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Adjuvanted nanoparticulate seasonal influenza vaccines

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Chapter 1

Introduction, aim and outline of this thesis

The current public health strategy for the containment of influenza is annual vaccination. However, due to a lack of efficacy of current influenza vaccines in some populations, research is needed on improving the immune response to vaccination with the use of adjuvants. This thesis describes investigations of the adjuvant effect of two nanoparticle systems for influenza subunit vaccine: cationic liposomes and peptide-based nanoparticles.

1.1. Influenza virus and flu

Seasonal outbreaks of influenza infections (also known as “seasonal flu”) are caused by influenza viruses. Although influenza can affect people of all ages, hospitalization and death are more frequent in the elderly and in populations with chronic diseases or immune-deficiency. Thereby influenza viruses are a substantial cause of morbidity and mortality worldwide each year, with three to five million cases of annual hospitalization and up to 500,000 annual deaths worldwide [1] [2] [3].

In temperate climates, influenza exhibits a seasonal pattern with a peak activity during the winter months (December through March), which is less apparent in tropical countries. The virus is stable at low humidity and at low temperatures, conditions that favor its transmission, and it grows in respiratory secretions [4].

The virus belongs to the Orthomyxoviridae family (single-stranded RNA viruses). The viral core consists of eight single stranded RNA segments, and nucleoproteins (PB1, PB2, PA, NP) surrounded by a capsid composed of the M1 protein. This core is enveloped by an outer lipid membrane in which several viral glycoproteins are inserted: hemagglutinin (HA), neuraminidase (NA) and matrix proteins (M) (Fig. 1).2. The virus is roughly spherical, with a size between 80 to 120 nm. The different influenza virus types (A, B, and C) are characterized by important antigenic differences [5] [6].

HA is the major influenza envelope glycoprotein. This trimeric protein forms spikes at the virus surface and facilitates viral entry and exit from human respiratory epithelial cells. More precisely, HA is responsible for the attachment of the virus to sialic acid-containing proteins on the host cell surface, the fusion with the endosomal membranes and the release of viral RNA into the cytoplasm. NA also plays a strong role in viral proliferation, as its enzymatic activity results in cleavage of the sialic acid residues on the host cell surface to facilitate release and spread of newly formed virus particles [7].

Sixteen HA and nine NA subtypes have been identified in influenza A, which helps to characterize the circulating viruses and the causes of human infections. Most seasonal local outbreaks in humans are caused by influenza A and B, and H1-3 and N1 and 2 are the principal antigenic types found in humans.

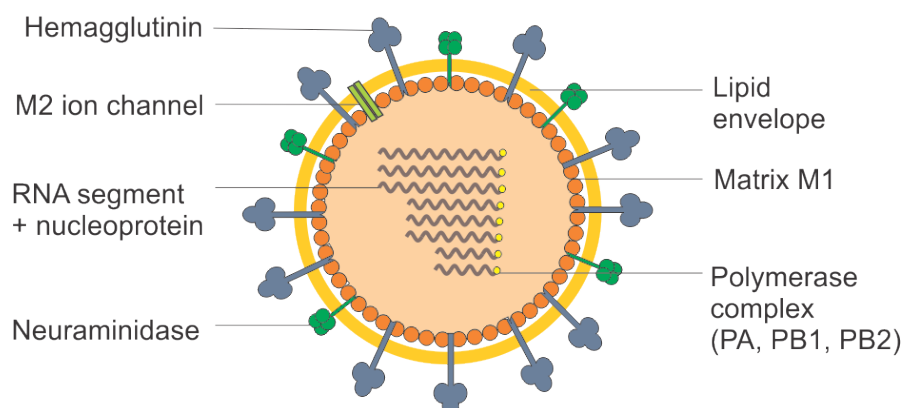


Fig. 1: Schematic representation of the structure of influenza A virus with viral proteins.

These viral proteins are recognized as foreign by a host's immune system, thus eliciting an immune response. However, each of these viruses undergoes antigenic modifications through the accumulation of point mutations in the surface proteins, allowing the virus to escape from the host's existing immunity. This phenomenon is referred to as antigenic drift [8]. This mechanism allows the influenza viruses to evade from adaptive immune responses, which consequently results in reduced clearing of the virus and virus-infected cells, and more severe infection. It is the main cause of the yearly seasonal influenza outbreaks.

In addition, a second mechanism of variation, known as antigenic shift [9], is less frequent but more dramatic and can occur either through cross-species transmission (e.g., poultry to humans) or by mixing of viral genes when there is simultaneous infection by more than one strain of influenza in a single human or nonhuman host. This is the main origin of influenza pandemics and cause of important morbidity and mortality among persons of all ages worldwide.

1.2. Immune response against influenza virus

The understanding of immunity induced by seasonal influenza viruses, not only helps to define the needs for a better protection against an influenza infection, but also the requirements for more potent vaccines. The immune system can be divided into innate and acquired parts. The innate immune system can be described as the non-specific and rapid response to infection but the results are short lived and non-protective against future infection. In contrast, the acquired immune system is specific and can offer long-term protection, which however takes more time to establish.

1.2.1. Innate immune response

The targets for influenza virus are the epithelial cells from the respiratory tract. Therefore, the primary line of defense is formed by the physical barrier (mucus, epithelium) and the innate cellular immune response.

The presence of influenza viruses is sensed by receptors of the innate immune system through recognition of pathogen-associated molecular patterns (PAMPs) of influenza A viruses by pattern-recognition receptors (PRRs) that initiate antiviral signaling cascades, resulting in the production of interferons, cytokines and chemokines. The PRRs involved in the recognition of influenza viruses are toll like receptors (TLRs), retinoic acid inducible gene-I (RIG-I) and the nucleotide oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) protein. The TLR ligands that recognize the influenza virus are: TLR7, an intracellular receptor which binds single-stranded viral RNA; TLR3, another intracellular receptor that recognizes double stranded viral RNA; and TLR2 & TLR4, which are able to recognize viral surface glycoproteins and lipoproteins. The activation of these receptors induces the production of proinflammatory cytokines and type I interferons.

Innate immune cells such as alveolar macrophages and monocytes are attracted by the infected cells. They enhance the pro-inflammatory response and also play a direct role in the phagocytosis of apoptotic infected cells and opsonized viral particles. Furthermore, the dendritic cells (DCs), which are professional antigen-presenting cells, are a link between the innate and the adaptive immune system. They initiate an adaptive immune response after infection by presenting viral antigens to B and T lymphocytes. In case of influenza infection, DCs can be infected either directly or by phagocytosis of viral particles or apoptotic infected cells. Viral proteins can be processed intracellularly and the derived peptides can then be presented to CD8+ T-cells or CD4+ T cells by MHC I or MHC II complexes, respectively. Moreover, type I interferons stimulate the DCs and enhance the antigen presentation to CD4+ and CD8+ T cells.

1.2.2. Adaptive immune response

The adaptive immune response against influenza is based on virus specific antibodies produced by B cells (humoral response) and virus specific CD4+ and CD8+ T cells (cellular response). This response is faster and stronger after the second encounter with the same pathogen, and contributes to the clearance of the influenza virus from the host.

1.2.2.1. Humoral response

Following an influenza virus infection, specific antibodies against the two surface glycoproteins HA and NA are produced by the host, which enable virus neutralization. The HA-specific antibodies inhibit virus attachment and entry into the host cell; and antibodies against NA have protective potential as they limit spreading of the virus. However, most antibodies directed against HA are influenza virus strain-specific and fail to neutralize intrasubtypic drift variants and viruses of other subtypes [9], due to the high variability of the HA globular head. Antibodies are also induced against other viral proteins, such as the highly conserved NP, which could potentially contribute to heterosubtypic immunity [10].

1.2.2.2. Cellular response

DCs degrade viral proteins and the derived peptides are presented on their surface by MHC class I or class II molecules. For MHC class II presentation, viral proteins are degraded in endosomes/lysosomes and the resulting peptides associate with MHC class II molecules. These complexes are then transported to the cell membrane for recognition by CD4⁺ T helper (Th) cells. For class I presentation, the influenza virus-derived peptides are liberated in the cytosol and transported to the endoplasmic reticulum where they associate with MHC class I molecules. Then the complexes are transported to the cell membrane for recognition by specific CD8⁺ T cells (CTLs).

Some CD4⁺ T cells display cytolytic activity to infected cells [11]. However, the most important phenotype of these cells is that of T helper (Th) cells. Different subsets of Th cells are distinguished based on their cytokine expression profiles. Th2 cells produce interleukin 4 (IL-4), interleukin 5 (IL-5) and interleukin 13 (IL-13) and stimulate B cells, resulting in antibody secretion (predominantly IgG1 subtype in mice). Besides, Th1 cells produce interferon gamma (IFN- γ) and interleukin 2 (IL-2), are involved mainly in promoting cellular immune responses (CTL), and are essential for memory CD8⁺ T cells. In addition, the production of IgG2a/c antibodies in mice is dependent on the presence of IFN- γ . Therefore, higher IgG2a/c or IgG1 antibody secretion suggests the induction of Th1 or Th2 response, respectively.

After activation of the CD8⁺ T cells in the draining lymph nodes and differentiation into CTLs, they migrate to the site of infection where they recognize and eliminate influenza infected cells. However, little is known about the specific role played by CTLs response in protection of human individuals against influenza infection.

1.3. Influenza vaccines

The aim of a flu vaccine is to induce an immune response similar to that after a primary viral infection (without causing the associated pathogenicity), which should

elicit a strong immune memory response to protect the host upon future infection [12]. As mentioned above, the circulating influenza viruses undergo antigenic drift through the accumulation of point mutations in the surface proteins, allowing the virus to escape from the host's existing immunity. Thereby, the seasonal influenza vaccine requires yearly modification, based on a mix of the three most current influenza strains (mostly: two viruses type A, and one virus type B).

1.3.1. Inactivated influenza vaccines

The different kinds of parenteral seasonal flu vaccines are: whole inactivated virus (WIV) vaccines, split virus vaccines, and subunit vaccines. WIV vaccines were first developed in the 1940s, consisting of beta-propiolactone inactivated whole virus particles. However, the use of these vaccines caused some adverse effects (particularly among children), such as local reactions at the site of injection and febrile illness [13]. In the 1970s, WIV vaccines were replaced by split vaccines. The production of split vaccines is similar to that of WIV vaccines, but is followed by the addition of detergent (such as Triton X-100) to extract proteins from the lipid membrane. Compared to WIV vaccines, split vaccines are claimed to be less reactogenic. Finally, subunit vaccines are prepared similarly to split vaccines, but include an extensive purification step, resulted in products containing relatively pure HA with small amounts of NA, but lacking most of the viral RNA. Therefore, subunit vaccines are considered safer and are therefore preferred over WIV and split vaccines.

The effectiveness of current inactivated influenza virus vaccines can be determined by means of protection, which corresponds to the percentage reduction in influenza infection frequency among people vaccinated compared with the frequency among those who are not vaccinated. The target groups for annual vaccination include: people over the age of 50, pregnant women, immunodeficient patients, patients with chronic pulmonary or cardio-vascular diseases, residents of long-term care facilities, and health care personnel [14]. The results show that 70–90% of vaccinated healthy young adults attain protection against the virus-specific illness. However, the protection rate is lower among the elderly, with 30–60% protection [15]. This difference in efficacy has been related to the senescence of the immune system and reduced production of protecting antibodies and cytotoxic lymphocytes in response to vaccination in elderly people [16]. Vaccines are not fully protective, but even being imperfect they still prevent substantial morbidity and mortality worldwide. Subunit vaccines are based on purified protein antigens, which implies a loss of (1) other viral molecules which would have typically alerted the host to the dangerous nature of the pathogen and (2) particulate nature. These features appear to be a major drawback of subunit vaccines, as they are too 'clean' and do not resemble pathogens sufficiently to elicit an adequate immune response by themselves [17]. However, the use of adjuvants, i.e., components or delivery systems that are added to a vaccine formulation to increase the immune response

against the antigen(s), can raise the vaccination efficacy (as described in more detail in section 1.3.3.)

1.3.2. Induction of protective immunity with influenza vaccine

Typical assays used to measure vaccine responses against influenza antigens include hemagglutination inhibition (HI) and microneutralization (MN) assays, tested in vitro with red blood cells (RBCs) and live viruses, respectively. These standardized tests are easy to perform and provide a quantitative measure of antibodies based on their ability to neutralize viral particles [18].

However, protective immunity against influenza has been described to occur in influenza vaccine trials in the absence of measurable neutralizing antibodies [19] [20]. This indicates an important contribution of cellular responses to protection against influenza, which has also become apparent in preclinical studies. For instance, BALB/c mice typically respond to inactivated influenza vaccines and subunit vaccines with a Th2-type immune response, which is associated with the stimulation of IgG1 antibodies [21]. However, the major antibody isotype present in the sera of mice that survive viral infections is IgG2a [22], which is stimulated during Th1-type immune responses [21].

One of the explanations could be that the antibodies have the potency to interact with Fc receptors, located on NK cells and macrophages. When such antibodies are bound to HA expressed on infected cells, they mediate cell lysis. This process is known as antibody-dependent cell cytotoxicity (ADCC) [23]. However, the ability to contribute to the influenza virus clearance [23] is different between the IgG isotypes [24]. IgG2a antibodies demonstrate more efficient protection in mice than IgG1 antibodies, whereas the IgG1 isotype is preferentially induced in humans during natural infection [25].

1.3.3. Adjuvanted influenza vaccines

Adjuvants can be used to improve the immune response to subunit influenza vaccines in many different ways. They can be used to enhance the speed and duration of the immune response; they can enhance immune responses in individuals with immature or senescent immune systems; they can decrease the dose of antigen and reduce vaccine costs; or they can help overcome antigen competition in combination vaccines [26]. The effect of an adjuvant addition to a subunit influenza vaccine can lead to stronger hemagglutination inhibition (HI) and microneutralization titers, which theoretically should allow a better protection of the vaccinee compared to an individual vaccinated with a non-adjuvanted vaccine.

Adjuvants mechanisms are diverse and not fully understood. They differ from one adjuvant to another. For instance, cationic compounds, like a natural infection,

induce a “danger signal” [27] [28]. It results in localized tissue damage, and cells will be ruptured releasing their intracellular contents (such as uric acid, heat shock proteins), which are termed ‘alarmins’ [29]. Other adjuvants such as TLR ligands (e.g., lipopolysaccharide [LPS], dsRNA CpG motifs and bacterial lipoproteins [30] [31]) can bind to PRRs and be recognized by the innate immune system as PAMPs, which can directly activate the DCs. This will result in co-presentation by the antigen-presenting cells (APCs) of the antigenic peptide with a co-stimulatory molecule such as CD80 and CD86, which is important for successful T cell activation [32]. Furthermore, immunostimulatory adjuvants such as saponins and ISCOMS can activate T cells independent of TLR activation [33].

Other mechanisms are linked to the action of the adjuvant itself. Firstly, colloidal adjuvants can be used to ‘carry’ antigen (e.g., through entrapment or adsorption); secondly, many of them are based on particulate structures with a size comparable to pathogens, which enable endocytosis by immune cells. Intracellular delivery of the antigen can also be promoted by the adjuvant composition or its surface charge. Moreover, particulate adjuvants enable protection of the antigen against enzymatic destruction and physiological clearance, can be maintained at the injection site for longer periods, and allow extended antigen presentation to APCs (“depot effect”). However, all these effects are dependent on numerous factors such as the route of administration, the antigen, the characteristics of the formulation itself (such as particle size, zeta potential, antigen loading). Only few adjuvants are licensed for use in humans and inclusion in commercial flu vaccines.

1.3.3.1. Aluminum salts

Aluminum salts were the first licensed adjuvants. They include, amongst others, aluminum hydroxide, aluminum phosphate and aluminum potassium sulfate. They have an accepted safety profile in humans for almost one century [34]. Their adjuvanticity was initially attributed to prolonged retention of antigen at the injection site. More recent research resulted in a better understanding of the mechanism [35]. The cellular uptake of aluminum salts (or of uric acid produced from necrotic cells in response to the salts) leads to lysosomal disruption and activation of the NLRP3 inflammasome. The rapid inflammatory mediator release recruits innate cells to the site, including eosinophils secreting IL-4 and monocytes that differentiate into DCs. The IL-4 environment of CD4+ T cell activation is expected to produce a Th2-type response.

