cosmetics and dermatology, anti-infection and anticancer therapy and diagnostics up to human as well as veterinary vaccines (Gregoriadis, 1995).

Liposomes are classified in terms of number of bilayers enclosing the sequestered aqueous volume as follows: unilamellar, oligolamellar, and multilamellar. Unilamellar vesicles can be further divided into small unilamellar vesicles (SUVs) with a large curvature, and large unilamellar vesicles (LUVs) with a low curvature and hence, with properties similar to those of a flat surface. Multilamellar vesicles (MLVs) are liposomes that represent a heterogenous group in terms of size and morphology (Cullis et al., 1987). Lipid composition, size and morphology are variables determining the fate of liposomes in biological milieu; therefore, the selection of suitable method for the preparation of liposomal drugs and vaccines is of importance in respect to subsequent animal experiments and future successful marketing of the product. Schematic structures of various types of liposomes as well as realistic picture obtained by cryoelectron microscopy are presented in Fig. 4.

3.2 Liposome-based vaccines

The use of liposomes as vaccine adjuvants was first described by Allison and Gregoriadis in 1974 (Allison & Gregoriadis, 1974). Since that time, numerous studies were performed and proved that liposomes can be used to enhance the immune response towards a large variety of peptide and protein antigens derived from various microbial pathogens as well as tumours.



A) Photograph of various liposomal structures by cryoelectron microscopy. B) Schema of types of liposomes: SUV – small unilamellar vesicle, LUV - large unilamellar vesicle,, MLV – multilamellar vesicle, MVV – multivesicular vesicle

Fig. 4. Schematic representation of various morphological classes of liposomes and their real image obtained by cryoelectron microscopy.

The potential for the participation of liposome-based recombinant vaccines on the human and veterinary vaccine market is very promising (Adu-Bobie et al., 2003). Liposomal vaccines have been around for about 30 years and plenty of liposome variants have been developed; some of them with evident immune-stimulating properties and an attractive safety profile which resulted in registered products on the market or preparations in advanced stages of clinical testing. Liposomal hepatitis A vaccine is the first formulation of liposomes to become licensed for clinical use in humans (Gluck et al., 1992) (Hepatitis A - HepA, Epaxal http://www.crucell.com/Products-Epaxal). Epaxal liposomes contain influenza hemagglutinin protein which facilitates their binding and endocytosis by specific receptor on antigen presenting cells. Such forms of liposomes are called virosomes. Liposomes represent almost ideal carrier system for the preparation of synthetic vaccines due to their biodegradability and versatility as regards the incorporation of quite a number of various molecules having different physico-chemical properties (the size of the molecule, hydrophilicity or hydrophobicity, the electric charge).

The molecules and antigens can be either sterically entrapped into the liposomes (the internal aqueous space), or embedded into the lipid membrane (e.g., membrane-associated proteins/antigens) by hydrophobic interactions. Further, they can be attached to either the external or the internal membrane by electrostatic, covalent or metallo-chelating interactions. It is possible to encapsulate simultaneously various compounds into the liposomes: hydrophilised/lipophilised adjuvants (e.g., MPL A, CpG oligonucleotides, MDP and its analogues), soluble or membrane protein antigens, and ligands for the targeting to specific receptors on the antigen-presenting cells. Further, liposomes can be coated with mucoadhesive biopolymers, or undergo surface-charge modifications (e.g., by cationic lipids) (Altin & Parish, 2006).

As a great advantage, liposomes can be used for the preparation of self-assembling hybrid supramolecular nanosystems such as proteoliposomes, which can combine liposomal

nanoparticles with suitable immunopotentiating/adjuvant molecules (e.g., MPL A, CpG oligonucleotides, MDP, and its analogues).

3.3 Liposomes as antigen carriers

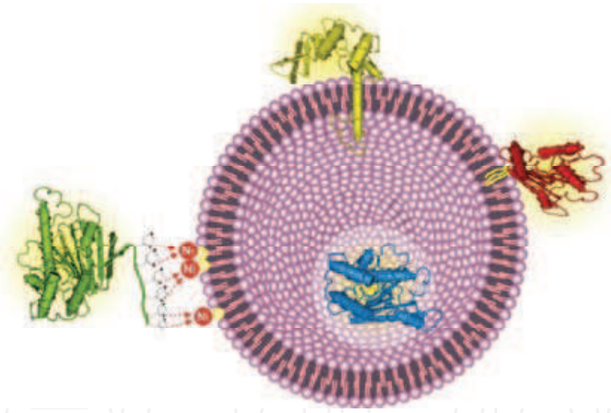
Structural diversity of liposomes permits tailoring of liposome-based vaccines to obtain an optimal adjuvant effect for a particular antigen. Their safety profiles and ability to induce an immune response makes them likely to be included in vaccine formulations. Liposomal formulations offer several major advantages. They can (1) prevent degradation of the delivered antigens and adjuvants; (2) allow membrane proteins to reconstitute and preserve their antigen structure; (3) increase the antigenic effect of weak immunogens; (4) target antigen-presenting cells (APCs) and direct the antigen to MHCI or MHCII presentation; (5) contain the antigen and adjuvant molecules in one particle, thus functioning simultaneously as a delivery system and a vaccine adjuvant; and (6) reduce the antigen and adjuvant doses required for an immune response and controlled release. Therefore, liposomes provide a safe and effective platform for construction of subunit vaccines.

With respect to the physico-chemical nature of liposomes and phospholipid bilayers, the liposomes represent one of the most versatile structures for the preparation of drug delivery systems. Both hydrophobic and hydrophilic protein or peptide antigens can be associated with liposomes. Generally, antigens can be associated with liposomes in two ways and it is known that the encapsulated liposomal antigens induce a different immune response than the surface-linked antigens in both humoral (Shahum & Therien, 1988) and cell-mediated immunity (Fortin et al., 1996). If entrapped into the internal aqueous space of a liposome, the protein or peptide antigen is protected against proteolytic degradation and the antigen clearance is decreased. On the other hand, the liposomal membrane represents a barrier restricting the interaction of the antigen with and its recognition by B-cells. Especially, the stable multilamellar liposomes were found to be low immunogenic (Shek & Heath, 1983) and the antibody response reached is low or absent when the liposomes are made of lipids with a high transition temperature; in other words, when they are composed of saturated phospholipids (Gregoriadis et al., 1987). These liposomes are very stable in body fluids as well as in digestive tract and prevent a release of the entrapped antigen. Also the interaction of the encapsulated antigens with B-cells is limited. The fluidity of liposomes was found to be an important parameter also for the immune response towards a surface-linked antigen. Again, more fluidic liposomes composed of unsaturated phospholipids are more efficiently phagocytosed by APC and induce a one order of magnitude higher immune response than rigid liposomes composed of saturated phospholipids (Uchida & Taneichi, 2008).

Liposomes are potentially very useful for the construction of vaccination systems given their facile biodegradability and versatility as carriers for varieties of molecules having different physico-chemical properties (such as size, hydrophilicity, hydrophobicity, or net electrical charge). Liposomes also offer the possibility to associate or entrap simultaneously more than one type of molecules. Of particular interest to us has been the co-association of hydrophilic or lipophilic adjuvants (e.g., monophosphoryl lipid A [MPL A], CpG oligonucleotides, muramyl dipeptide (MDP), and/or MDP lipophilic analogues) with soluble or membrane protein antigens or ligands for the targeting of specific receptors on antigen-presenting cells. The molecules and antigens can be either sterically entrapped into the liposomes (the internal aqueous space), or embedded into the lipid membrane (e.g., membrane-associated proteins/antigens) by hydrophobic interactions (Fig. 5). The ligands for the targeting to specific receptors on the antigen-presenting cells can significantly enhance the intensity of

the immune response (Altin & Parish, 2006). For the mucosal application, the liposomes can be coated with mucoadhesive biopolymers or modified with surface-charge modifiers (e.g., cationic lipids). In this way, liposomes become a versatile platform that represents a real multifunctional vaccination carrier.