Some studies have shown that one-dose seasonal WIV H1N1 and H3N2 vaccines adjuvanted with aluminum phosphate induced relevant HI titers in elderly and adult populations (at two dosages: 6 and 15 µg). However, no comparison with non-adjuvanted vaccine was performed in this trial [36,37]. In fact, the addition of aluminum salts to split or subunit influenza vaccines has induced only marginal improvements and other, more potent adjuvants are required for these vaccines [38] [39]. Among the explanations found in the literature, this lack of efficacy was

attributed to either partial denaturation of epitopes due to adsorption to aluminum salts [40] or conformational changes [41].

1.3.3.2. Oil-in-water emulsions

The MF59 adjuvant was originally approved for inclusion in a licensed influenza vaccine to be used in the elderly in Europe in 1997. The Novartis MF59 adjuvanted seasonal influenza vaccine (Fluad) is now licensed in more than 20 countries worldwide and more than 85 million doses have been administered. This oil-in-water (o/w) emulsion is composed of 5 % squalene (a naturally occurring oil) combined with the surfactants sorbitan trioleate (Span 85) and polyoxyethylenesorbitan monooleate (Tween 80). The adjuvant mechanism attributed to the emulsion is its ability to improve cell recruitment to the site of injection and to trigger the production of chemokines by cells at the site of injection [42] [43]. Clinical trials showed that MF59-adjuvanted vaccine is well-tolerated and has an efficacy of 86% against influenza infection, compared to 43% efficacy for unadjuvanted vaccine [44,45]. Several other o/w emulsion-based adjuvants are now available, such as AS03 (Glaxo SmithKline Biologicals), which is used in a pandemic influenza vaccine, Pandemrix [46].

However, despite the efficacy of emulsions to raise the immunogenicity of influenza vaccines, they still lack the ability to modulate the immune response toward a strong T cell response. The availability of several immune modulators and the potential association of those with vaccine delivery systems (such as nanoparticles) offer many attractive opportunities in terms of new influenza adjuvanted vaccines.

1.4. Nanoparticulate antigen delivery systems

Antigen delivery systems have proved their ability to enhance the immune response against many subunit vaccines. They present the advantage of enabling easy change in their composition and physicochemical characteristics.

Virosomes are a good example of an efficient nanoparticulate adjuvant system for influenza vaccines. They consist of reconstituted virus-like particles, including the virus envelope and surface antigens (HA and NA) mixed with phospholipids, but lack the genetic material of the virus. Virosomes are approved by regulatory authorities for human vaccines and several products are licensed such as Isiflu® (Johnson & Johnson). Virosomal influenza vaccines were found to be efficient in the elderly and in healthy, chronically ill, and immune compromised adults [47]. However, the immune response induced by virosomal vaccines was reported in several studies as inferior to that raised by other licensed adjuvanted influenza vaccines [48] [49], which illustrates the current need for new adjuvant systems.

There are numerous lipids and polymer based nanoparticulate delivery systems which exhibit a spherical and/or vesicular form, thereby allowing antigens to be

entrapped or surface-associated depending on the physicochemical properties of each component. For antigens such as HA, localized in the outer membrane of influenza virus, the most straight forward delivery systems consist of a nanoparticulate delivery system that can be mixed with the antigen(s). Following this approach, the work described in this thesis explored two types of nanoparticulate delivery systems: liposomes and polymer-peptide block copolymer nanoparticles.

1.4.1. Liposomes

Liposomes are artificial vesicles, with particle sizes ranging between 30 nm to several microns. They can incorporate hydrophilic drugs in the aqueous compartment(s), hydrophobic drugs in the lipid bilayer(s), or amphiphilic compounds that partition between both phases. The composition of the lipid bilayer consists of (natural or synthetic) phospholipids and cholesterol, and can be manipulated to influence the physicochemical characteristics of the liposomes (e.g., size, bilayer rigidity, surface charge, pH sensitivity) [50] [51].

Liposomes are often composed of lipids that occur naturally in cell membranes, such as phosphatidyl choline (PC) and cholesterol; these formulations are completely biodegradable. A number of parenterally administered liposomal products were approved by the FDA for use in humans, such as Doxil® (Johnson & Johnson, USA) and AmBisome® (Gilead Sciences, USA). Besides, there are numerous liposomal vaccine formulations which are currently in clinical trials (Table 1). The immune response generated by liposomal vaccines can be further enhanced by incorporation of immune modulators and/or targeting moieties in the liposomes.

Table 1: Selected liposome and lipid-based vaccines in clinical trials. Adapted from Watson et al. [52]

Name (company)	Disease	Antigen	Composition	Status	References
Stimuvax (Merck)	Non-small cell lung cancer	BLP25 (palmitoylated MUC1),	MPL, DPPC, DMPG, Chol	Phase 3	[53-55]
AS01a (GSK)	Malaria	Recombinant fusion of P. falciparum circumsporozoite protein and Hepatitis B surface antigen	MPL, QS21PC, Chol	Phase 3	[56,57]
Vaxisome (NasVax)	Influenza	Inactivated influenza vaccine	CCS, Chol	Phase 2	[58,59]
JVRS-100 (Juvaxis BioTherapeutics)	Influenza	Inactivated influenza vaccine	DOTIM, Chol, non-coding plasmid DNA	Phase 2	[60]
CAF01 (Statens Serum Institut)	Tuberculosis	protein antigen Ag85B-ESAT	DDA, TDB	Phase 1	[61]

The adjuvant effect of liposomes is highly dependent on their physicochemical properties. In particular, the surface charge has a major influence on the adjuvant effect. Cationic liposomes have proved to be superior adjuvants compared to neutral and negatively charged liposomes [50] [51]. The adjuvant effect of cationic

liposomes has been attributed to several mechanisms: i) nonspecific cell damage (inducing inflammation) at the site of injection, ii) electrostatic interaction between the cationic liposomes and negatively charged groups on the surface of APCs, iii) formation of an antigen depot at the injection site, and iv) activation of DCs [32] [62]. Liposome characteristics influence the antigen's immunogenicity, which can be easily customized, e.g., through the preparation method, the composition, and the co-encapsulation of immune modulators. A high density of positive charges at the cell surface is likely to be recognized as a signal of danger for cells and/or contributes to activation of cascades that are classically activated by endogenous cationic compounds [63].

1.4.2. Polymer-peptide block copolymer nanoparticles

Polymer-peptide block copolymer nanoparticles are formed from high-molecular-weight amphiphilic block copolymers composed of distinct hydrophilic and hydrophobic blocks. Carefully choosing the polymer architecture and chemistry yields a broad and tunable range of carriers, with control over its self-assembly into different defined structures like micelles, vesicles (polymersomes), and nanoparticles. Peptide copolymer nanoparticles are of particular interest as there is a profusion of choice regarding block composition and length, and consequently a large variety of particle properties. Furthermore, some studies have found several potential applications in drug delivery and vaccine research, as self-assembled peptides have been shown to be able to enhance immune responses by acting as an adjuvant [64] [65].

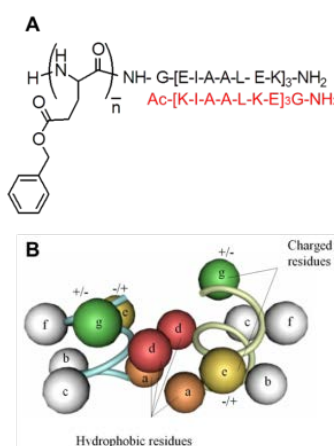


Fig. 2: (A) Molecular structure of the poly(γ-benzyl L-glutamate)-E (in black) and peptide “K” (in red); (B) coiled-coil liaison between the two α-helical peptides “E” and “K”.

In this thesis a new class of polypeptide-b-designed peptides will be tested, consisting of a hydrophobic poly(γ -benzyl l-glutamate) (PBLG) block conjugated to a hydrophilic peptide block with an exact amino acid sequence. The PBLG block adopts a well-defined rod-like α -helical secondary structure, while the hydrophilic peptide block has been designed to form a coiled-coil motif with its complementary peptide partner. In this study the well-studied heterodimeric coiled-coil peptide pair E/K was used (Fig. 2) [66].

The synthesis and the self-assembly abilities of this class of amphiphilic peptides has been described in previous studies [67] [68]. These peptides were applied to create versatile systems, based on the hydrophobic polypeptide PBLG blocks and the functionality of the designed hydrophilic peptide. Specifically, the different PBLG block lengths could be connected non-covalently with various hydrophilic blocks via the specific coiled coil interactions of peptide “E” with peptide “K” [69]. The size, morphology, and surface functionality of the peptide-based nanoparticles can be tailored by combining different building blocks (Fig. 3). Moreover the coiled-coil liaison is non-covalent, highly specific and potentially reversible and allows the functionalization of the peptide polymer nanovesicle’s surface with poly(ethylene glycol) (PEG) molecules [66].

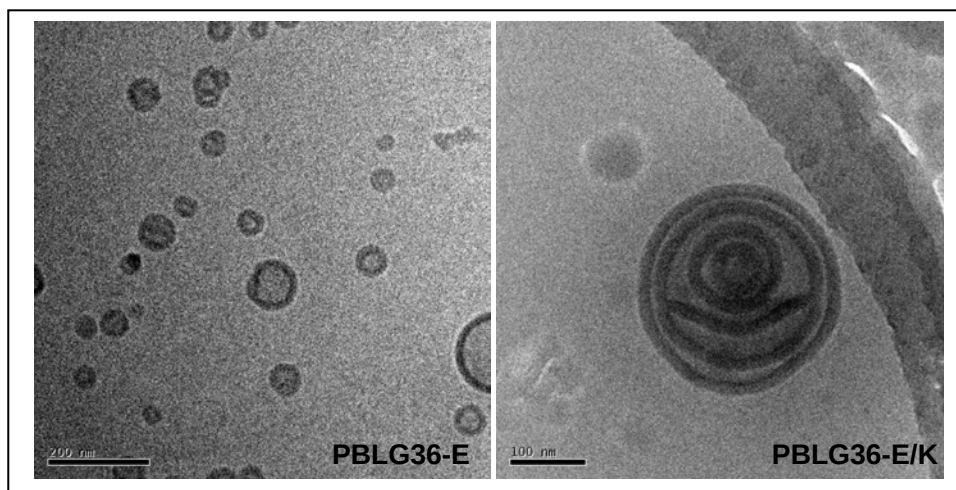


Fig. 3: Cryo-TEM images of PBLG36-E and PBLG36-E/K polymersomes [66].

1.4.3. Nanoparticulate systems and immune modulators

The encapsulation of TLR ligands in nanoparticle vaccine delivery systems can be an efficient strategy to increase the strength and modulate the quality of the immune response against influenza vaccines. Table 2 lists some examples of studies

with TLR ligands co-delivered with nanoparticulate systems (Table 2). Many of such studies have been conducted with cationic liposomes, e.g., to investigate the influence of their positive charge and the association with immune modulators on the immunogenicity of associated antigens. The insights obtained with these studies can also partially be applied to amphiphilic peptide nanoparticles. This will enable studies of their adjuvant effect and optimization thereof, in order to assess their potential use for future adjuvanted subunit vaccines.

Table 2: Examples of inactivated influenza vaccines formulated with nanoparticulate delivery systems combined with TLR agonist.

TLR ligand (μg / dose)	TLR	Nanocarrier (composition)	Antigen (μg / dose)	Size (diameter)	Exp. model	Response	Ref.
Poly ICLC (20 μg)	3	Liposomes (PC:Chol:Stearylamine [7:2:1])	WIV H5N1 (0.1 μg)	300-2000 nm	Balb/c mice	↗ antibody titers, IFN- γ secretion	[70,71]
GLA (20 μg)	4	O/W emulsion (SE)	Split (0.2 μg)	100 nm	Balb/c mice	↗ Th1 (IFN- γ , TNF and IL-2) Broadened serological responses	[72,73]
MPL	4	Liposomes: (DMPC:Chol [7:3])	Split H1N1, split H3N2	200 nm	Balb/c mice	↗ IFN- γ secretion and CTL responses	[74]
MPL (37.5 μg) R837 (60 μg)	4, 7	PLGA nanoparticles	WIV (10 μg)	350 nm	Balb/c & C57BL/6 mice Macaque	↗ antibody and T cell responses	[75]
3M-052 (20 μg)	7	Liposomes (PC)	Recombinant HA (10 μg)	-	Balb/c mice	↗ Th1 response ↗ serum neutralization	[76]
CpG (10 μg)	9	Liposomes (DMPC:DMPG [9:1])	Subunit	1.3–1.5 μm	Balb/c mice	↗ IFN- γ secretion	[77]

1.5. Aim and outline of this thesis

1.5.1. Aim

As detailed above there is a current lack of effective vaccines against flu, and a need for new adjuvanted vaccines, such as nanoparticulate adjuvanted vaccines. However, despite all the research on nanoparticulate adjuvant mechanisms, we still do not have a clear and complete understanding about how these systems can make the host mount a sufficiently strong immune response against an antigen to protect this host against seasonal influenza infection.

The aim of this thesis is to gain a better insight into the role of the formulation of nanoparticulate adjuvants on the immunogenicity of subunit influenza vaccines. For this purpose, a monovalent subunit influenza vaccine (H3N2), which is one of the most common strains responsible for seasonal flu outbreaks, was used as a model. Two different approaches of nanoparticulate adjuvanted vaccines were investigated: polymer-peptide block copolymer nanoparticles and cationic liposomes.

In the first track (chapters 2-4) the suitability of a new class of material, peptide-based nanoparticles, as adjuvants was explored. Little is known about this class of

nanoparticulate delivery systems and their efficacy as adjuvant. Focusing on the formulation, the major aim of this work was to investigate how these nanoparticles act as an adjuvant for subunit influenza vaccine. In the second track (chapters 5 & 6) cationic liposomes were studied in order to gain fundamental insight into the relationship between their physicochemical characteristics and immunogenicity. To this end, a range of formulations was screened to investigate the immunogenicity of these systems in mice. In particular, the effect of nanoparticle composition, surface charge, and encapsulation of immune modulators on the immune response against associated HA was studied.

1.5.2. Outline of this thesis

Chapter 2 introduces a new technique to produce polymersomes based on PBLG-E. A detergent removal technique that has been used for three decades to form liposomes is adapted to prepare PBLG-E polymersomes. This method will be particularly useful for incorporating antigens into the vesicles, as it is a benign process.

In **chapter 3**, subunit influenza vaccine is formulated with PBLG50-K polymersomes to evaluate the feasibility of a polymersome adjuvanted subunit influenza vaccine. The polymersome formulations are physicochemically characterized and administered intramuscularly to mice to evaluate their immunogenicity.

In an attempt to enhance the Th1 response induced with peptide polymer nanoparticles (NPs), in **chapter 4** the synthesis of a new copolymer peptide is presented: PBLG-TAT. TAT peptide is known to be a cell-penetrating peptide. Moreover, a TLR ligand, CpG, is encapsulated in the new NP formulation and the NPs are associated with subunit influenza vaccine. The ability of the formulations to modulate the immune response is tested in vitro (with human dendritic cells [DCs]) and in vivo (with mice).

The use of cationic liposomes is explored in **chapter 5 & 6**. To better understand the mechanisms of the adjuvanticity of cationic liposomes comparisons are made between: (1) formulations based on four different cationic compounds, DDA, eDPPC, DPTAP and DC-Chol (**chapter 5**); and (2) formulations based on 100% saturated lipids vs. lipids mixed with cholesterol in a 1:1 molar ratio (**chapter 6**). The formulations are physicochemically characterized and tested in vitro for their ability to enhance antigen uptake by DCs. Moreover, the immunogenicity of the formulations is tested in mice.

Furthermore, the ability of cationic liposomes to modulate the quality of the immune response against HA is explored in **chapter 6** by: (1) varying the HA antigen loading method and (2) the encapsulation of two different TLR ligands, imiquimod and CpG in the liposomes. The DC-stimulating properties of these formulations are investigated, as well as the humoral and cellular immune

responses after immunization of mice.

The **chapter 7** contains a summary of the work achieved in this thesis and discusses some perspectives for further research on nanoparticulate adjuvanted subunit influenza vaccines.

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Chapter 2

Detergent-aided polymersome preparation

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Abstract

Until now, most preparative methods used to form polymeric vesicles involve either organic cosolvents or sonication. In this communication, we demonstrate for the first time a detergent-aided method to produce polymersomes. Peptidic polymersomes were formed from the rod-rod block copolymer PBLG₃₆-E, where PBLG is hydrophobic poly(γ -benzyl L-glutamate) and E is a hydrophilic designed peptide. The block copolymer was first solubilized by detergent micelles in aqueous buffer, after which the concentration of detergent was reduced by dilution, transforming the particle morphology in solution from mixed micelles to polymersomes. The polymersome formation was monitored with dynamic light scattering and confirmed with transmission electron microscopy. Polymersomes with average diameters of approximately 300 nm were obtained as well as discs with average diameters of approximately 100 nm. This detergent-based method can be used to create polymersomes with a range of properties, as verified by its application to another biocompatible block copolymer, the flexible polybutadiene₄₆-b-poly(ethylene glycol)₃₀. The technique will be particularly useful when delicate biomacromolecules such as (membrane) proteins, peptides, or nucleic acids are to be encapsulated in the polymersomes because the detergents used are compatible with these compounds, and the possible denaturing effect of sonication or organic solvents on the biological activity of the molecule of interest is avoided.

Introduction

Polymersomes are structurally similar to viral capsids in many ways and are now being designed to perform in a similar way as viruses: to carry, protect, target, and release cargo.[1] Biological cargo, such as proteins, peptides, or nucleic acids, is becoming increasingly common, intended for biomedical activity in the body.[2] The advantage of polymersomes over the traditional nanocapsules, liposomes, is that their membranes are more stable, leading to an enhanced ability to carry and protect cargo. The targeting and release properties of polymersomes also have more potential to be tailored to the intended application than liposomes, owing to the wide range of block copolymers available.

There are currently two classes of polymersome preparation: solvent free and with organic solvents.[3] In the first class, the block copolymer is hydrated to form polymersomes. Some block copolymers require no agitation during hydration, whereas others require stirring, vortexing, extrusion, electric current, or sonication. Other block copolymers are too hydrophobic to undergo controlled aggregation in aqueous solution and first need to be dissolved in an organic solvent, which is then mixed with/ exchanged for an aqueous solution.

In the growing number of cases in which biomacromolecules, whose functions depend on intra- and intermolecular structures, are to be incorporated into the polymersome membrane or aqueous interior, organic solvents or high energy input (that is, sonication) cannot always be used because they would degrade the activity of the cargo. Therefore, a dilemma remains: for polymersomes that are intended to incorporate sensitive biomacromolecules but are unable to form vesicles directly in water or cannot be sonicated, there is currently no suitable method available. However, a third method for vesicle formation has been used for nearly 40 years to create liposomes: the detergent removal technique.[4] This has been the preferred preparation method for liposomes incorporating sensitive membrane proteins with preserved structure and function.[5-7] The first step of this method is to solubilize the water insoluble phospholipid that is going to constitute the liposomes in a detergent (water-soluble surfactant). Low-molecular-weight detergents typically have a large hydrophilic section in comparison with the hydrophobic section and form micellar structures with highly curved interfaces. Amphiphiles such as phospholipids and certain block copolymers have a larger hydrophobic component in comparison with the polar section and form lamellar assemblies, such as vesicles. When bilayer-forming phospholipids are solubilized by a large excess of high curvature detergent molecules above the critical micelle concentration (cmc) of the detergent, mixed micelles are formed composed of the detergent and the phospholipid. The detergent in the micelles is in equilibrium with the detergent monomers in the aqueous phase, with the exchange rate in the microsecond range for medium chain detergent molecules.[7] The exchange rate of the lipid between

aggregates is dramatically lower than that of the detergent, on the order of seconds to hours,[8-10] because of the poor solubility of the larger hydrophobic block in water. The second step is to alter conditions such that the morphology of the self-assembled particles is no longer directed by the molecular properties of the detergent but rather by the phospholipid. The mixed micelles are slowly diluted below the cmc of the detergent by adding aqueous solution, and as the micellar-monomer equilibrium is maintained, the amount of detergent in the micelles is reduced. As the proportion of bilayer-forming molecules in the mixed micelles increases, new, lower curvature structures evolve. With lipids, the departure from high curvature micelles passes through sheets, which near the cmc of the detergent close to eliminate exposure of the hydrophobic edge to water, culminating in vesicles.[11]

Although certain phospholipids and block copolymers share molecular characteristics such that they each assemble into vesicles, their interaction parameters between the hydrophilic and hydrophobic components and the aqueous solvent are very different, which affects the self-assembly process.[8] In this article, we adapt the detergent removal method to block copolymers for the first time to create polymersomes. The polymer specific adaptations are explained, making this technique readily applicable to the creation of biomacromolecule-containing polymersomes in the future.

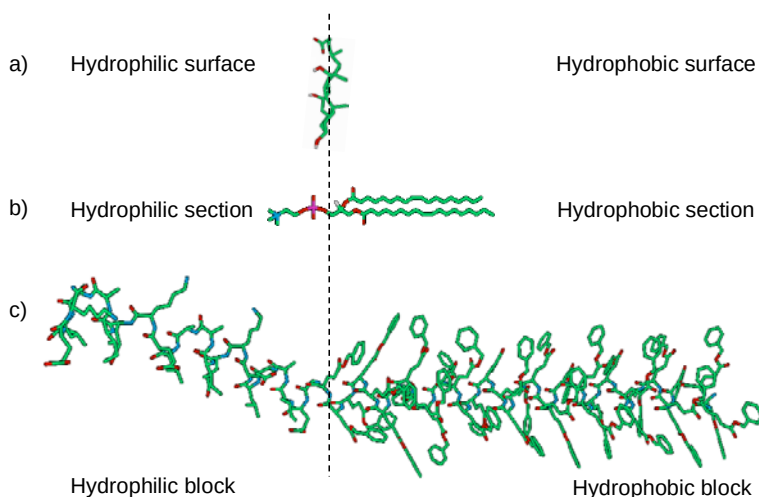


Fig. .: Molecular shape, amphiphilic nature, and relative size of (A) cholate, (B) DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), and (C) PBLG₃₆-E [12] are illustrated. The molecules are depicted approximately to scale, with the hydrophilic sections of the molecules aligned on the left side of the dashed line and the hydrophobic sections on the right.

Experimental section

Block Copolymers. PBLG₃₆-E was synthesized as previously described.[12] The amino acid sequence of the E block was G(EIAALEK)₃-NH₂. The average molecular weight of PBLG₃₆-E was 10230 g.mol⁻¹, and the polydispersity index (PDI) was 1.1. Polybutadiene₄₆-b-poly(ethylene glycol)₃₀ (PB46-b-PEG30, Polymer Source Inc.) had an average molecular weight of 3800 g.mol⁻¹ and a PDI of 1.05. The polybutadiene was polymerized by 1,2-addition.

Preparation of Mixed Micelles. A uniform polymer film was created in a 100 mL round-bottomed flask using 0.01 μmol of block copolymer (100 μL of a 0.1 mM block copolymer stock solution in tetrahydrofuran was added to the round-bottomed flask, and the solvent was removed by rotary evaporation under reduced pressure). Detergent (40 μmol) was added to the round-bottomed flask (200 μL of 200 mM sodium cholate or octyl glucoside in phosphate buffered saline (PBS, 10 mM phosphate, 137 mM NaCl, 3.35 mM KCl, pH 7.4 buffer)). The flask was then gently agitated until the polymer film was solubilized and the suspension was homogeneous.

Detergent Dilution. The mixed micelle suspension was diluted by the addition of PBS over 1/2 h using a syringe pump (NE-300, just infusion, Prosense B.V.) with stirring at 700 rpm.

Detergent Removal. The majority of the detergent was removed by means of dialysis. Slide-A-Lyser dialysis cassettes (Therm Scientific) with a molecular weight cutoff of 3000 g.mol⁻¹ were used. The cassettes were thoroughly rinsed with water and then PBS. Samples were dialyzed against PBS for at least 48 h with two changes of buffer.

PBLG₃₆-E and Cholate Quantification. After dialysis (against water), the amount of PBLG₃₆-E and cholate in the samples was quantified by nuclear magnetic resonance spectroscopy. ¹H NMR spectra were recorded on a Bruker AV-400 spectrometer, in 7:3 (v/v) dimethylformamide-d₇/trifluoroacetic acid to prevent block copolymer aggregation. A residual dimethylformamide proton resonance was used to calibrate the chemical shifts, and dioxane was used as an internal calibrant to quantify the amount of PBLG₃₆-E and cholate. It was found that 80% of the original polymer material was present after dialysis, and no cholate could be detected (sensitivity ~0.1 μmol).

Characterization. Experimental diffusion coefficients, *D*, were measured at 25 °C by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS equipped with

a Peltier-controlled thermostatic cell holder. The laser wavelength was 633 nm and the scattering angle was 173°. The Stokes-Einstein relationship $D = k_b T / 3\pi\eta d_h$ was used to estimate the hydrodynamic diameter, d_h . Here k_b is the Boltzmann constant and η is the solvent viscosity.

Transmission electron microscopy (TEM) was conducted on a JEOL 1010 instrument with an accelerating voltage of 60 kV. Samples for TEM were prepared by placing 5 μ L of solution on carbon-coated copper grids. After $\sim$ 5 min, the droplet was removed from the edge of the grid. A drop of 2% phosphotungstic acid (PTA) or 2% osmium tetroxide (OsO₄) stain was applied and removed after 2 min.

Results and Discussion

Whereas phospholipids are typically $\sim$ 2 nm long and somewhat flexible, the amphiphilic block copolymer PBLG36-E used in this study has an average length of $\sim$ 8 nm and is relatively rigid.[12] Both the poly(γ -benzyl L-glutamate) block, denoted PBLG36, and the peptide E (amino acid sequence G(EIAALEK)₃) can adopt an α -helical conformation. In this conformation, the hydrophobic PBLG36 block has an average length of 4.5 nm, whereas the water-soluble peptide E block is $\sim$ 3.5 nm long. Recently, we have shown that PBLG36-E forms bilayered vesicles in aqueous solution.[12] Because of the large hydrophobic block size, none of the common solvent-free polymersome preparation methods, that is, bulk/film hydration, sonication, and so on, which all require hydration of a macroscopic phase of the block copolymer, have been successful. In this communication, we use sodium cholate as the detergent to solubilize PBLG36-E. Sodium cholate is often used to incorporate proteins in liposomes.[7] It is a low-molecular weight, rigid, disk-like anionic detergent with a cmc of $\sim$ 10 mM in 100 mM NaCl aqueous solution at 25 °C.[13] The relative sizes of cholate, a phospholipid typically used to prepare liposomes by the detergent removal method, and PBLG36-E are illustrated in Figure 1. From the size disparity between the block copolymer and both the phospholipid and detergent, it can readily be appreciated that the balance of self-assembling forces between the micelle- and vesicle-forming molecules is very different when using this method to prepare liposomes or polymersomes. The process of making polymersomes starts with forming mixed micelles of the detergent molecule and the block copolymer from a PBLG36-E film and an aqueous solution of cholate micelles. The aggregation number of cholate micelles is quite variable, with micelles containing between 2 and 30 molecules.[13-15] Cholate micelles (200 mM in PBS, 25 °C) were determined by DLS to have an average hydrodynamic diameter (d_h) of 2 nm, in agreement with reported values,[16] and a size range of about 1-5 nm (Figure 2A). Using the detergent dilution method to produce liposomes, lipid/cholate molar ratios on the order of 1:2 are typically employed.[17,18] In comparison with lipids, the block copolymer has a much larger surface area to be encapsulated;

therefore, a PBLG36-E/cholate molar ratio of 1:4000 (0.05 mM PBLG36:200 mM cholate) was chosen. Thin films of PBLG36-E were hydrated in aqueous cholate solutions for 24 h with occasional gentle agitation to solubilize the block copolymer. Because many cholate molecules are required to shield the large hydrophobic PBLG block from the aqueous solution, a departure from the morphology of pure cholate micelles is expected.[19,18] This was observed by TEM, with images of the mixed micelle stage containing a majority of pure cholate micelles and some larger particles between 5 and 20 nm in diameter (Figure 2B). With the low block copolymer/cholate ratio employed under the current preparation conditions, the size distribution of the mixed micelle population as determined by DLS does not vary significantly from that of pure cholate micelles (Figure 2A). The size distributions of mixed micelle solutions were stable for at least 4 days, as determined by DLS.

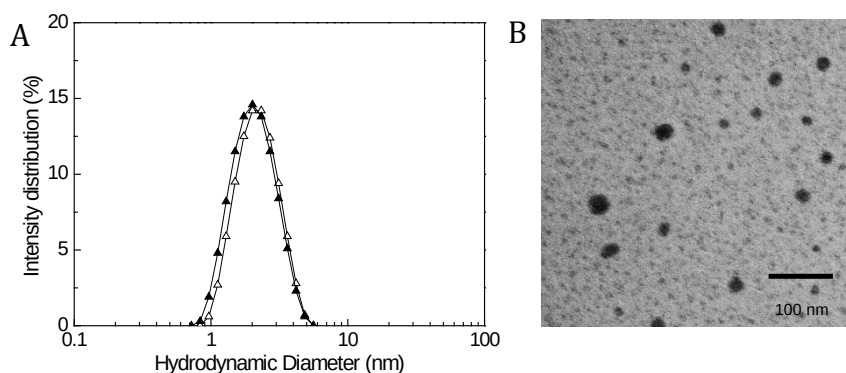


Fig. 2: (A) DLS intensity distributions of sodium cholate (Δ) and sodium cholate/PBLG₃₆-E 4000:1 ($\blacktriangle$). (B) PTA-stained TEM image of a sodium cholate/PBLG₃₆-E 4000:1 mixture showing micelles and mixed micelles. Conditions: 200 mM cholate, 0.05 mM PBLG₃₆-E in PBS, 25 °C.

The second step in the formation of vesicles is the dilution of the mixed micelles such that detergent molecules are gradually removed from the micelles and the morphology of the structures shifts from being dominated by the selfassembling properties of the detergent to those of the block copolymer. This was achieved by diluting the mixed micelle solution from 200 mM cholate, well above the cmc, to 2 mM, which is well below the cmc. The solution was stirred rapidly during detergent dilution to prevent uncontrolled aggregation, similar to the preparation of liposomes using the detergent dilution method.[20] Moreover, well-defined size distributions were only observed when the aqueous solution was gradually added (over 30 min or longer).

After this dilution step, the particle sizes as observed with DLS had increased from 2 nm to larger structures with a bimodal distribution. The average d_h of the

predominant structure was ~ 350 nm, and the average d_h of the second population was ~ 100 nm (Figure 3A). This size distribution is most likely due to the range of molecular lengths and self-assembling characteristics of the block copolymer (PBLG $_{\geq 22, \leq 46}$, PDI = 1.1).

For detergent/phospholipid systems, the initial mixed micelles increase in dimension upon dilution and finally form liposomes around the cmc of the detergent.[11] This means that the intrinsic self-assembly of the lipids only fully emerges and liposomes assemble when the detergent concentration becomes too low to form micelles. The energetic determinants of vesicle formation are different for block copolymers and lipids; therefore, it may be expected that aspects of the micelle to vesicle transition also differ.