The importance of liposomes for the effective co-administration of adjuvants could be demonstrated using MDP as an example. MDP has a weak immunoadjuvant activity in aqueous solution due to its rapid excretion into urine. Appropriate formulations of hydrophilic MDP in "water in oil" emulsions (Parant et al., 1979) or liposomes were used to harness its full adjuvant potenital (Tsujimoto et al., 1986). Some lipophilic derivatives of MDP like B30-MDP and MDP-Lys (L18) were synthesised and tested as adjuvants for recombinant hepatitis B surface antigen (Tamura et al., 1995) or influenza surface antigens hemagglutinin and neuraminidase (Nerome et al., 1990). We used new synthetic nonpyrogenic lipophilic analogues of norAbu-MDP modified at a peptide part by hydrophobic ligands (Fig. 2) and these well defined synthetic molecules were used for the first time in combination with metallochelating liposomes to construct an experimental recombinant vaccine. Surprisingly, we have found that at certain surface density of lipophilic analogues of norAbu-MDP (about 5 mol % of total liposomal lipid), the liposomes are promptly recognised and phagocytosed by human dendritic cells. The phagocytosis is about one order of magnitude higher than that of proteoliposomes or liposomes lacking norAbu-MDP adjuvant. This finding implicates an existence of receptors on dendritic cells, which can recognise some molecular pattern formed by the hydrophilic part of norAbu-MDP exposed on the liposomal surface (illustration of this phenomenon is in Fig. 12B).



a) physical entrapment inside the liposome (blue protein); b) reconstitution of membrane protein in lipid bilayer via hydrophobic transmembrane domain (yellow protein); c) anchoring of lipidised protein onto liposomal surface or attachment of recombinant protein onto the liposomal surface by covalent bond using activated lipids (red protein); d) attachment of recombinant protein onto the liposomal surface by non-covalent bond using metallochelating lipids

Fig. 5. Association of protein antigen with liposome.

3.4 Methods of liposome preparation

The laboratory and industrial procedures for the liposome preparation have been established and liposomes have been approved by FDA for biomedical applications. A variety of procedures for the preparation of various types of liposomes has been developed and reported in several reviews and monographs (Gregoriadis, 1992; Woodle &

Papahadjopoulos, 1988). To classify these methods, they were arranged in three categories: 1) mechanical dispersion methods such as hand shaking or vortexing, sonication, and high pressure homogenisation; 2) detergent-solubilizing dispersion methods including solubilized lecithin dispersion with sodium cholate or octylglucoside; and 3) solvent dispersion methods such as ethanol injection, ether infusion, and reverse-phase evaporation. These primary processes can be linked with secondary processes such as high-pressure homogenisation or extrusion through polycarbonate filters of various pore size, which are easy ways to prepare liposomes of a desired size and morphology (Barnadas-Rodriguez & Sabes, 2001; Berger et al., 2001; Cullis, 1987; Hope et al., 1985; Perrett et al., 1991; Turanek, 1994; Woodle & Papahadjopoulos, 1988; Schneider et al., 1995).

The stability of proteins is limited and not all the methods are useful for the preparation of proteoliposomes, especially if the protein is to be entrapped inside the liposome. The detergent dilution method is characterized by very mild conditions during the process and is suitable for the reconstitution of membrane proteins. Because many recombinant proteins tend to precipitate, this method is also useful to work with these protein antigens. Next paragraph describes in detail a modified detergent dilution method for the preparation of proteoliposomes.

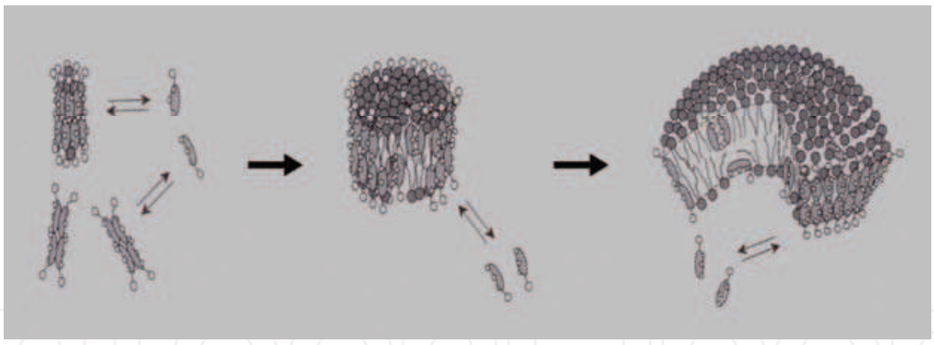
3.5 Preparation and characterization of metallochelating liposomes

Because the preparation of metallochelating liposomes represents a post-forming modification of liposomes, it avoids a denaturation of proteins owing to the process used for the liposome production. Therefore, nearly all the methods mentioned above could be used to prepare more or less monodisperse liposomes. Here we describe in brief a modification of the detergent removal method, which is suitable for the preparation of very monodisperse unilamellar liposomes that are useful for structural studies by various techniques (e.g., TEM, dynamic light scattering, and gel permeation chromatography).

When essentially unilamellar monodisperse liposomes of spherical shape are needed (which is a prerequisite for a precise monitoring of the proteoliposome formation by dynamic light scattering), the detergent removal method is preferred to the other methods. The method is based on the transformation of phospholipid micelles stabilized by detergent with high critical micellar concentration (CMC) (e.g., cholate) to desk micelles and finally to vesicles during the process of detergent removal (Zumbuehl & Weder, 1981) (Fig. 6). The mild conditions provided by this method are advantageous for the preparation of proteoliposomes, especially for the reconstruction of membrane proteins (Rigaud & Levy, 2003) like viral or bacterial antigens or recombinant his-tagged proteins that are often prone to precipitation.

There are many variants of the detergent removal method, e.g., the dilution of the solution of mixed micelles, gel permeation chromatography, a simple dialysis or the controlled one in a special apparatus, cross-flow filtration, adsorption on beads, etc. (Schubert, 2003). The application of the flow-through ultrafiltration cell represents a new approach to the detergent removal method (Masek et al., 2011a). The linkage of the cell with systems like FPLC facilitates automation of the whole procedure and manipulation with the sample. The full control over the dialysis rate and the removal of the undesired residua (e.g., detergent, organic solvents, protein solubilizers) is ensured and various steps like an addition of required components through an injection valve during various stages of the liposome formation are easy to perform without breaching the sterile conditions. In this case, the sterile filter inserted in front of the cell inlet ensures that the sterility is kept during the whole process (Fig. 7). The low dead volume of the cell is of great importance for the preparation of liposomes and proteoliposomes in small laboratory scale. However, this arrangement enables also very easy up-scaling of the whole technology. A precise control