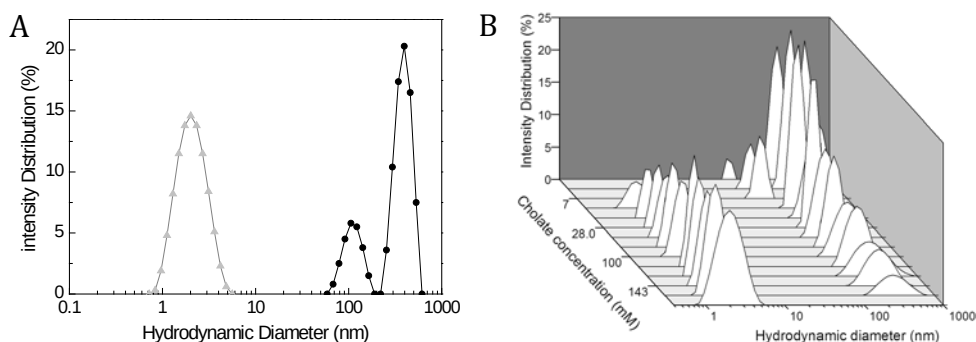


Fig. 3: (A) DLS intensity distributions of cholate/ PBLG₃₆-E mixed micelles (▲) and of polymersomes formed after diluting the mixed micelles to 2 mM detergent (●), (B) Evolution of micelle/polymersome diameters as a function of cholate concentration during dilution from 200 to 2 mM. Initial conditions: 200 mM cholate, 0.05 mM PBLG₃₆-E in PBS, 25 °C.

DLS was conducted during the detergent dilution step to gain insight into the route of vesicle formation. PBS was added incrementally to mixed micelles (200 mM cholate, 0.05 mM PBLG₃₆-E), and the size distribution was monitored after each dilution step. Upon the first PBS addition (170 mM cholate), a transition from micelles to large structures was observed (Figure 3B), which is in marked contrast with the temporal pathway of liposome formation. As more PBS was added, the amount of large structures gradually increased, and there was a simultaneous decrease in the amount of micelles. As the detergent concentration passed below the cmc of cholate (10 mM), micelles were no longer detected. It should be noted, however, that because DLS is intrinsically biased toward the detection of large particles, it is expected that micelles are present beyond the detection limit of DLS. The cholate concentration at which the large structures are first detected is approximately 15 times its cmc, implying that the determining factor in the micelle-to-vesicle transition for this polymer is not the dispersion of the micelles at the cmc of the detergent.

In fact, it was not necessary to dilute the samples below the cmc of the detergent because the 350 nm population was stable before all micelles (many of which would be pure cholate micelles) had dispersed, as seen in Figure 3B. To avoid unnecessary dilution of the suspensions, it was preferred to dilute the cholate from 200 to 20 mM, with the vesicle size distribution not significantly different from samples that had been diluted below the cmc of the detergent (Figure 4A). After dilution to 20 mM cholate, TEM revealed polymersomes with diameters matching the DLS distribution and with membrane thicknesses of ~ 15 -20 nm, which is in close agreement with the calculated average thickness of 18 nm[12] (Figure 4B). In addition to polymersomes, another bilayered structure, discs, was observed. The diameter of the discs was ~ 100 nm, which is also consistent with the DLS results. If polymersomes are required as the exclusive morphology, parameters such as the dilution rate or the concentrations of the detergent and block copolymer should be adjusted, or an alternative block copolymer/detergent should be used.

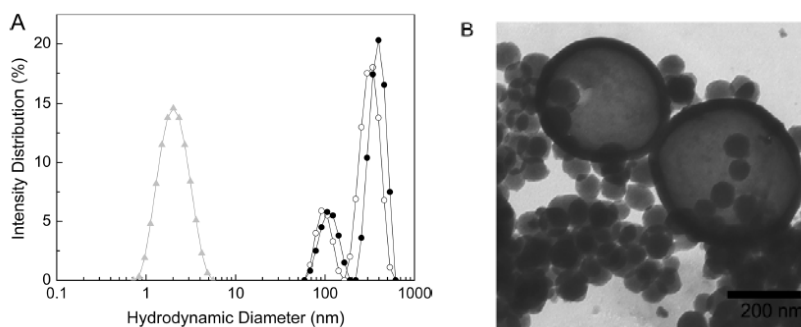


Fig. 4: (A) DLS intensity distributions during vesicle preparation: cholate/ PBLG₃₆-E mixed micelles ($\blacktriangle$), polymersomes formed after diluting the mixed micelles to 2 mM detergent ($\bullet$), and polymersomes formed after diluting the mixed micelles to 20 mM detergent ($\circ$). (B) OsO₄-stained TEM image of polymersomes and polymer discs after diluting the mixed micelles to 20 mM. Initial conditions: 200 mM cholate, 0.05 mM PBLG₃₆-E in PBS, 25 °C.

These results show that the relative influence and function of the detergent on the vesicle self-assembly process is clearly different for phospholipids and this block copolymer. To verify that it is dilution, that is, removal of detergent from the mixed micelles, not only stirring that induces self-assembly of the block copolymer, a sample with 200 mM cholate and 0.05 mM PBLG₃₆-E was stirred without dilution. A population of particles did emerge, although the detected size distribution varied haphazardly during stirring, with the average d_h ranging between 300 and 1000 nm. Additionally, the rate of formation was reduced at least four-fold, with large particles still forming after 2 h. In contrast, when samples are diluted and stirred, the entire polymer population assembles into stable polymersomes within 0.5 h. This shows that with a PBLG₃₆-E/cholate ratio of 1:4000, each polymer is effectively

isolated from one another, and removal of cholate molecules from the mixed micelles facilitates complete conversion to well-defined vesicles.

A possible explanation of the observed results is as follows. In the mixed micelle stage, the large hydrophobic PBLG block is shielded from the aqueous environment by a layer of disk-like cholate molecules. [15] Because of the high exchange rate of cholate between micelles and solution, [21,22] detergent depleted “sticky patches” temporarily appear, allowing the block copolymer to exert its native self-assembling propensities and leading to coalescence between detergent-coated block copolymers. Because of its large hydrophobic block, PBLG₃₆-E exhibits very strong phase separation in comparison with phospholipids in aqueous solution, with similar block copolymers having exchange rates ranging from hours to being nondetectable. [23-25] Once a number of PBLG₃₆-E molecules self-assembles, it is unlikely that the reverse process would occur. As a control experiment, PBLG₃₆-E polymersomes were prepared, and cholate was added to a final concentration of 200 mM. The polymersome/ micelle suspension was stirred for 30 min (the standard duration of dilution), and no significant changes in the polymersome population were observed with DLS. In essence, for this polymersome assembly process, the important aspect of the detergent is that it provides a means of solubilizing the block copolymer and dampening its strong aggregation tendency en route to polymersomes. The micelle, to monomer transition of the detergent does not induce polymersome formation. In more general terms, the initial detergent concentration should be high enough to solubilize completely the block copolymer, and to trigger the structural conversion the mixed micelles should be diluted until all of the block copolymer has assembled into vesicles, with the precise detergent concentration dependent on the block copolymer and detergent used.

The polymersome preparation method was also applied to the flexible, unstructured, neutral block copolymer polybutadiene₄₆-b-poly(ethylene glycol)₃₀. Although PB₄₆-b-PEG₃₀ and PBLG₃₆-E have very different physical properties, in comparison with lipids they both contain very large hydrophobic blocks that aggregate strongly in aqueous solution. Mixed micelles of PB₄₆-b-PEG₃₀ and the detergent octyl glucoside were diluted, generating polymersomes above the cmc of the detergent (Figure S1 of the Supporting Information). These results reveal the generality of this detergent based method and indicate that it is applicable to a diverse range of block copolymers and detergents.

Because detergents may interact with other molecules in the environment to which the polymersomes are applied, in some instances, detergent removal may be desired. Therefore, dialysis was used to reduce the detergent concentration outside the PB₄₆-b-PEG₃₀ and PBLG₃₆-E polymersomes from 20 mM to ~0.1 μ M. The distributions of polymersome diameters did not change significantly during dialysis (e.g., Figure 5A), and the polymersomes were stable for at least 1 week at 4 °C.

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the PB₄₆-b-PEG₃₀ and PBLG₃₆-E polymersomes from 20 mM to ~ 0.1 μ M. The distributions of polymersome diameters did not change significantly during dialysis (e.g., Figure 5A), and the polymersomes were stable for at least 1 week at 4 °C. After vesicles have formed in solution, the enclosed detergent will not diffuse out of the assembly as readily as from micellar or lamellar sheet morphologies. Therefore, the rate of detergent removal depends on how readily the detergent diffuses through the vesicle membrane and the rate of flip-flop of the amphiphile between the bilayers.[7] Polymersomes have relatively thick and rigid bilayers, and the rate of flip-flop is expected to be insignificant; therefore, the rate of detergent removal depends almost entirely on the diffusion of entrapped detergent through the block copolymer membrane, and it is expected to be more difficult to remove residual detergent from polymersomes than from liposomes.²⁶ Following dialysis for 48 h, PBLG₃₆-E polymersomes were solubilized, and it was observed with NMR spectroscopy that <0.5% of the cholate remained after dialysis. From the NMR spectra, it was also seen that the recovery of PBLG₃₆-E after dialysis was nearly quantitative (80%).

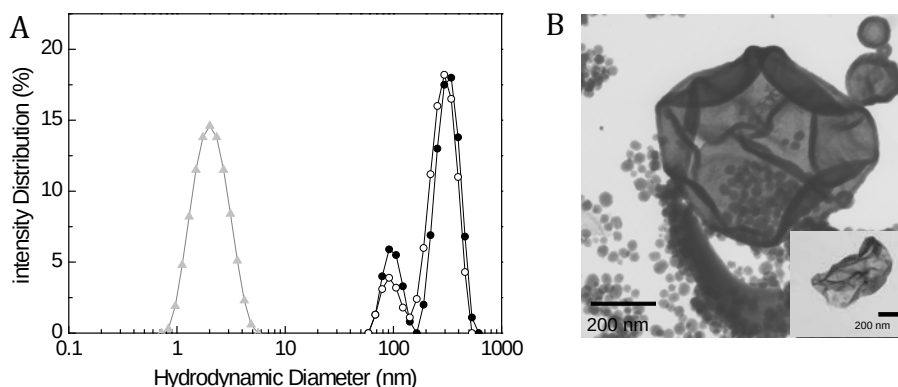


Fig. 5: (A) DLS intensity distributions of cholate/PBLG₃₆-E mixed micelles (▲), polymersomes formed after diluting the mixed micelles to 20 mM detergent (○), and polymersomes after detergent removal by dialysis (●). (B) TEM image of OsO₄-stained sample after diluting the mixed micelles to 20 mM. Insert: after dialysis. Initial conditions: 200 mM cholate, 0.05 mM PBLG₃₆-E in PBS, 25 °C.

Conclusions

We have demonstrated that the detergent removal technique, which has been used to produce liposomes for four decades,[4,27] can also be used to produce polymersomes. The dilution of cholate/ PBLG₃₆-E or octyl glucoside/PB₄₆-b-PEG₃₀ mixed micelles leads to a controlled transition from micelles to polymersomes. Whereas in the case of liposome formation, the micelle-to-vesicle transition is controlled by the breakup of detergent micelles, these block copolymers dictate the self-assembled structures of the two-component systems more forcibly, and the

micelle-to-vesicle transition is determined by the self-assembly of predominantly detergent-covered hydrophobic polymer blocks well above the cmc of the detergent. The detergent is an agent to modulate the force of the phase separation such that well-ordered nanophase separation can occur in aqueous solution rather than the uncontrolled aggregation that occurs without a shielding layer. In this respect, the role of the detergent is reminiscent of the role of organic solvent in the commonly used solvent-replacement technique.[28-30] Because the utility of the detergent is restricted to its ability to solubilize the polymer, the method is termed “detergent-aided polymersome preparation”. This new pathway to produce polymersomes increases their possible applications because it does not require high energy input (e.g., sonication) or possibly damaging organic solvents, and thus it is compatible with labile biomacromolecules. Other than the benign nature of the detergent removal method, another advantage of this route has traditionally been that it is possible to control the liposome size and homogeneity. This can be achieved by varying the rate of detergent dilution[20,11] using different classes of detergent[31,32,18] or vesicle-forming lipid,[20,27] varying the initial detergent/lipid ratios[18] and concentrations,[11] and by changing the pH[27] and ionic strength[33] of the aqueous solution. The effect of these parameters on the properties of the polymersomes and the structural evolution during the formation of vesicles will be the subject of future publications.

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Chapter 3

Polymersomes enhance the immunogenicity of influenza subunit vaccine

Barnier-Quer C *, Robson Marsden H *, S, Zope H, Kros A, Jiskoot W. Polymersomes enhance the immunogenicity of influenza subunit vaccine.

* Authors contributed equally

Abstract

In this study, peptide polymersome based on the peptide polymer -K are tested as an immune adjuvant for an antigen, influenza hemagglutinin (HA). The polymersomes are loaded with HA antigens and the in vivo immunogenicity of the resulting hybrid assemblies was tested in vivo, resulting in an improvement of the immune response for the influenza antigen co-administered with the polymersomes.

Introduction

Vaccination against influenza remains the most effective method to prevent infection by the virus and to reduce the associated morbidity and mortality.¹ Seasonal influenza vaccines currently in use are mostly subunit formulations, consisting of hemagglutinin antigens (HAs) from a mixture of strains. The downside of these vaccines is their relatively low immunogenicity, which can necessitate their administration with an adjuvant, i.e. a component, added to the antigen to enhance its immunogenicity, although the currently marketed seasonal influenza vaccines do not contain any adjuvant. Well known examples of adjuvants that are licensed for use in humans are colloidal aluminum salts and emulsions, such as MF59. Adjuvants can act in several different ways, e.g. by creating an antigen depot at the injection site, by protecting the antigen from enzymatic degradation, by improving the delivery of the antigen to dendritic cells (DCs) or by activating DCs.^[2] Various types of nanoparticles have been shown to be able to act as antigen delivery systems which can combine several of these mechanisms.^[3,4] Polymer as well as lipid based nanoparticles with HA have been successfully tested, enhancing antigen uptake by the DCs and resulting in enhanced antigen-specific acquired immune responses.^[5,6] Nanoparticles can range in size from 10 to 1000 nm, and some studies have shown that the uptake of particles by DCs and their immune-stimulating effect is dependent on their size.^[7] Nanoparticles can vary in several other properties, such as composition, surface charge and hydrophobicity. The nanoparticles can be loaded with antigens by adsorption, covalent attachment, or encapsulation. However, the elicited immune responses of the current formulations do not offer adequate protection and there is still a need for new alternatives. Polymersomes are self-assembled polymer shells composed of block copolymers.^[8–10] These block copolymers have amphiphilic properties similar to lipids, but they have much larger molecular weights, and for this reason they have been compared with viral capsids, composed of large polypeptide chains. Depending on the choice of the block copolymer, its molecular weight and biocompatibility, polymersomes can be used as delivery systems with a broad range of tunable properties.^[11–13] Polymersomes have shown to be stable, in terms of size and structure,^[14] they enable the encapsulation of both hydrophilic and hydrophobic species and can carry functional moieties, such as structures with cell penetrating capabilities.^[15] Moreover, polymersomes based on the degradable di-block polymer polyethylene glycol-polybutadiene functionalized with an HIV-derived Tat peptide successfully enhanced, *in vitro*, the cellular delivery of nanoparticles to DCs.^[16] These results highlight the potential use of polymersomes as robust, virus like antigen delivery systems, but they have not been tested for vaccination yet. Recently, we developed a new class of polypeptideblock-peptides which self-assemble into polymersomes.^[17–19] These particles were shown to be stable for several months. The hydrophilic peptide block is composed of a specific amino acid sequence that is

able to form a coiled-coil complex [20,21] allowing for the non-covalent functionalization of the polymersome surface with functional moieties. The ability of this recognition motif was indicated by the development of non-covalent triblock copolymers and model systems for membrane fusion.[22,23] The hydrophobic block is composed of poly(γ -benzyl-L-glutamate) and both blocks adopt an α -helical conformation when the amphiphiles are assembled into polymersomes.

The aim of this study was to investigate whether polymersomes can enhance the immunogenicity of a HA subunit vaccine. The polypeptide- block-peptide used in this study was the rod-rod block copolymer PBLG₅₀-K,[17] where PBLG₅₀ is the hydrophobic poly(γ -benzyl-L-glutamate) block with an average degree of polymerization of 50, and K is a hydrophilic designed peptide with amino acid sequence G(KIAALKE)₃-NH₂. This amphiphilic block copolymer has been shown to self-assemble into polymersomes with a size of 250 nm. The HA antigen was from a H3N2 A/Wisconsin strain, which is currently used for seasonal vaccination in combination with HA from two other strains. The association of the antigen with the polymersomes was investigated, and the DC-stimulating capacity in vitro and the immunogenicity in mice of the HA-polymersomes were compared with that of plain HA.

PBLG₅₀-K has been shown previously to assemble into well-defined polymersomes in aqueous buffered solutions.[17–19] The polymersome size can be tuned from 200 to 2000 nm with low polydispersity by varying the conditions during the self-assembly process, such as ionic strength and temperature, or the preparation method used.[18,19] As previously stated the interaction of nanoparticles with DCs and the resulting immune-stimulating effect is dependent on their size,[24] with optimum DC uptake for particles with a diameter of 0.5 micron and below. Therefore for this study, polymersomes at the lower end of this size range were selected. In HEPES sucrose at 20 °C PBLG₅₀-K self-assembled into polymersomes with a hydrodynamic diameter of about 250 nm, a polydispersity index of 0.1 (Fig. 1) and a zeta potential (ZP) of -40 mV. The polymersomes were stable, with no sign of turbidity/sedimentation and no detectable aggregation, as observed with dynamic light scattering (DLS) and electron microscopy, for at least 4 weeks. The HA proteins are elongated molecules that extend 13 nm from the exterior of influenza viruses, being anchored in the viral membrane by means of a hydrophobic domain. This hydrophobic domain causes free HA to aggregate in aqueous solutions. The HA used in this study formed clusters with an average hydrodynamic diameter of about 50 nm in PBS, as measured by DLS. The binding of HA in these clusters is proposed to be relatively weak, as no clear aggregates were observed with transmission electron microscopy (TEM) when samples were stained with either OsO₄ (pH7), or PTA (pH 2 or 7.4) (Fig. 2B inset). Upon the addition of the HA solution to the preformed PBLG₅₀-K polymersomes an immediate particle size

increase was observed by DLS. Higher final concentrations (from 0.5 to 50 $\mu\text{g.mL}^{-1}$) of HA resulted in larger HA/polymersome aggregates (Fig. 1).

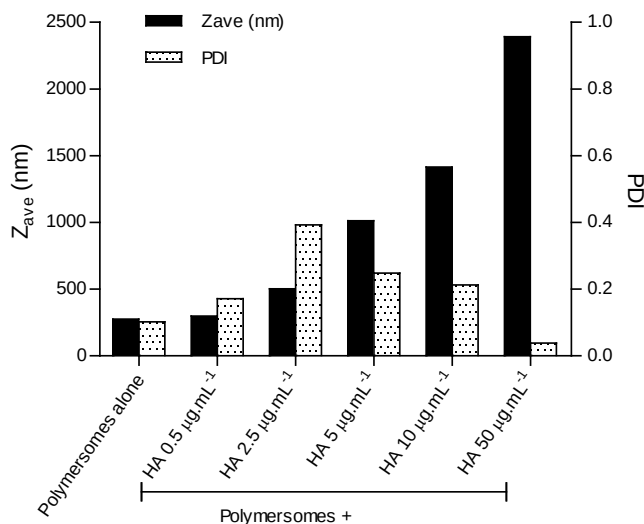


Fig. 1: Average hydrodynamic diameter (Z_{ave}) and polydispersity index (PDI) of a fixed amount of PBLG₅₀-K polymersomes ($100 \mu\text{g.mL}^{-1}$), mixed with a raising amount of HA (final concentration ranging from 0.5 to 50 $\mu\text{g.mL}^{-1}$).

A comparative study with another kind of polymersomes, based on a PBLG-E polymer,[18] with a different hydrophilic peptide sequence $\text{G(EIAALEK)}_3\text{-NH}_2$, was conducted and showed no sign of aggregation (data not shown), indicating that the nature of the peptide has a direct impact on the HA/polymersome interaction. TEM revealed that the plain PBLG₅₀-K polymersomes did not aggregate which is in accordance with the DLS data (Fig. 2A). For the polymersome/HA mixtures, clustering was observed, with the HA presumably acting as a non-covalent crosslinker (Fig. 2B and D). The TEM images also revealed that the HA proteins interact with the polymersomes in a relatively weak manner as the shape and size of individual polymersomes did not change. The association of HA with the polymersomes was further investigated by filtering the suspension through 0.1 mm filters. Under these conditions the PBLG₅₀-K polymersomes were retained on the filter. The filtration of free HA showed a recovery of 88%, whereas for the polymersome/HA complexes it dropped to 28%, showing that most of the HA was associated with the polymersomes.

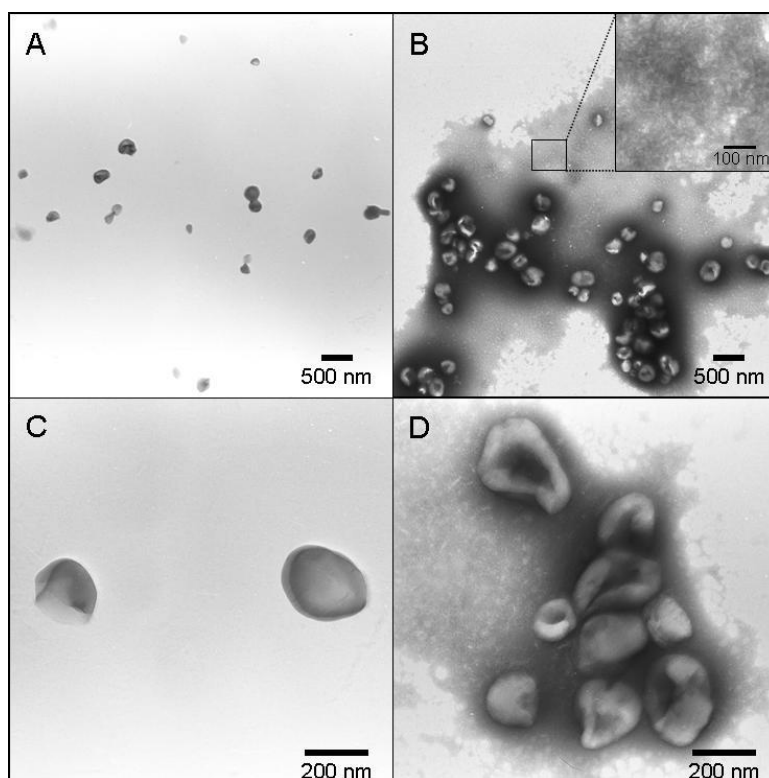


Fig. 2: TEM images (PTA staining) of the polymersomes (A, C) and the mixture of polymersome with 10 $\mu\text{g.mL}^{-1}$ HA (B, D).

The adjuvant effect of PBLG₅₀-K polymersomes (mixed with HA antigen) was investigated in an immunization study with mice. In order to study the effect of the polymersomes without any masking from the antigen alone, we used HA doses of 0.5 and 2.0 mg per immunization (corresponding to HA concentrations of 2.5 and 10 mg.mL^{-1} in the formulations). The polymersome concentration was kept constant for all the formulations (100 mg.mL^{-1}), resulting in a final HA/polymersome weight ratio of 1/40 and 1/10, respectively. The two doses of HA were tested in the presence and absence of polymersomes. The HA-specific serum IgG, IgG1 and IgG2a were assessed after the first (prime) and the second (boost) immunization, and hemagglutination inhibition (HI) titers, as a measure for the level of functional antibodies, were measured after the boost. After both the prime and the boost (Fig. 3A and B), PBLG₅₀-K polymersomes significantly enhanced the IgG titers compared to non-adsorbed HA for the high-dose (2 mg HA) group. In the low-dose group (0.5 mg) there was also a trend toward higher IgG responses for the polymersome formulation as compared to free HA, although the differences were not statistically significant. The IgG1 titers closely followed the total IgG titers, while the IgG2a titers (after prime and boost) were below the detection limit for all groups (results not shown). The HI titer was assessed by measuring the inhibition

by the mouse sera of HA-induced red blood cell agglutination. The sera from mice immunized with non-adjuvanted HA showed a dose dependent HI titer, which was close to the detection limit of the assay for the low-dose group (Fig. 3C). For both HA doses polymersomes acted as adjuvant, as higher HI titers were found, ca. 20 fold for the low HA dose and 8 fold for the high dose, although the latter increase was not statistically significant.

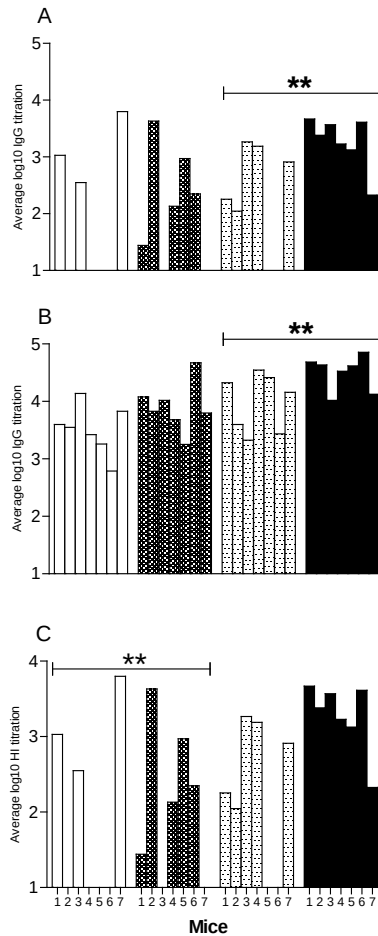


Fig. 3: Immune response in mice after subcutaneous injection of HA formulations: HA-specific serum IgG titers after prime (A) and boost (B), and HI titers after boost (C). Each bar represents the titer of an individual mouse. The formulations tested are: $\square$ 2.5 $\mu\text{g.mL}^{-1}$ HA, $\square$ 2.5 $\mu\text{g.mL}^{-1}$ HA + PBLG₅₀-K, $\square$ 10 $\mu\text{g.mL}^{-1}$ HA, $\blacksquare$ 10 $\mu\text{g.mL}^{-1}$ HA + PBLG₅₀-K. (**: significant difference between the average titers of each group, for $p < 0.01$).

The cytotoxicity of the polymersomes has been tested in vitro. The cell viability was evaluated in Caco-2 cells using the MTT assay. The cells were exposed for 48 hours to a polymersome concentration range of 0.5 to 10 mg.mL^{-1} (the necessary

dilution in the cell culture media did not allow the testing of higher polymersome concentrations). The resulting percentages of cell viability showed no sign of toxicity for any of the polymersome concentrations tested (results not shown).

In summary, the immunization study shows an increase of the serum IgG and HI titers, when the antigen is co-administered with the polymersomes. The improvement of the immune response against HA, when associated with the polymersomes demonstrates that the polymersomes can act as an adjuvant. The HA/polymersome hybrid has been characterized with DLS and electron microscopy showing that HA forms complexes with the polypeptide-block-peptide based polymersomes. The HA/polymersome association is presumably a combination of both electrostatic and hydrophobic interactions, arising from the hydrophobic membrane-anchoring domain of the HA, the localized charge on the HA, and the charged corona of the polymersomes. This is confirmed by the difference observed in the DLS study between PBLG-K and PBLG-E. The presence of three protonated lysine residues (at pH 7.4) in K, instead of three glutamic acids in E, had changed the electrostatic interactions between HA and the polymersome surface, resulting in different aggregation behaviors. The mechanism by which polymersomes act as an adjuvant is unknown, but could include a depot effect,[5] the ability to target the antigen presenting cells (APCs) with the antigen/adjuvant complexes and enhancement of the antigen uptake by APCs.

As detailed in the introduction, the antigen used in this study is one of the main components of the current subunit seasonal influenza vaccines (which consist of a mixture of HA from different strains). Since neutralizing antibody levels expressed as HI titers are considered to be the main protective immune component for parentally administrated subunit vaccines,[25,26] and as this assay is also the test of reference according to the industry standards, the increase of the systemic immune response against HA formulated with PBLG₅₀-K polymersomes, as observed in our study, is a clear improvement compared to the current formulation (HA alone). However, in order to provide a superior level of protection against the influenza infection than HA alone, it has been shown previously that an IgG2a response (indicative of a Th1 immune response) is the strongest isotype in response to viral infection.[27] Our formulations induced low IgG2a titers, therefore our future research will be directed at enhancing the IgG2a response of the polypeptide-*block*-peptide polymersomes by coencapsulation of immuno-modulators.

In conclusion, the work detailed in this paper demonstrates, for the first time, that polymersomes are able to enhance the immunogenicity of an antigen. Moreover, it shows the proof of concept that they can be used as a delivery tool for influenza subunit vaccine with enhanced immune response and no sign of cellular toxicity.

Experimental section

The polypeptide-block-peptide block copolymer PBLG₅₀-K was prepared and characterized as described previously.¹⁷⁻¹⁹ Influenza hemagglutinin (HA) antigen (H3N2 Wisconsin strain) was obtained from Solvay (Weesp, The Netherlands). Bovine serum albumin (BSA) was purchased from Merck (Darmstadt, Germany). ELISA plates were obtained from Greiner (Alphen a/d Rijn, The Netherlands). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (γ chain specific), IgG1 (γ 1 chain specific) and IgG2a (γ 2a chain specific) were ordered from Southern Biotech (Birmingham, USA). Chromogen 3,3',5,5'-tetramethylbenzidine (TMB), substrate buffer for ELISA, GM-CSF and interleukin-4 (IL4), were provided by Biosource-Invitrogen (Breda, the Netherlands). Hank's Balanced Salt Solution (HBSS), fetal bovine serum (FCS) and all culture media, including penicillin/streptomycin (PEST) and trypsin were supplied from Gibco (Invitrogen, Carlsbad, CA). Nimatek® (100 mg/ml Ketamine, Eurovet Animal Health B.V., Bladel, The Netherlands) and Rompun® (20 mg/ml Xylazine, Bayer B.V., Mijdrecht, The Netherlands) were obtained from the pharmacy of Leiden University Medical center. 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), dichloromethane, dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other chemicals were acquired from Sigma- Aldrich (Zwijndrecht, NL), unless stated otherwise.

PBLG₅₀-K polymersomes were prepared by a solvent evaporation method as was previously described.¹⁹ Briefly, PBLG₅₀-K (0.02 μ mol) was dissolved in 2 ml tetrahydrofuran (THF) in a 50 ml round flask, then 3 ml of HEPES sucrose buffer (Hepes 20 mM, sucrose 10% (w/w), pH 7.4) were added all at once to the polymer solution, and the mixture was homogenized by vortexing for 1 minute (200 rpm). Finally, the THF was removed by rotary evaporation at 30 kPa, 25°C for 10 minutes. HA loaded polymersomes were prepared by adding the HA stock solution (453 μ g.mL⁻¹) to the preformed PBLG₅₀-K polymersome suspension (100 μ g.mL⁻¹), resulting in a final HA concentration varying between 2.5 to 50 μ g.mL⁻¹.

Particle size distributions were determined by means of dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments). The zeta potential of the particles was measured by laser Doppler velocimetry on the same instrument. The PBLG₅₀-K concentration was determined with a BCA protein assay (Pierce) according to the manufacturer's instructions, using albumin standard.

Transmission electron microscopy (TEM) was conducted on a JEOL 1010 instrument with an accelerating voltage of 60 kV. Samples for TEM were prepared by placing 5 μ L of solution on carbon-coated copper grids. After 5 min, the droplet was removed from the edge of the grid. A drop of 2% phosphotungstic acid (PTA) or 2% osmium tetroxide (OsO₄) stain was applied and removed after 2 min. The

degree of HA association to the polymersomes was determined by filtration as described previously [28].

For this study we used labeled HA (IRDye® 800CW, Licor). Briefly, the polymersomes/HA complexes and free HA were filtered through polycarbonate membranes (Whatman, Nucleopore) of 0.1 μm pore size, using an extruder (T001 10 ml, Thermobarrel Extruder Lipex Biomembrane). Under these conditions the polymersomes are retained on the filter and free HA passes through. The amount of HA in the filtrate was quantified with an Infinite M100 microplate reader (Tecan).