over the rate of the detergent removal yields a final liposomal preparation of high monodispersity (PDI within the range of 0.05 - 0.06), which is shown to be reached routinely (Fig. 8). This monodispersity is better than those obtained by the dialysis method performed in the dialysis bags or slides (produced by Pierce) (PDI ≈ 0.08-0.12).
The size of the mixed micelles (≈5-6 nm; see Fig. 8) used by us for the preparation of liposomes is in good correlation with the Small's mixed micellar model proposing the structure of a small phospholipid bilayer disc stabilised at its hydrophobic edges by the molecules of cholate (Small, 1971; Schubert, 2003).
The process of the formation of the monodisperse liposomes is in good accordance with the proposed kinetic model of the micelle-vesicle transition based on a rapid formation of disk-like intermediate micelles followed by a growth of these micelles up to their critical size and their subsequent closure to form vesicles. The final size of the liposomal preparation could be controlled by ionic strength of the buffer used for the preparation of the micelles (Fig. 9). An increase of the NaCl concentration reduces CMC of cholate and shields the negative charge of the mixed micelles. These two factors are responsible for the formation and stabilisation of the large discoid bilayer micelles that are transformed into the larger liposomes (Schubert, 2003).
Various additives like bilayer stabilising sugars (e.g., sucrose) or recombinant protein solubilizers (e.g., urea, guanidine) are compatible with this method and can shift the size of the liposomes into the required range (Walter et al., 2000). Some recombinant proteins (e.g., circovirus envelope protein), which tend to precipitate in the absence of stabilizing buffers (imidazole and urea stabilizing buffer) were successfully linked onto metallochelating liposomes by one-step procedure based on the addition of the protein into the mixed micelle solution prepared in protein stabilizing buffer and transforming into proteoliposomes during the ultrafiltration procedure (Turánek, unpublished results).

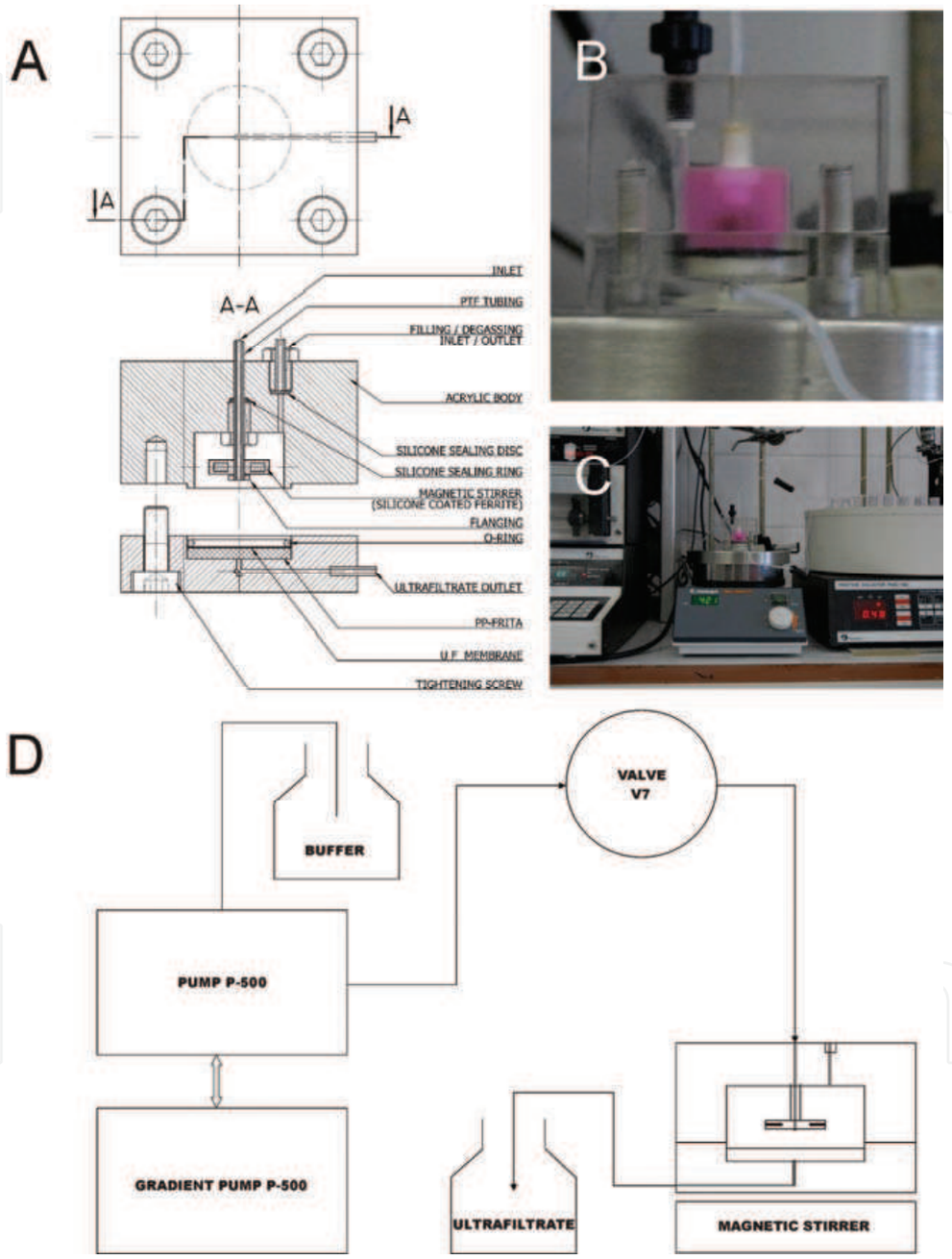


Adapted: R. Schubert, Methods Enzymol. 367 (2003) 46–70.

Fig. 6. Principle of detergent removal method and formation of liposomes from mixed bile salt-phospholipid micelles.

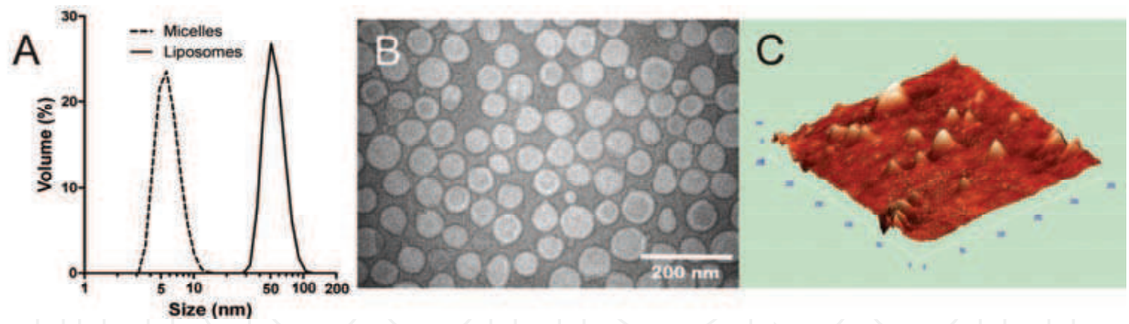
Small mixed micelles are fused in disc phospholipid micelles stabilized at edges by detergent. Further removal of detergent induces formation of large disc micelles which spontaneously vesiculate after reaching a critical size.
The transformation of micelles into liposomes during the ultrafiltration removal of cholate is a critical step affecting the final quality of liposomes. This process is easy to be monitored by DLS. The removal of cholate induced a formation of disc micelles, which was reflected by an increase of the micelle size and eventually by a formation of liposomes (Fig. 10). The process of liposome formation had been completed before the CMC of cholate was reached, as shown by the dashed vertical line. This line divides the flow-through volume axis into the

left part, where micelles do predominantly exist and are transformed into liposomes, and the right part, where liposomes represent the main lipid form, while the residual detergent and other low molecular weight contaminants (e.g., traces of ethanol or tetrahydrofuran used to solubilize the lipids) are continuously removed by the process of ultrafiltration.



(A) Schematic illustration of the ultrafiltration cell. (B) Photograph of the ultrafiltration cell in detail (pink: LR-PE-labelled liposomes inside the cell). (C) Schematic illustration of the linkage of the ultrafiltration cell with the FPLC system. (D) Photograph of the system

Fig. 7. System for preparation of liposomes by removal of detergent using ultrafiltration.



A) Size distribution of micelles and liposomes. The hydrodynamic diameters of the micelles and liposomes were determined by dynamic light scattering instrument NanoSizer NS (Malvern, UK) at 25 °C. Silica cuvette of 45-µl volume (Hellma, Germany) was used. (B) TEM micrograph of monodisperse liposomal preparation. (C) AFM micrograph of monodisperse liposomal preparation

Fig. 8. Size distribution of micelles and metallochelating liposomes analysed by dynamic light scattering and visualization of liposomes by TEM and AFM.

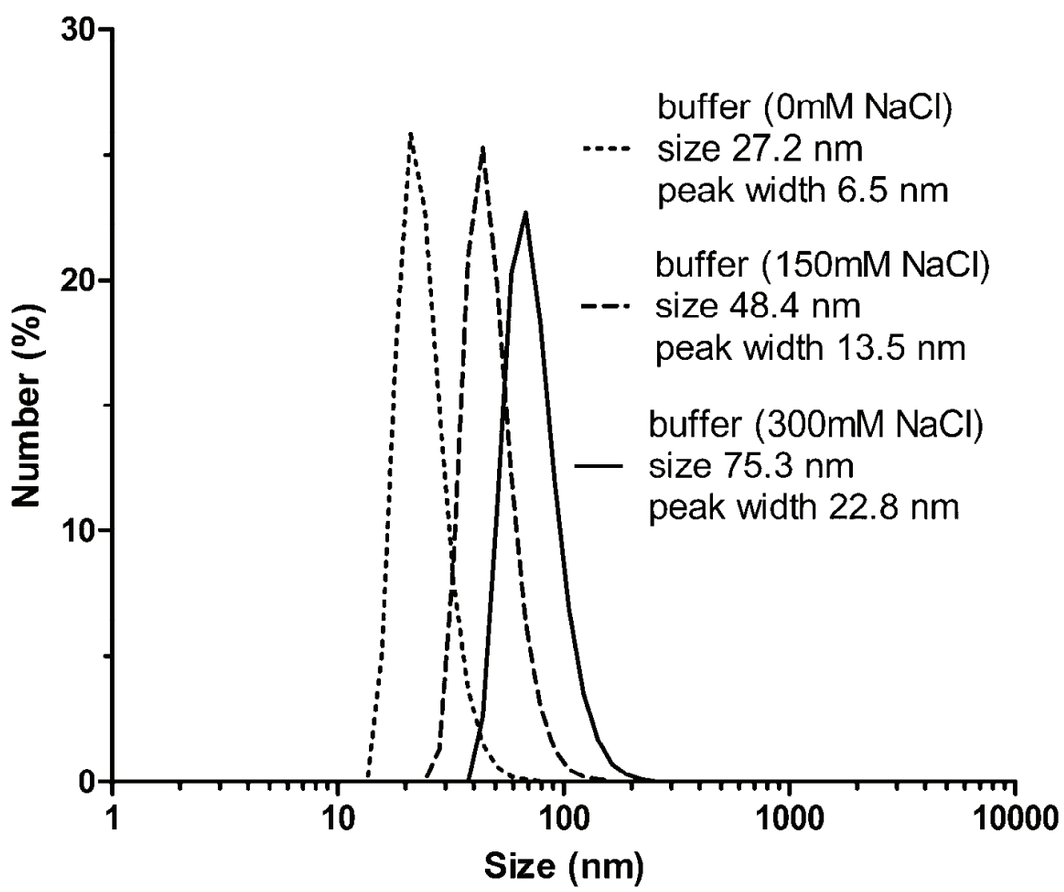


Fig. 9. Effect of ionic strength on the size distribution of liposomes prepared by detergent removal method.

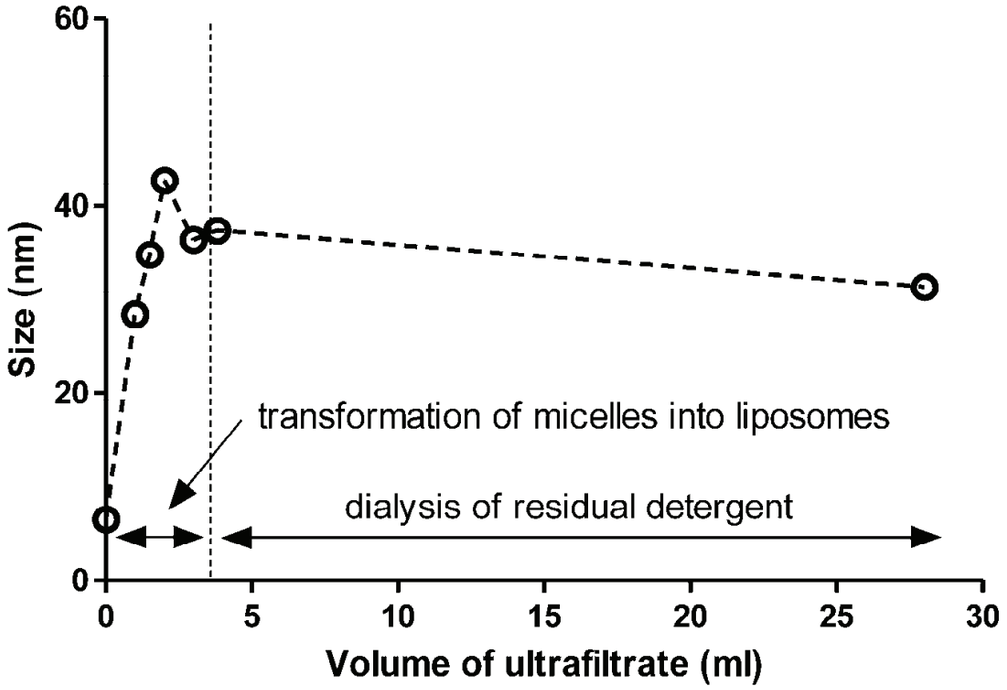


Fig. 10. Transformation of micelles into liposomes during ultrafiltration monitored by DLS. The dashed vertical line indicates the ultrafiltrate volume, when the CMC of sodium cholate was reached. This line divides the flow-through volume axis into the leftpart, where micelles do predominantly exist and are transformed into liposomes, and the right part, where liposomes represent the main lipid form, while residual detergent and ethanol/THF are continuously removed by the process of ultrafiltration.

4. Metallochelating bond and its application for construction of proteoliposomes

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. 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. It is beyond the scope of this chapter to address thoroughly this particular question. However, the GPC data indicate a good *in vitro* stability of the proteoliposomes containing recombinant His-tagged Outer surface protein C from *Borrelia burgdorferi* (rOspC) designated rOspC–HisTag during the chromatographic process, within which they experience a shear stress and dilution.