Toxicity of the formulations on Caco-2 cells was assessed using the MTT method.[29] Caco-2 cells (10,000/well) were seeded in a 96-well plate (Nunc) and maintained for 2 days at 37 °C and 5% CO₂. After 48 h exposure to a range of concentrations of the PBLG₅₀-K polymersomes, the cells were washed twice with Hank's Balanced Salt Solution (HBSS) and incubated for 3 h with 0.5 $\mu\text{g.mL}^{-1}$ MTT in DMEM. Medium was removed and the purple formazan crystal was dissolved in 100 μL DMSO. Absorbance at 570 nm was measured using a μQuant ELISA plate reader (Biotek).

The immunogenicity study was achieved with female C57BL/6 mice, 8-weeks old at the start of the vaccination study, were purchased from Charles River, and maintained under standardized conditions in the animal facility of the Leiden/Amsterdam Center for Drug Research, Leiden University. The study was carried out under the guidelines compiled by the Animal Ethic Committee of The Netherlands. The mice received two subcutaneous injections of 200 μl vaccine: a prime (day 1) and a boost (day 21). We used two different HA dosages: 0.5 μg and 2 μg HA/injection. The antigen was either injected alone or mixed with polymersomes (100 $\mu\text{g.mL}^{-1}$). Blood samples were taken one day before prime and boost, and 3 weeks after the boost. IgG titers were determined by ELISA. The IgG subtype profile of influenza-specific antibodies was checked on day 20 and 42 by sandwich ELISA as previously described.[30] Briefly, ELISA plates (Greiner) were coated overnight at 4°C with 100 ng/well of influenza subunit antigen (H3N2) in coating buffer (0.05 M sodium carbonate/bicarbonate, pH 9.6). Plates were subsequently washed twice with PBS containing 0.05% Tween 20, pH 7.6 (PBST) and then blocked by incubation with 1% (w/v) BSA in PBST for 1 h at 37°C. Thereafter the plates were washed three times with PBST. Two-fold serial dilutions of sera from individual mice were applied to the plates and incubated for 2 h at 37°C. Plates were incubated with HRP-conjugated goat antibodies against either mouse IgG, IgG1 or IgG2a (Invitrogen) for 1.5 h at 37°C. After washing, plates were incubated with TMB and the reaction was stopped with sulfuric acid (2M). The detection was done by measuring optical density at 450 nm. Antibody titers were expressed as the reciprocal of the sample dilution that corresponds to half of the maximum absorbance at 450 nm of a complete s-shaped absorbance–log dilution curve. Hemagglutination inhibition (HI)

titers in serum were determined as described by Amorij et al. [31] Briefly, serum was inactivated at 56°C for 30 min. In order to reduce nonspecific hemagglutination, 25% kaolin suspension was added to inactivate sera. After centrifugation at 1,200×g, 50 µL of the supernatant was transferred in duplicate to 96-well round bottom plates (Greiner) and serially diluted twofold in PBS. Then, four hemagglutination units of A/Wisconsin influenza inactivated virus were added to each well, and the plates were incubated for 40 min at room temperature. Finally, 50 µL of 1% guinea pig red blood cells were added to each well and incubated for 2 h at room temperature. The highest dilution capable of preventing hemagglutination was scored as the HI titer. Antibody and HI titers were logarithmically transformed before statistical analysis.

Unpaired Student's t-test analysis was performed for each antigen dosage, in order to demonstrate significant differences between the two experimental groups (HA alone and the polymersome/HA mix). The statistical analysis was carried out using Prism (Graphpad) and a p value less than 0.01 was considered to be significant

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Chapter 4

Peptide amphiphile nanoparticles enhance the immune response against a CpG adjuvanted influenza antigen

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* Authors contributed equally

Abstract

We developed a new class of polypeptide-b-peptide amphiphiles (PbP-A), based on a hydrophilic peptide conjugated to a hydrophobic poly- γ -benzyl-L-glutamate (PBLG) block. These peptide amphiphiles are able to self-assemble into nanostructures. In this study we have shown that properties such as size, morphology and surface charge of the resulting nanoparticles can be tuned. These polypeptide nanoparticles were tested for their adjuvant capacity. Immunogenicity of the hybrid assemblies was tested in vivo and showed an improvement of the immune response for the influenza antigen co-administered with polypeptide nanoparticles or CpG containing polypeptide nanoparticles. This advanced formulation demonstrated enhanced efficacy when compared to HA alone, or with commonly used commercial adjuvant "Alum".

Peptide and protein-derived materials have come into the spotlight for supramolecular chemists in recent years. Sequence-depending, peptides can fold into secondary structures such as β -sheets, α -helices or random coils that can further direct the hierarchical self-assembly of these macromolecules.[1] Over the last decade, self-assembly of peptide amphiphiles has been studied extensively and more recently as potential biomaterials.[2] These self-assembling peptide amphiphiles can be divided into three classes: [3] (a) amphiphilic peptides composed solely of a specific amino acid sequence [4] (b) hydrophilic peptides conjugated to a hydrophobic anchor like alkyl chains[5] and lipids,[6] and (c) hybrid peptide-polymers.[7] Conjugation of a peptide to a hydrophobic anchor strongly affects the supramolecular assembly and various morphologies have been reported, such as spherical and worm-like micelles, bicelles and vesicles.[8] These peptide amphiphile-based materials have found several potential applications in drug delivery,[9] in vivo imaging,[10] and regenerative medicine.[11] Only recently these materials have entered the field of vaccine research[12] where self-assembled peptides have been shown to enhance immune responses by acting as an adjuvant. Pioneering work by Collier and team demonstrated the capacity of self-assembling peptide motifs to provoke an immune response in mouse models against conjugated ovalbumin (OVA) peptide or protein antigens.[13] Tirrell and coworkers used peptide amphiphiles based micelles as a “self-adjuvanting” vaccine.[12] A dialkyl tail was conjugated to a peptide containing a cytotoxic T-cell epitope derived from the model tumor antigen OVA. These initial studies aimed at understanding the in vivo immune modulation using these micellar peptide-based materials. In both studies an enhanced Th2 response was observed, while the Th1 response was rather low. In our laboratory we have a long-term interest in developing effective adjuvants for influenza hemagglutinin (HA). Ideally, a viral vaccine should induce, besides antibodies, a substantial Th1 immune response, as this helps to protect against intracellular pathogens. Optimization of peptide amphiphile-based vaccines can be achieved by varying the amino acid sequence or the hydrophobic element, or by the addition of low molecular weight immune modulators.[14]

In order to improve cellular delivery, antigen-containing particles have been decorated with cell-penetrating peptides such as the TAT peptide.[15] Its arginine rich amino acid sequence results in a positive surface charge. Cationic nanoparticles (NP) generally show better efficacy for enhancing the immunogenicity of the carried antigen than neutral or negatively charged particles, presumably due to an enhanced interaction between the negatively charged cell membranes and the cationic particles.[16] [17] For example, poly(ethylene glycol)-polybutadiene polymersomes functionalized with TAT peptides successfully enhanced the cellular delivery of these NP to antigen-presenting cells (APC).[18] Furthermore, in order to enhance and modulate the immune response towards a higher Th1 response, co-delivery of an immune potentiator such as the TLR9 ligand CpG (bacterial cytosine phosphodiester guanine oligomer) is a promising approach.[19] CpG helps to

initiate a rapid innate immune response characterized by the secretion of a variety of cytokines, for example interferon- γ . [20]

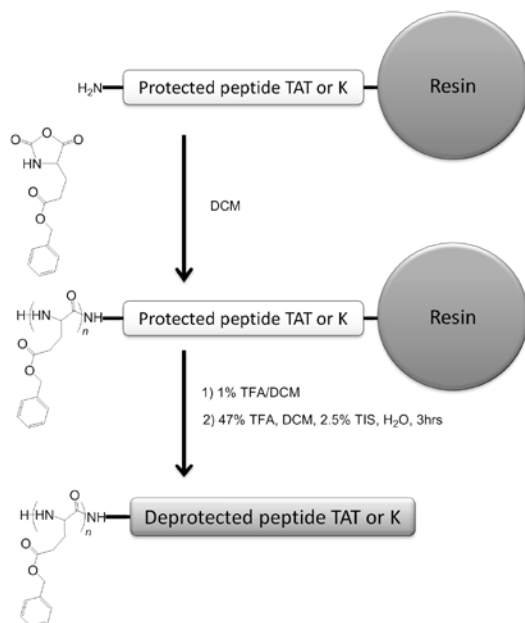


Fig. 1: Synthesis of PBLG-TAT and PBLG-K using solid phase peptide synthesis followed by N-carboxyanhydride ring-opening polymerization initiated from the N-terminal amine of the resin bound peptide.

We have previously reported a new class of polypeptide-b-peptide amphiphiles (PbP-A), based on a hydrophilic peptide block (denoted peptide “K”) with a specific amino acid sequence conjugated to a hydrophobic poly- γ -benzyl-L-glutamate (PBLG) block.[14b] [21] [22] In this design peptide “K” is able to form a coiled-coil motif with the complementary peptide “E”. These peptide amphiphiles self-assemble into micellar or vesicular structures depending on their composition. Control over both shape and size of the assemblies was achieved utilizing this coiled-coil motif[14b, c] The efficacy of peptide amphiphile PBLG₅₀-K [amino acid sequence peptide “K”; (KIAALKE)₃] was tested as a vaccine delivery system for an HA influenza subunit vaccine.[23] Injection of HA mixed with PBLG₅₀-K NP in adult mice resulted in an enhanced IgG1 antibody response against HA and an increased hemagglutination inhibition (HI) titer as compared to HA alone.[23] Unfortunately, the Th1 response remained low, as measured by the IgG2a titer. These initial results showed that the PbP-A NP might be used as an adjuvant, however a redesign is required to evoke a strong Th1 response in order to create an effective viral vaccine.

In this contribution, we aimed to design an effective vaccine containing two key features: 1) efficient delivery and uptake of the antigen (HA) by APC and 2) the incorporation of an immune potentiator that modulates the immune response towards an enhanced Th1 response. We designed PbP-A using the peptide “TAT” and peptide “K” sequence as hydrophilic head group for cellular delivery as well as to capture CpG by ionic interactions. PBLG-K was also part of this formulation enabling functionalization of the resulting NP through specific coiled coil peptide binding in future studies.[14c] PBLG-TAT and PBLG-K mixed with CpG were used to form NPs and these NPs were used to adsorb HA on the surface. This approach enables the co-delivery of the antigen together with an immune modulator aiming to induce a strong immune response.[24] The NP were examined in vitro for their capability of stimulating dendritic cells (DC) and their immunogenicity was studied in vivo by subcutaneous administration in mice and compared with that of HA formulated with aluminum salt $[\text{Al}(\text{OH})_3]$, a frequently used adjuvant in commercial vaccines.

Synthesis of the polypeptide-b-peptides was performed as shown in Fig. 1. Peptides “TAT” [Amino acid sequence: GLRKKRRQRRR] and “K” $[\text{G}(\text{KIAALKE})_3]$ were synthesized on a sieber amide resin using standard Fmoc solid phase peptide synthesis protocols. The N-terminal amine was used to initiate solid phase ring opening polymerization (ROP) of γ -benzyl-L-glutamate N-carboxyanhydride (BLG-NCA). The reaction was carried out in dichloromethane (DCM) under an inert atmosphere for two days to obtain resin-bound polypeptide-b-peptides. The resin was washed thoroughly with DCM to remove any residual homopolymers formed during the polymerization and the protected polypeptide-b-peptides were released from the solid support using 1:99 (v/v) TFA/DCM (TFA; trifluoroacetic acid) for two minutes in several fractions with subsequent precipitation in cold methanol. Polypeptide-b-peptides with high molecular weight are cleaved first and therefore macromolecules with different molecular weight can be separated. The purity of each fraction was ascertained with gel permeation chromatography (GPC) and fractions with a similar polydispersity index (PDI 1.3-1.4) and molecular weight were combined. The protecting groups were removed from the hydrophilic peptide sequence using a mixture of TFA/DCM/water/TIS (TIS; triisopropylsilane). Under the used conditions, the benzyl protecting groups of the PBLG block are unreactive. The resulting product was precipitated in cold methanol and washed several times resulting in the desired amphiphilic polypeptide-b-peptides. The removal of the protecting groups from the hydrophilic peptide and the degree of polymerization (DP) of the hydrophobic PBLG block were determined by ^1H -NMR analysis in DMF-d_7 at 60°C . The peak integral of the benzylic protons in the PBLG block was compared with the integral arising from the leucine and isoleucine methyl protons of the K or TAT block and revealed that the average degree of polymerization for the PBLG block was 30 for both peptide amphiphiles (PBLG₃₀-K and PBLG₃₀-TAT, Fig. S2 Supporting Information). The average molecular weights of the polymers (Table 1)

determined using ^1H -NMR spectroscopy are in good agreement with the data obtained by GPC.

Table 1: Molecular characteristics of the peptide compounds used in this study

Molecules	Structure ^{a)}	M_n [g.mol^{-1}]	PDI ^{d)}
K	G(KIAALKE)-NH ₂	2335.8 ^{b)}	
TAT	G(LRKKRRQRRR)-NH ₂	1508.8 ^{b)}	
PBLG ₃₀ -K	PBLG ₃₀ -G(KIAALKE)-NH ₂	8906 ^{c)} /9912 ^{d)}	1.4
PBLG ₃₀ -TAT	PBLG ₃₀ -G(LRKKRRQRRR)-NH ₂	8079 ^{c)} /10301 ^{d)}	1.3

^{a)} The sequence for the peptide “K” and “TAT” are written using the one letter amino acid code. PBLG; poly(γ -benzyl L-glutamate). ^{b)} Determined by MALDI-TOF mass spectrometry. ^{c)} Determined by comparing ^1H NMR spectroscopy. ^{d)} Determined for protected polypeptides using GPC calibrated with polystyrene standards.

Peptide amphiphile NP were prepared by the rapid water addition-solvent evaporation (WASE) method,[22] as this procedure is fast and has been proven to be compatible with in vivo applications.[23] Four NP formulations were designed with different physicochemical properties (Table 2). Two formulations, NP1 and NP2, were composed of PBLG₃₀-K and PBLG₃₀-TAT, respectively. The third formulation, NP3, consisted of a PBLG₃₀-TAT:PBLG₃₀-K (9:1) mixture, combining peptide “TAT” to enhance cellular uptake and peptide “K”. Moreover, this will enable future modification of the NP through coiled-coil formation with the complementary peptide “E”, allowing further surface functionalization if desired.[7c] The 9:1 molar ratio of peptide amphiphiles PBLG₃₀-TAT:PBLG₃₀-K was selected after an initial screen by zeta potential and dynamic light scattering (DLS) measurements, which revealed that it is the most cationic mixture of all formulations that assembled into NP with reproducible size (data not shown). We expected that triggering the immune response by an immune modulator was desired and thus formulation NP4 contained the TLR9 ligand CpG oligonucleotide (10 $\mu\text{g.mL}^{-1}$). For this formulation, CpG in HEPES sucrose buffer was added to the THF solution containing peptide amphiphile PBLG₃₀-TAT:PBLG₃₀-K (9:1), ensuring the efficient incorporation of CpG into the PBLG₃₀-TAT/K (9:1) polypeptide NP based on attractive electrostatic interactions. All NP formulations were subsequently mixed with HA and used in all in vivo and in vitro experiments (Table 2). The final HA concentration and NP concentration in all formulations were always 10 $\mu\text{g.mL}^{-1}$ and 6.77×10^{-6} M, respectively.

The self-assembled NPs were first characterized by DLS and zeta potential measurements. DLS revealed that the NP1 and NP2 formulations had comparable hydrodynamic diameters (Z_{av} 200-240 nm), with a zeta potential of -30 mV and +20 mV, respectively (Fig. 2A). Immune modulator CpG was added during the self-assembly of NP3 to obtain NP formulation NP4. This resulted in a drop of the zeta potential from +20 mV (NP3) to -41 mV in presence of CpG (NP4), and also resulted in smaller NP (Z_{av} of 216 nm and 160 nm, respectively). This decrease in both size and zeta potential is indicative of the tight binding of the negatively charged CpG oligonucleotides with the positively charged NP. The CpG loading was determined by measuring the adsorption of fluorescently-labeled CpG (ODN 1826-FITC, Invitrogen), showing a CpG loading efficiency between 55% and 60%. [19c]

Table 2: Formulations used in *in vitro* and *in vivo* studies, hereafter referred to as annotated.