Also, the data on the incubation of rOspC-HisTag proteoliposomes in serum at 37 °C demonstrated the stability of the metallochelating bond linking the protein to the liposomal surface. In fact, *in vivo* fate of liposomes after the intradermal administration is different than that following an intravenous injection. First, dilution of proteoliposomes 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. Moreover, the flow rate of the tissue fluid within intradermal extracellular

matrix is considerably lower than that of the muscle tissue or blood vessels. This fact is often overlooked. The stability of metallochelating bond probably depends also on the character of a particular protein. The study by Ruger shows that the single-chain Fv Ni-NTA-DOGS liposomes are unstable in human plasma and the majority of single-chain Fv fragments (anti CD 105) are released from the liposomal surface, which results in a lost of the specific targeting performance to the cells expressing a surface protein endoglin (CD 105) (Ruger et al., 2006). On the other hand, Ni-NTA3 –DTDA liposomes 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 the three-functional chelating lipid Ni-NTA3 –DTDA probably endows the metallochelating bond with a higher *in vivo* stability (van Broekhoven et al., 2004). Application of Ni-NTA-DOGS liposomes for the construction of experimental vaccine against sytemic *Candida* infection based on *Candida* Heat shock protein 90 kDa (rHSP90-HisTag) showed good stability in serum as well as strong immune response against recobinant rHSP90-HisTag antigen in mice (Masek et al., 2011b).

In vivo activity (immunogenicity) was also demonstrated for antigens associated with ISCOM particles via metallochelating lipid dipalmitoyliminodiacetic acid (Malliaros et al., 2004) and a peptide antigen associated with liposomes via Ni-NTA-DOGS (Chikh et al., 2002). Generally, metal ions, physico-chemical character of the metallochelating lipids and their surface density on the particles belong to the factors that could be optimized to get a required *in vivo* stability and, therefore, a strong immune response. The design and synthesis of new metallochelating lipids might accelerate a development and application of metallochelating liposomes for the construction of drug delivery systems and vaccines. Besides Ni^{2+}, other divalent ions such as Zn^{2+}, Co^{2+}, Fe^{2+}, and Cu^{2+}, have to be considered and experimentally tested as well.

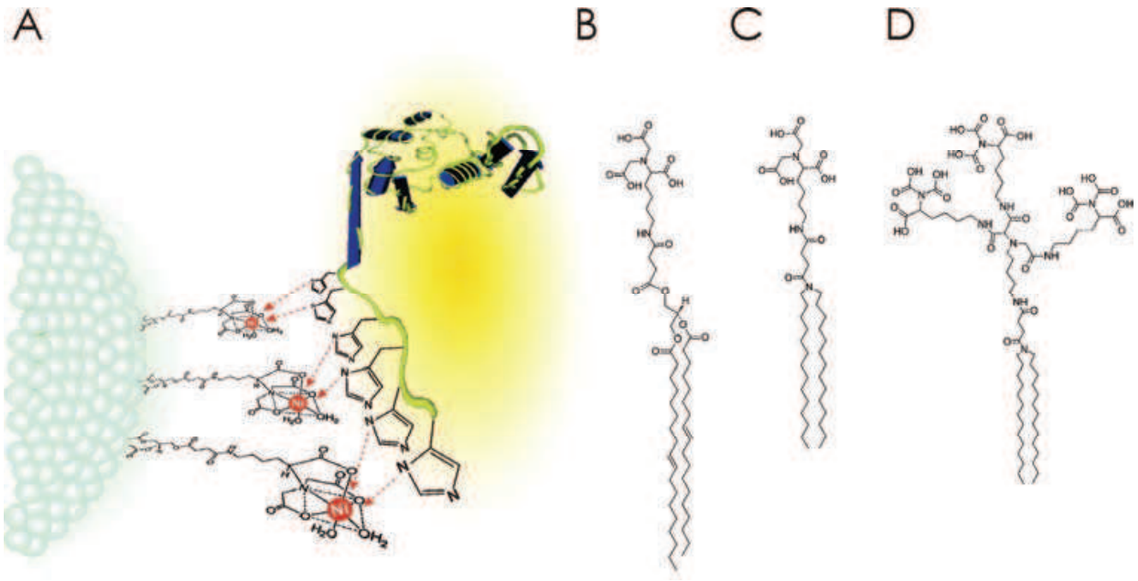


Fig. 11. Schematic illustration of recombinant His-Tagged protein bound onto the surface of metallochelating liposome (A) and formulae of metallochelating lipids NTA-DOGS (B), NTA–DTDA (C), trivalent NTA3 –DTDA (D).

5. Metallochelation liposomes for construction of experimental recombinant vaccines

Only few papers report the implementation of the metallochelating lipids in the attachment of the recombinant proteins or synthetic peptides with His-Tag anchor (short peptide consisting of 4 to 6 molecules of histidine). Both reversible character and high affinity of the metallochelating bonds are very useful for the preparation of various self-assembling supra-molecular structures useful for the construction of experimental vaccines (Chikh et al., 2002; Malliaros et al., 2004; Masek et al., 2011a; Masek et al., 2011b). As an example of synthetic liposome-based recombinant vaccine we can use metallochelating liposomes and recombinant antigen rOspc-6HisTag derived from the pathogen Borrelia burgdorferi.

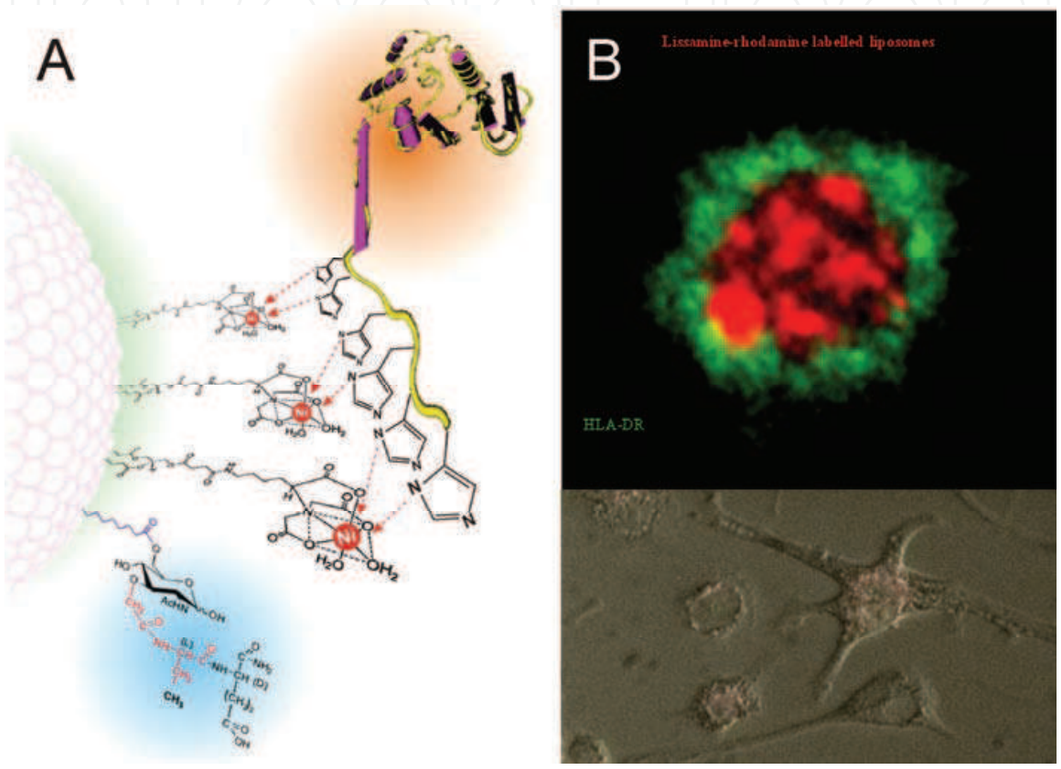
5.1 rOspC antigen *Borrelia* as an example for construction of metallochelation liposome-based vaccines

Lyme disease or Lyme borreliosis is an infectious disease caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex vectored by ticks of the genus *Ixodes*. At least three species are pathogenic for humans, *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*. The initial stage of Lyme disease is commonly associated with skin rash occurring within few weeks after the tick bites. Later the infection can spread to bloodstream and insult of joints, heart, and nervous system. Although not common, some patients experience late stage symptoms like arthritis, nervous system complications, or acrodermatitis chronica atrophicans. Here, slower response to antibiotics therapy, sometimes taking weeks or months to recover, or eventually, incomplete resolution is observed. In a few cases, antibiotic-refractory complications persist for months to years after antibiotic therapy, most likely due to infection-induced autoimmunity. Therefore, alternative approaches such as preventive immunisation are needed, mainly in the endemic areas (Krupka M, 2007; Tilly et al., 2008).

Protective immune response to *Borrelia* involves non-specific activity of complement, phagocytic cells and *Borrelia*-specific Th1-dependent response leading to production of complement-activating antibodies, in mouse presented mostly by IgG2a (IgG2b). During natural infection, nevertheless, *Borrelia* and tick saliva modulate the immune response toward non-protective Th2 type response, associated with production of neutralizing, poorly opsonizing *Borrelia*-specific antibodies (Vesely et al., 2009). *Borrelia* outer surface proteins OspA and OspC are among the most promising antigens for elicitation of opsonizing antibodies. The applicability of OspA antigen is limited because *Borrelia* expresses it mainly in the tick and the antibodies thus should act outside of the vaccinee's organism (Pal et al., 2000). Therefore continuously high level of OspA-specific antibodies is required to prevent *Borrelia* transfer. In contrast, OspC is expressed during the transfer and the initial stage of infection. In this case the vaccine-induced immune memory has enough time to initiate the production of opsonizing antibodies preventing *Borrelia* spreading (Tilly et al., 2006).

OspC antigen can be used here as an example of reverse vaccinology approach. Full length recombinant OspC is difficult to prepare in high yield and purity. Production of Osp-s for vaccination purposes is hindered by low yield of fully processed lipidized Osp antigens or low immunogenicity of their non-lipidized versions. In our experiments, removing of N′ terminal lipidation signal was associated with an increase of the recombinant protein yield and purity but, as demonstrated also for other *Borrelia* lipoproteins, a decrease in immunogenicity (Erdile & Guy, 1997; Gilmore et al., 2003; Lovrich et al., 2005; Weis et al., 1994). Induction of OspC-specific opsonizing antibodies to non – lipidised OspC could be

enhanced by appropriate adjuvants and carriers like such as various modification of liposomes. It was reported that immunisation of mice with non-lipidated OspC in strong adjuvants (Complete Freund's Adjuvant, TiterMax, or Alum) could induce intense OspC-specific antibody responses (Earnhart et al., 2007; Earnhart and Marconi, 2007; Gilmore et al., 1996; Gilmore and Mbow, 1999; Ikushima et al., 2000). Here we demonstrated that similarly strong response could be elicited by immunisation of experimental mice with metallochelating nanoliposomes with entrapped lipophilic derivatives of norAbu-MDP as a potent adjuvant molecule.

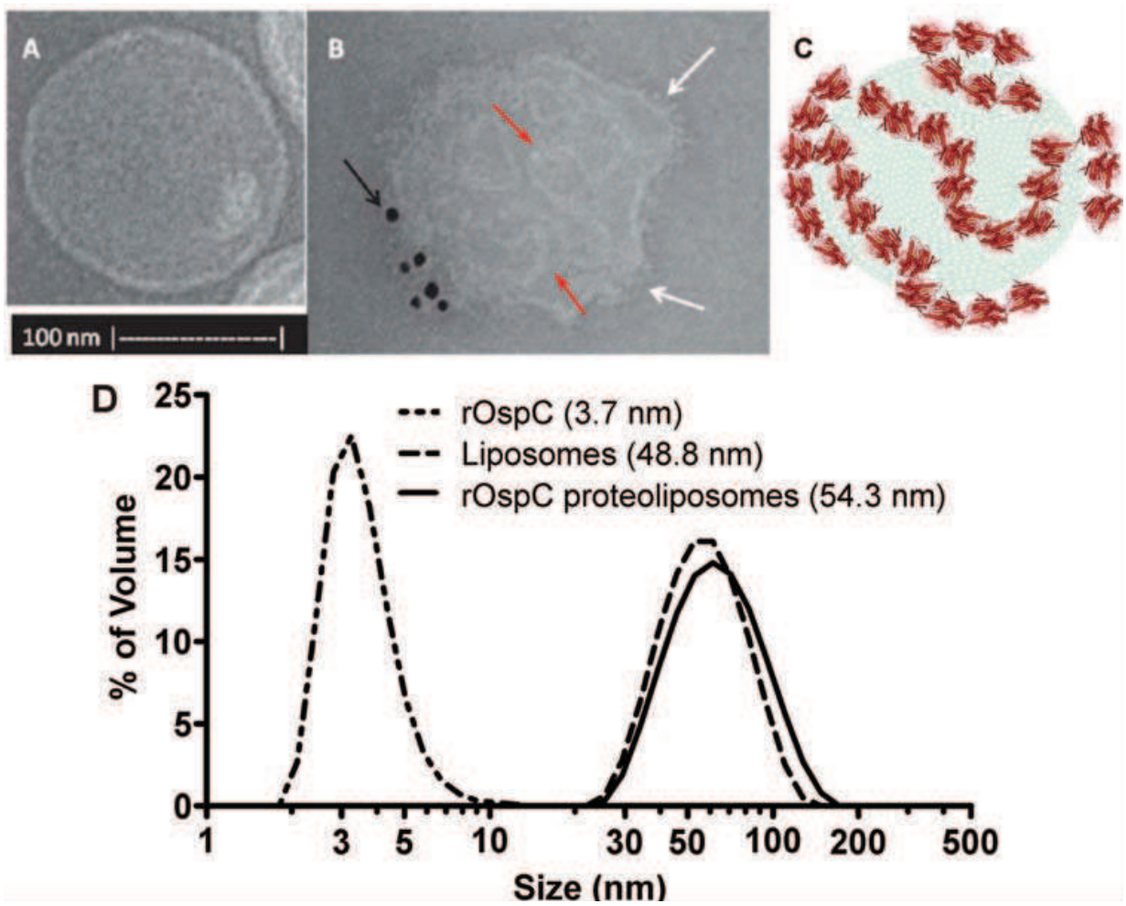


A) Scheme of metallochelating liposome with His-Tag recombinant protein antigen bound to the surface. Lipophilic nor-AbuMDP analogue as adjuvant is exposed on the surface of proteoliposome; B) Upper photograph (confocal microscop) shows human dendritic cell with phagocytosed prototypical vaccination nanoparticles cumulated in giant lysozomes (green – HLA-DR marked with antiHLA-DR antibodies; red – Lyssamine-rhodamine labelled vaccination particles). Lower photograph (Hofman modulation contrast and fluorescence microscopy) shows dendritic cells with accumulated vaccination particles inside (red fluorescence)

Fig. 12. Schematic drawing of prototypical recombinant protein vaccination nanoparticle and interaction with human dendritic cells.

5.2 Structure of OspC metallochelating proteoliposome

Protein-His-Tag/metallochelating lipid complex is anchored in phospholipid bilayer by its lipid moiety. Egg yolk or soya phosphatidyl choline contain large portion of unsaturated fatty acids, therefore DOGS-NTA lipid is freely miscible with these lipids and phase separation do not occur in liposomal bilayer. In other words, contribution of lipids to the formation of various protein structures on the liposomal surface is negligible.