	Short name	Composition	Z_{ave}	pdi
Nanoparticle formulations without HA	NP1	PBLG ₃₀ -K	210 ± 42	0.07 ± 0.03
	NP2	PBLG ₃₀ -TAT	233 ± 69	0.13 ± 0.09
	NP3	PBLG ₃₀ -TAT: PBLG ₃₀ -K (9:1)	216 ± 45	0.14 ± 0.07
	NP4	PBLG ₃₀ -TAT: PBLG ₃₀ -K (9:1) + CpG	160 ± 68	0.09 ± 0.03
Nanoparticle formulations with HA	HA/NP1	HA/PBLG ₃₀ -K	753 ± 393	0.24 ± 0.04
	HA/NP2	HA/PBLG ₃₀ -TAT	799 ± 314	0.31 ± 0.17
	HA/NP3	HA/PBLG ₃₀ -TAT: PBLG ₃₀ -K (9:1)	1210 ±	0.33 ± 0.13
	HA/NP4	HA/PBLG ₃₀ -TAT: PBLG ₃₀ -K (9:1) + CpG	430 688 ± 457	0.31 ± 0.07
Control formulations		HA HA/CpG HA/Al(OH) ₃		

Z_{ave} ; Z-average hydrodynamic diameter and pdi; polydispersity index obtained from dynamic light scattering measurements (n=3)

Addition of HA to the preformed NP1-4 resulted in a size increase and a significant drop in zeta potential for the two cationic nanoparticles NP2 and NP3 (Fig. 2 A). HA is a negatively charged protein that interacts with the cationic surface of the NP, resulting in the drop of the zeta potential. The adsorption efficiency of HA correlated well with the surface charge of the NP. A fluorescent labeled HA (IR Dye 800CW, Licor) was used to quantify the amount of HA associated with each NP formulation. [23] The highest amount of HA was adsorbed on cationic NP2 and NP3 (adsorption efficiency 79% and 76%, respectively), while HA association with negatively charged NP1 and NP4 was significantly lower (39% and 36%, respectively) (Fig. 2 B).

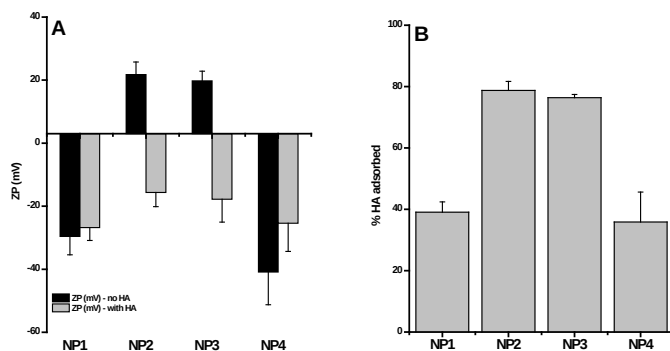


Fig. 2: **(A)** Zeta potential (ZP) of polypeptide-*b*-peptide NP1-4; before (■) and after HA adsorption, HA/NP1-4 (□). Each bar represents the average of 3 different batches. **(B)** Percentage of the fluorescently labeled HA associated with HA/NP1-4. Error bars represent the standard deviation (n=3).

The morphology of the resulting assemblies was visualized using cryo electron microscopy (Cryo-EM). Cryo-EM imaging showed that NP1 self-assembled into micellar structures of approximately 200 nm in diameter (Fig. 3 A). Self-assemblies of NP2 resulted in 20-30 nm micelles and aggregation of these micelles with a typical size of 200 nm (Fig. 3 B). Next, we studied the self-assembling behavior of NP3 and we found structures resembling to those observed for NP1 as well as NP2 (Fig. 3C). Interestingly, the addition of the negatively charged CpG resulted in a better defined and more homogeneous population of NP with a diameter around 200 nm (Fig. 3 D). Moreover, a clear contrast between (CpG-containing) NP4 (Fig. 3 D) and NP3 (without CpG) (Fig. 3 C) was found, most likely because of electrostatic interactions between the surface bound CpG and the positively charged polypeptide NP. As expected, the addition of HA to the four NP formulations induced aggregation as observed by DLS. Within these aggregates, the individual NP morphology and size were maintained (Fig. 3 F-I). We also observed 10-15 nm structures on the surface of NP which are similar to those observed for free HA in solution (Fig. 3 E), presumably due to association of HA with the NP surface.

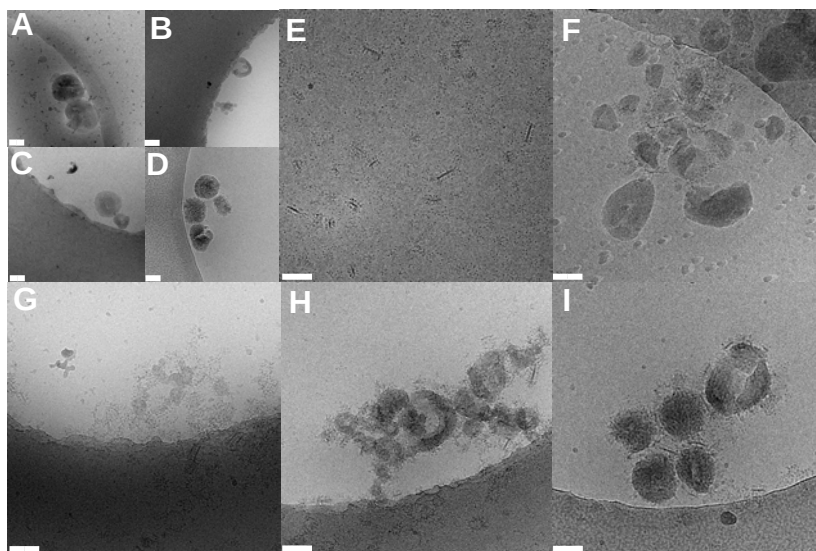


Fig. 3: Cryo-EM imaging of polypeptide-b-peptide NP. Plain NP: A) NP1, B) NP2, C) NP3, D) NP4; HA alone (E); NP mixed with $10 \mu\text{g}.\text{ml}^{-1}$ HA: F) HA/NP1, G) HA/NP2 H) HA/NP3, I) HA/NP4. Scale bar is 100 nm.

To assess the immunogenicity of the HA/NP formulations *in vivo*, these were injected subcutaneously in C57BL/6 mice. All mice received a first (prime) injection at day 0, and a second (boost) injection after 21 days with the same mixture. Serum was collected on day 20 and 42. HA-specific serum IgG1 (Fig. S3, Supporting Information) and IgG2a/c titers (Fig. 4 A, B) were assessed after the first (prime) and the second (boost) immunization, and HI titers, as a measure for the level of functional antibodies, were measured after the boost (Fig. 4 C). Interestingly, after boost, all HA/NP formulations induced superior levels of IgG1 compare to the antigen alone, which is consistent with the observations made in our first paper (Fig. S3-A, Supporting Information). Although, important differences were noticed, after both, prime and boost injections of the HA/NP4 formulation, as they elicited high levels of IgG2a/c compared with all the other groups, including HA mixed with the commercially available adjuvant $\text{Al}(\text{OH})_3$ (Fig. 4 A, B), resulting in a shift of the IgG2a/c/IgG1 ratio (Fig. S3-B, Supporting Information). Increased levels of IgG2a/c indicate an enhanced Th1 response, which is in line with the superior level of interferon- γ secretion from the spleen cells collected from the mice injected with the HA/NP4 formulation (Fig. S4, Supporting Information). Moreover, the sera from mice immunized with HA/NP4 had an enhanced HI titer compared with all other groups with a significant difference compared with the non-adjuvanted HA control ($p < 0.05$) (Fig. 4 C).

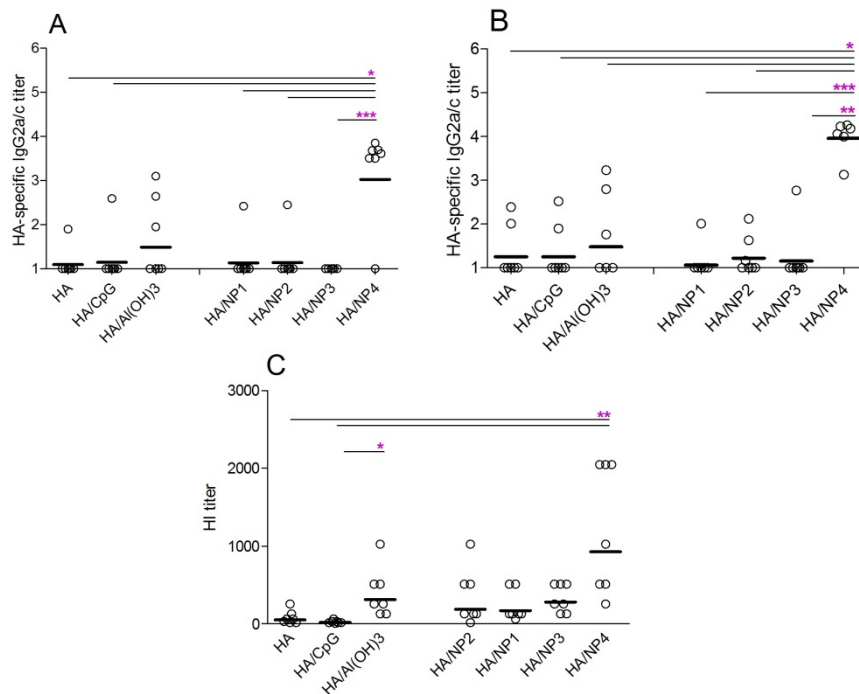


Fig. 4: Immune response in mice after subcutaneous injection of 2.0 µg HA, free or mixed with CpG or Al(OH)₃ or NP formulations: HA-specific serum IgG2a/c titers after prime (A) and boost (B), and HI titers after boost (C). For panels A & B each dot represents the log serum titer of an individual mouse (non-responding mice were given an arbitrary titer of 10) bars represent the geometric mean. For panel C, each dot represents the log HI titer in serum of an individual mouse and bars represent the geometric mean. Significant difference between the groups were indicated with *, ** and *** (Respectively: $p < 0.05$, $p < 0.01$, $p < 0.001$).

Stimulation of antigen uptake by APC is important to generate a robust antiviral immune response; however, it is not the only prerequisite. Adjuvant systems should also enable a potent stimulation of the innate immune response. Therefore DC activation by the HA/NP was studied. DC derived from monocytes isolated from human donor blood were used to measure the upregulation in vitro of two maturation surface markers (MHCII and CD86) upon stimulation by the HA/NP. Cells exposed to the different HA/NP formulations showed upregulated MHCII marker levels as compared with HA. However, the increase was only significant when the DC were stimulated by NP3 containing the TAT peptide sequence. For the CD86 markers a similar trend was observed: the CpG-containing NP (HA/NP4) induced a significant upregulation as compared with HA alone. In contrast, injection of a HA/CpG solution did not yield an increase in CD86 marker expression (Fig. 5), showing the cooperative effect of CpG and PbP-A NP.

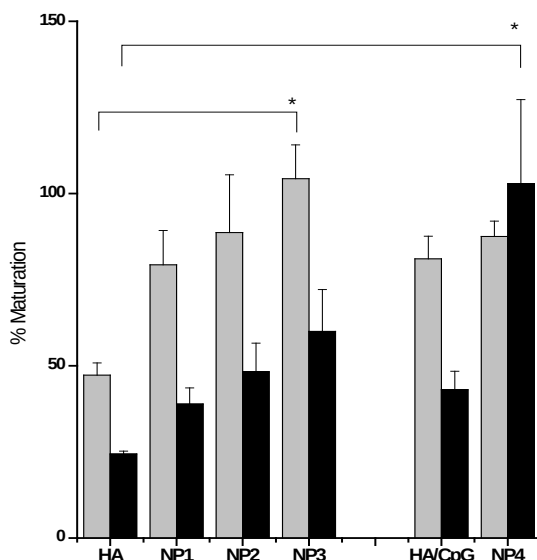


Fig. 5: Upregulation of the MHC II (□) and CD86 (■) DC maturation markers induced by the various polypeptide-b-peptide NP formulations versus free HA; relative to the 100 ng.mL⁻¹ LPS control group. Error bar represents SEM (n=3). Significant difference between the polypeptide-b-peptide NP formulation and free HA are indicated with * ($p < 0.05$).

In summary, we have synthesized two new peptide amphiphiles, PBLG₃₀-TAT and PBLG₃₀-K, and demonstrated their ability to self-assemble into cationic NP in various formulations. Co-delivery of CpG with HA/NP (HA/NP4) resulted in an enhanced immune response in mice against the HA antigen, towards a Th1 response (as reflected by elevated levels of IgG2a/c). A Th1 response is important to target intracellular pathogens, thus opening an avenue for vaccine development against viral pathogens. This formulation also demonstrated a higher immunogenicity when compared with the commonly used adjuvant Al(OH)₃. Further studies are required to obtain a better understanding of the self-assembly of the polypeptide amphiphiles and the effect of the resulting NP on the immunogenicity of associated antigens.

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Supplementary information

1. Abbreviations

ANOVA, analysis of variance; APC, antigen-presenting cells; BLG-NCA, γ -benzyl L-glutamate N-carboxyanhydride; BSA, bovine serum albumin; CpG, bacterial cytosine phosphodiester guanine oligomer; DIPEA, N,N-diisopropylethylamine; DC, dendritic cells; DCM, dichloromethane; DIPEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; NMP, N-methyl-2-pyrrolidone; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; Fmoc, 9-fluorenylmethoxycarbonyl; GPC, gel permeation chromatography; HA, hemagglutinin; HBSS, Hank's balanced salt solution; HCTU, O-(6-chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; $^1\text{H-NMR}$, ^1H nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; HRP, horseradish peroxidase; Ig, immunoglobulin; LC-MS, liquid chromatography-mass spectrometry; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMP, 1-methyl-2-pyrrolidinone; NP, nanoparticles; PbP-A, polypeptide-b-peptide amphiphiles; PDI, polydispersity index; THF, tetrahydrofuran; Cryo-EM, cryogenic electron microscopy; TFA, trifluoroacetic acid; TIS, triisopropyl silane.

2. Materials and Methods

Fmoc protected amino acids and peptide synthesis grade DMF, DCM and NMP solvents were obtained from BioSolve Ltd. Sieber amide resin was purchased from Iris Biotech GmbH, Germany. Influenza hemagglutinin antigen (H3N2 Wisconsin strain) was obtained from Solvay (Weesp, The Netherlands). BSA was purchased from Merck (Darmstadt, Germany). ELISA plates were obtained from Greiner (Alphen a/d Rijn, The Netherlands). HRP conjugated goat anti-mouse IgG (γ chain specific), IgG1 (γ 1 chain specific) and IgG2a (γ 2a chain specific) were ordered from Southern Biotech (Birmingham, USA). Chromogen 3,3',5,5'-tetramethylbenzidine (TMB), GM-CSF and interleukin-4 (IL4), were provided by Biosource-Invitrogen (Breda, The Netherlands). HBSS, FCS and all culture media, including penicillin/streptomycin (PEST) and trypsin were supplied from Gibco (Invitrogen, Carlsbad, CA). Nimatek® (100 mg.mL⁻¹ ketamine, Eurovet Animal Health B.V., Bladel, The Netherlands) and Rompun® (20 mg.mL⁻¹ xylazine, Bayer B.V., Mijdrecht, The Netherlands) were obtained from the pharmacy of Leiden University Medical Center. HEPES, DCM, DMSO, MTT and other chemicals were acquired from Sigma-Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

3. Solid-Phase Peptide Synthesis of Peptide “K” and “TAT”

Peptide “K” and “TAT” were prepared following standard Fmoc chemistry on sieber amide resin using Liberty-1 (CEM corporation, Matthews, NC, United States) microwave assisted automated peptide synthesizer. Scale of synthesis was 0.25

mmol and activation of amino acid derivatives was achieved using HCTU/DIPEA. After the peptide “K” and “TAT” were prepared, the resin was removed from the reaction vessel, The amount of successfully synthesized “K” and “TAT” was estimated using the mass added to the resin during the synthesis, and by integration of HPLC peaks from an LC-MS run of a test cleavage of 10 mg of resin-bound peptide using TFA/TIS/water; 95/2.5/2.5 (v/v).