A) Structure of plain metallochelating liposome (TEM) B) Structure of metallochelating liposome with OspC bound onto the surface (white arrows – OspC); black arrow – OspC marked by 10nm immunogold particles; small red arrow – bead chain of OspC molecules; C) Schematic presentation of bead chain model of proteoliposome D) Size distribution and hydrodynamic diameter of OspC, plain metallochelating liposomes and OspC proteoliposomes analysed by DLS. The size distribution of parent monodispersed metallochelating liposomes (dashed line) was compared with that of OspC proteoliposomes (full line). As a reference, size distribution of OspC (dotted line) is shown. Numbers in brackets represent mean hydrodynamic diameters of particles

Fig. 13. Characterisation of OspC preoteoliposomes.

This is advantageous for study of protein-protein interaction of proteins anchored on the surface of liposomes. Moreover, anchoring of proteins via metallochelating bond produces highly oriented binding of proteins because the proteins are attached to liposomes exclusively by His-tagged end of the polypeptide chain. If some interaction between liposomal surface bound proteins exists, we can observe formation of various structures which are conditioned by the character and number of protein-protein interactions. Another important feature of metallochelating proteoliposomes is relatively high surface concentration of proteins. This concentration could be set by changing the DOGS-NTA/phosphatidyl choline ratio in the lipid mixture used for preparation of liposomes.

The ultrastructure of the rOspC-His-tag proteoliposomes was revealed by TEM (Fig. 7). rOspC-His tag as an example of the preoteoliposomal structure "Bead chain" model. Binding of individual molecules of OspC protein onto the liposomal surface is clearly visible

on the rim of the liposome and immunogold staining confirmed the identity of OspC molecules as well as preservation of the epitopes recognised by polyclonal antibodies (Fig. 7b). In the case of recombinant OspC, TEM micrograph (Fig. 7b) showed that the individual molecules of OspC antigen were bound onto the surface of the liposome and some organisation in beads-like structures was revealed. This observation testifies against the simplification of the proteoliposomal structures and hence against accepting the simple schematic concept based on the random distribution on the liposomal surface.

Binding of OspC onto the surface of metallochelating liposomes was proved by an increase of hydrodynamic diameter as assayed by DLS. The increase of the size of proteoliposomes is well distinguished from plain liposomes, even if the increase of the size is only 5.5 nm (Fig.. 13D). This precise measurement was allowed by a preparation of parent monodispersed metallochelating liposomes and pointed to the importance of using monodispersive liposomal preparation for such a study (Fig. 8). In the case of homogenous coating of liposomes by OspC, the increase in the size should be of about 7.4 nm, theoretically. A lower increase of the size (5.5 nm) indicates only partial coating of the liposomal surface by the protein and this is in a good accordance with the structure revealed by TEM (Fig. 7b).

Binding of OspC onto metallochelating liposomes was confirmed also by GPC used as an independent method (Fig. 9) The liposomal fraction was separated from free protein and OspC was assayed by SDS PAGE followed by immunoblot (Fig. 9C). The vast majority of OspC was shown to be bound onto liposomes and was only slightly ripped from their surface by shearing forces taking place during penetrating through GPC column. The tailing character of the OspC elution profile is supportive to this explanation. Stability of the metallochelating bond in model biological fluid was studied by incubation in undiluted human serum. In spite of the presence of serum, it was estimated that more than 60% of OspC was still associated with liposomes. Based on this data, the half life of OspC proteoliposomes in serum was estimated to be at least 1 hour.

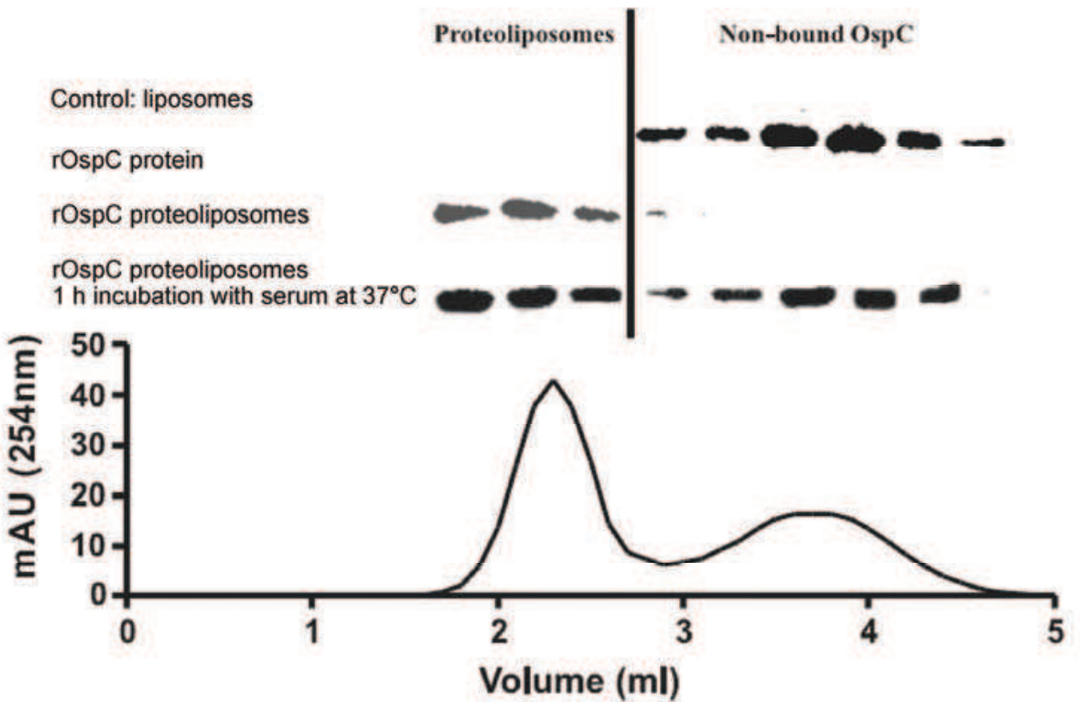


Fig. 14. Stability analysis of OspC proteoliposomes by GPC.

Non-bound OspC was separated from the proteoliposomes by gel permeation chromatography using Superose 6 column. OspC was detected in various fractions from GPC by immunoblot. OspC GPC elution profile is correlated with immunoblot assay.

5.3 Immunization experiments

New synthetic nonpyrogenic lipophilic analogues of NorAbu-MDP modified at a peptide part by two different hydrophobic ligands (Fig. 15) and for the first time these defined synthetic molecules were used in combination with metallochelating liposomes to construct an experimental recombinant vaccine. The important finding was that both MT06 and MT05 adjuvants exerted a high adjuvant effect comparable or better than MDP but proved itself as nonpyrogenic (rabbit pyrogenicity test) and safe. While alum induced stronger antibody response in IgG1 subtype, both MT06 derivatives and liposomal-MDP induced stronger immune response in both IgG2 subtypes. Interestingly, in comparison with MT06, the analogue MT05 induced stronger response in IgG1, IgG3 and IgM isotypes, respectively. This interesting finding pointed on the effect of lipophilic residues, which could not be supposed as the only accessory part of the molecule, but can significantly affect the quality of immune response. The position (peptide or sugar part) and the character (hydrophobicity and bulkiness) of the lipophilic function can affect the interaction with appropriate receptors as well as the metabolic degradation of the molecule. This aspect has not been described in the literature yet and is of interest for our understanding of the mechanism of action.

OspC itself did not elicit detectable OspC-specific antibodies of IgG, IgM, and IgA isotypes (Ig*). Similarly, when OspC bound onto the surface of liposomes was used for immunisation, only negligible increase of OspC-specific Ig* was detected after the second immunisation. In contrast, immunisation with OspC plus adjuvants (FCA, AlOH, MDP, MT05, and MT06) elicited strong OspC-specific antibody responses with ELISA titres of the same magnitude. The contributions of particular IgG isotypes for the immune response showed differences among various adjuvants (Fig. 15) (FCA was no tested for the IgG isotype response).

Mice (5 per group) were immunised by i.d. application of various liposome - adjuvant formulations of rOspC. Pooled sera from each group were used for ELISA analysis of specific antibodies titers. Naive sera were obtained before immunisation. ELISA plates were coated with 100 µl of rOspC (1 µg/ml), incubated with anti-mouse IgG1 (A), anti-mouse IgG2a (B), anti-mouse IgG2b (C), anti-mouse IgG3 (D), or anti-mouse IgM (E) and after addition of OPD plus H2O2, the absorbance was read at 490 nm on ELISA reader. The results are expressed as the end point titers +/- SD. Mean values are expressed in the table under each graph. Formulae of tested compounds in this experiment (F).

Although AlOH adjuvant induced strong OspC-specific antibody responses in total immunoglobulin level (Ig*) and IgG1 isotype (Fig. 15A), the response in complement-activating IgG isotypes (IgG2a and IgG2b; (Fig. 15B,C) was only modest. In comparison with AlOH, the synthetic adjuvant MT06 when combined with liposomes-bounded OspC induced strong OspC-specific response in isotypes IgG2a and at lesser extent in IgG2b (Fig. 15B). Application of another synthetic adjuvant MT05 was associated with dominancy of IgG3 and IgM (Fig. 15D,E). Furthermore, we compare responses to synthetic norAbu-MDP adjuvants with the response to liposomes-bounded OspC plus MDP, which elicits strongest OspC-specific antibodies in IgG2b and in lesser extent in IgG2a isotype (Fig. 15B,C).

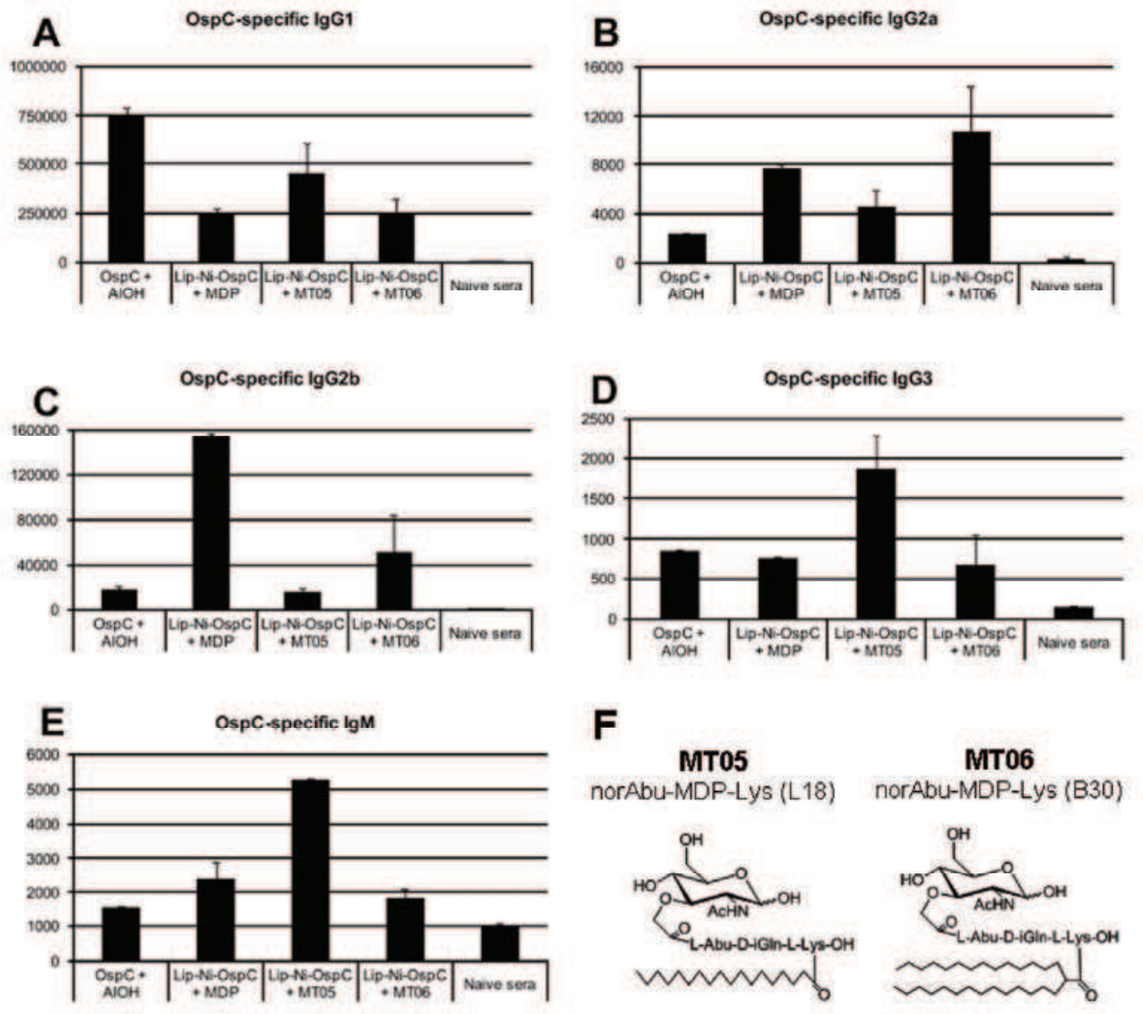


Fig. 15. ELISA analyses of specific antibody titers in sera of immunised mice.

6. Conclusion

Liposomes offer an interesting alternative to aluminium hydroxide and other adjuvant systems, especially in those cases, when the induction of cell immunity is of importance. Various methods for preparation of proteoliposomal vaccination particles are developed and metallochelating liposomes represent the newest and versatile approach to this problem. New nonpyrogenic lipophilic derivatives of norAbu-MDP have been shown to be potent adjuvants for week antigens like recombinant nonlipidized OspC and are suitable adjuvant components, together with other synthetic adjuvants like MPLA and CpG oligos, for construction of vaccines based on various liposomal platforms. Adjuvant potency of these new MDP analogues is comparable to MDP but they lack side effects related to MDP, like strong pyrogenicity and flu like syndromes. Also other synthetic adjuvans like (MPL-A and CpG oligonucleotides) or could be entrapped in metallochelating liposomes alone or in their combinations to precisely tailor the immune response towards particular antigen, especially if the Th1 response is of interest.

Moreover, liposomes are also applicable for non-invasive routes of application as mucosal and transdermal ones, and if rationally designed and applied with new synthetic adjuvants derived from PAMP, they are able to steer the immune system towards desired effective response. The most important observation was that in all vaccinated animals liposomal based vaccines did not induce any side effects. Application of modern physico-chemical and microscopic methods for study of the structure and stability of proteoliposomal vaccination particles is indispensable part of successful development of modern safe and effective vaccines.

7. Acknowledgment

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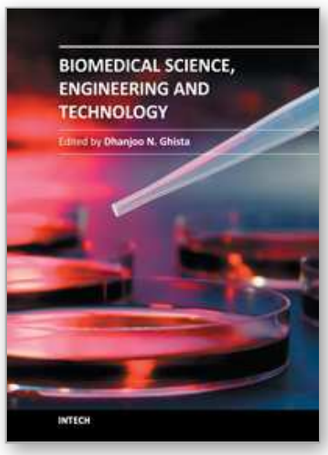

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