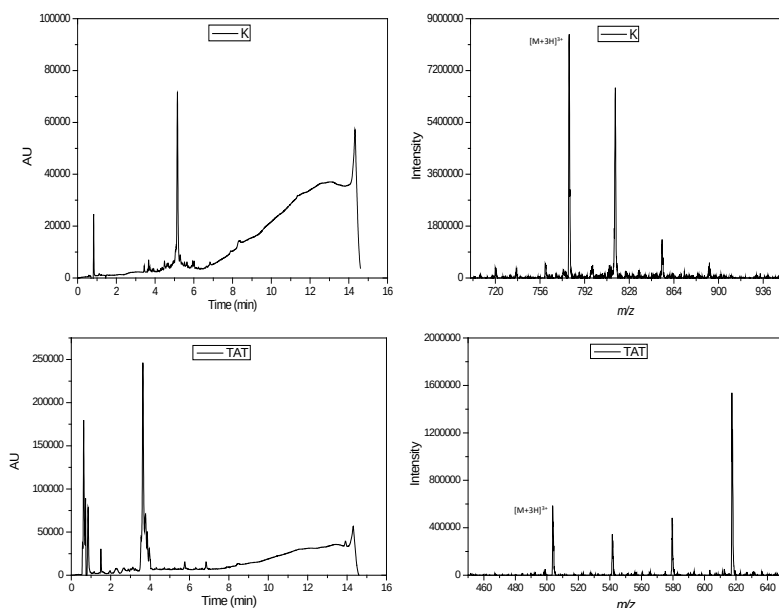


Fig. S1: HPLC traces of peptide K (with 67% purity) and peptide TAT (with 60% purity) along with mass observed for main peak and corresponding TFA adducts.

4. Solid-Phase Synthesis of Poly(γ -benzyl L-glutamate)-block-K (PBLG-K) and Poly(γ -benzyl L-glutamate)-block-TAT (PBLG-TAT)

γ -benzyl L-glutamate N-carboxyanhydride was synthesized and characterized as described previously.[1] The poly(γ -benzyl L-glutamate) block was synthesized on the solid phase using one-pot NCA polymerization of BLG-NCA initiated from the N-terminus amine of resin-bound peptide “K” or “TAT”. The resin-bound peptide was dried overnight in vacuum oven at 37 °C, and then placed in argon atmosphere for 5 h. Next, under an argon atmosphere, the peptide bound 0.25 mmol resin was swollen in dry DCM. Subsequently the appropriate weight of NCA (determined from the mass loading and HPLC peak integration) was added. [1] The flask was shaken for 48 h. The resin was drained and washed extensively with DCM to wash away the homopolymer formed during the reaction. The yields of the resin-bound block copolypeptides were 50-60%. While keeping the side chains of the peptides intact, PBLG-Peptide “K” or “TAT” hybrid block copolymer was cleaved from the resin using

TFA:DCM (1:99) (v/v) for 2 min, 10 times. Each cleavage mixture was precipitated in cold methanol and centrifuged to obtain pellets. Pellets were washed 3 times using cold methanol and vacuum-dried. The protecting groups of peptide “K” and “TAT” were removed using TFA:DCM:water:TIS; 47.5:47.5:2.5:2.5 (v/v) for 3 h. The product was precipitated drop wise in cold methanol and centrifuged. Further 3 washes of cold methanol were performed. The product was vacuum dried to obtain both block copolymers in 20-25% in yield.

5. Characterization of the PBLG-K and PBLG-TAT Polypeptide-b-Peptides

Molecular weights and polydispersity index (PDI) of the protected PBLG-K and PBLG-TAT PbP-A were determined using gel phase chromatography (GPC). GPC analyses were performed using a Shimadzu system and a Polymer Laboratories column (3M-RESI-001-74, 7.5 mm diameter, 300 mm length). To prevent aggregation, DMF was used as the eluent at 60 °C with a flow rate of 1 mL.min⁻¹. Molecular weights were calibrated using polystyrene standards.

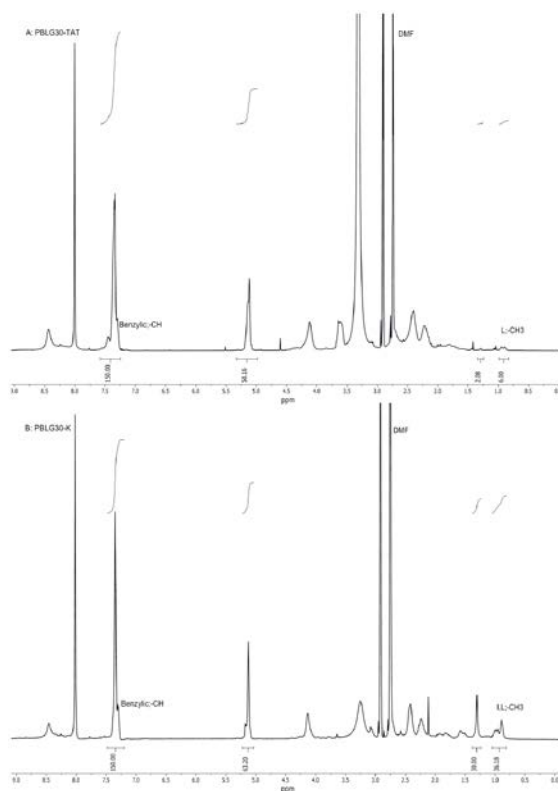


Fig. S2.: ¹H NMR spectrum of PBLG₃₀-TAT (A) and PBLG₃₀-K (B) in N,N-dimethylformamide-d₇ (DMF-d₇) at 60 °C. By comparing benzylic -CH with respective leucine or leucine/isoleucine -CH₃ peak, an average degree of polymerization of 30 was estimated for both block copolymers. The high intensity signal at 3.3 ppm for PBLG₃₀-TAT is due to residual water.

6. Preparation of PBLG₃₀-K and PBLG_{PBLG}-TAT Nanoparticles

The nanoparticles (NP) were prepared by a solvent evaporation method as described previously.[2] Briefly, the polymer (0.02 μmol) was dissolved in 2 ml THF in a 50 ml round flask, then 3 ml of HEPES sucrose buffer (HEPES 20 mM, sucrose 10% (w/w), pH 7.4) was added all at once to the polymer solution, and the mixture was homogenized by vortexing for 1 minute (200 rpm). Immediately, the THF was removed by rotary evaporation at 30 kPa, 25°C for 10 minutes. The CpG loaded NP were prepared by using the same method, but with HEPES sucrose buffer containing 10 $\mu\text{g.mL}^{-1}$ CpG. Finally, the HA/NP were prepared extemporaneously, by mixing (prior to the injection) the proper volumes of HA stock solution (453 $\mu\text{g.mL}^{-1}$) to the preformed polypeptide NP suspension (Unbound HA was not removed and the final HA concentration was 10 $\mu\text{g.mL}^{-1}$).

7. Size, Charge and Morphology Characterization of the Polypeptide Nanoparticles

Particle size distributions were determined by means of dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments). The zeta potential of the particles was measured by laser Doppler electrophoresis on the same instrument.

Samples for cryo-EM were concentrated by centrifuging using Amicon Ultra, Ultracel®-100K (regenerated cellulose 100,000 MWCO, Millipore, Ireland) at room temperature. HA was added to the samples just before the vitrification process. Typically 10 $\mu\text{g.mL}^{-1}$ concentration of HA is used during the experiments therefore for imaging concentration of HA was adjusted to the resulting centrifuged sample. Sample stability was verified by DLS and TEM. Cryo-EM measurements were performed on a FEI Technai 20 (type Sphera) transmission electronmicroscope or on a Titan Krios (FEI). A Gatan cryo-holder operating at $\sim -170^\circ\text{C}$ was used for the cryo-EM measurements. The Technai 20 was equipped with a LaB6 filament operating at 200 kV, and the images were recorded using a 1k $\times$ 1k Gatan CCD camera. The Titan Krios was equipped with a field emission gun (FEG) operating at 300 kV. Images were recorded using a 2k $\times$ 2k Gatan CCD camera equipped with a post column Gatan energy filter (GIF). The sample vitrification procedure was carried out using an automated vitrification robot: a FEI Vitrobot Mark III. TEM grids, both 200 mesh carbon coated copper grids and R2/2 Quantifoil Jena grids, were purchased from Aurion. Copper grids bearing lacey carbon films were homemade using 200 mesh copper grids from Aurion. Grids were treated with a surface plasma treatment using a Cressington 208 carbon coater operating at 25 A for 40 s prior to the vitrification procedure.

8. Quantification of HA and CpG Associated with the Nanoparticles

Estimation of the HA adsorption onto the nanoparticles was done as described previously [3]. For this study we used labeled HA (IRDye® 800CW, Licor). Briefly, the HA/NP complexes and the free HA were filtered through polycarbonate membranes (Whatman, Nucleopore) of 0.2 μm pore size, using an extruder (T001 10

ml, Thermobarrel Extruder Lipex Biomembrane). Under these conditions the NP are retained on the filter and HA passes through. The amount of HA, remaining in the filtrate, was quantified with an Infinite M1000 microplate reader (Tecan).

The amount of CpG present in the NP was determined using fluorescently labeled analogs (10% of used CpG were labeled), as described previously [4]. Free TLR ligand was separated from the NP by filtration using an Amicon Ultra, Ultracel®-100K (Regenerated cellulose 100000 MWCO, Millipore, Ireland) and quantified using a FS920 fluorimeter (Edinburgh Instruments, Campus Livingston, UK).

9. DC Maturation Assay

Monocytes isolated from buffy coats (Sanquin, Amsterdam, The Netherlands) were cultivated to differentiate into immature DC, as described previously. DC were incubated for 48 h at 37°C in RPMI 1640, containing 500 U/ml GM-CSF and 100 U/ml IL4, with 100 µl volume of HA/NP suspension in 1 ml cell culture medium, and LPS (100 ng.mL⁻¹ cell culture medium) was used as a positive control. Cells were washed 3 times with PBS containing 1% (w/v) BSA and 2% (v/v) FBS and incubated for 30 min with a mixture of 50× diluted anti-HLADR-FITC or anti-CD86-APC (Becton Dickinson) on ice, to measure the expression of MHCII or CD86, respectively, on the surface of the DC. Cells were washed and expression of the surface markers was quantified by using flow cytometry (FACSCanto, BD Biosciences, San Jose, CA, USA). Live cells were gated based on forward and side scatter. The upregulation of the two surface markers by 100 ng.mL⁻¹ LPS was set as 100%. A minimum of 10,000 gated cells were analyzed in each experiment.

10. In vivo Experiments

Female C57-BL/6 mice, 8-weeks old at the start of the vaccination study, were purchased from Charles River Laboratories International, Wilmington, MA, USA, and maintained under standardized conditions in the animal facility of the Leiden Academic Centre for Drug Research, Leiden University. The study was carried out under the guidelines compiled by the Animal Ethic Committee of The Netherlands. The mice received two subcutaneous injections of 200 µl vaccine (2 µg HA/injection): a prime (day 1) and a boost (day 21). The antigen was either injected alone or mixed with NP to the final concentration of 10 µg HA/ml of NP solution. Unbound HA was not removed and each mouse received the same amount of HA and NP. Blood samples were taken one day before prime and boost, and 3 weeks after the boost. IgG titers were determined by ELISA.

The IgG subtype profile of influenza-specific antibodies was checked on day 20 and 42 by sandwich ELISA as previously described. Briefly, ELISA plates (Greiner Bio-One B.V., The Netherlands) were coated overnight at 4°C with 100 ng HA/well in coating buffer (0.05 M sodium carbonate/bicarbonate, pH 9.6). Plates were subsequently washed twice with PBS containing 0.05% Tween 20, pH 7.2 (PBST) and then blocked by incubation with 1% (w/v) BSA in PBST for 1 h at 37°C. Thereafter the plates were washed three times with PBST. Two-fold serial dilutions

of sera from individual mice were applied to the plates and incubated for 1.5 h at 37°C. Plates were incubated with HRP-conjugated goat antibodies against either mouse IgG1 or IgG2a (Invitrogen, The Netherlands) for 1 h at 37°C. After washing, plates were incubated with TMB and the reaction was stopped with sulfuric acid (2 M). The detection was achieved by measuring optical density at 450 nm. C57BL/6 mice express the *Igh1-b* gene, which encodes the IgG2c isotype rather than IgG2a. However, here we used an anti-IgG2a isotype (which cross-reacts with IgG2c[5] and titers are reported as IgG2a/c titers. Finally, antibody titers were expressed as the reciprocal of the sample dilution that corresponds to half of the maximum absorbance at 450 nm of a complete s-shaped absorbance–log dilution curve.

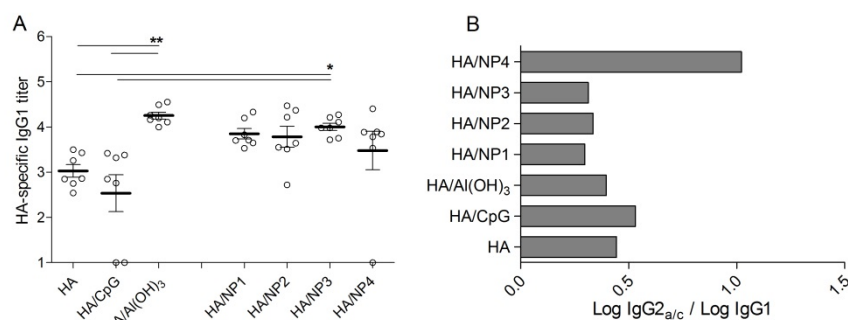


Fig.S3: Immune response in mice after subcutaneous injection of HA/NP; (A): HA-specific serum IgG1 titers after boost, each dot represents the log serum titer of an individual mouse (non-responding mice were given an arbitrary titer of 10) bars represent the geometric mean and significant difference between the groups were indicated with * and ** (Respectively: $p < 0.05$, $p < 0.01$). (B): Corresponding average Log IgG 2 a/c/Log IgG1 ratio, indicative of the quality of the immune response.

11. Hemagglutination Inhibition Assay

Hemagglutination inhibition (HI) titers in serum were determined as described previously. [3] Briefly, serum was inactivated at 56°C for 30 min. In order to reduce nonspecific hemagglutination, 25% kaolin suspension was added to inactivate sera. After centrifugation at 1,200×g, 50 μ L of the supernatant was transferred in duplicate to 96-well round-bottom plates (Greiner) and serially diluted twofold in PBS. Then, four hemagglutination units of A/Wisconsin influenza inactivated virus were added to each well, and the plates were incubated for 40 min at room temperature. Finally, 50 μ L of 1% guinea pig red blood cells (Harlan Scientific, The Netherlands) diluted in PBS, were added to each well and incubated for 2 h at room temperature. The highest dilution capable of preventing hemagglutination was scored as the HI titer.

All the data of the in vitro studies were analyzed with a one-way analysis of variance (ANOVA) with Bonferroni's post-test. Regarding the in vivo data, Antibody titers were logarithmically transformed before statistical analysis. A one-way ANOVA with

a Kruskal Wallis post-test analysis was performed in order to demonstrate significant differences between the experimental groups, the statistical analysis was carried out using Graphpad Prism software and a p value less than 0.05 was considered to be significant.

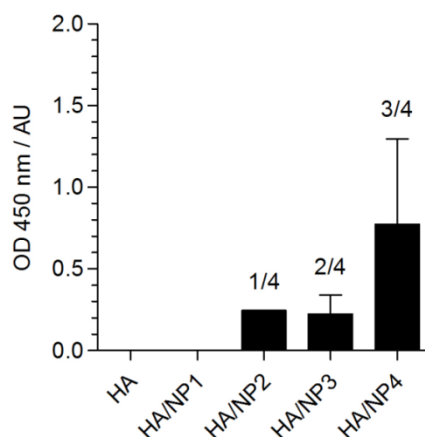


Fig.S4: IFN gamma levels secreted by spleen cells collected from mice exposed to HA alone and HA/NP formulations.

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Chapter 5

Cationic liposomes as adjuvants for influenza hemagglutinin: More than charge alone

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Abstract

Cationic liposomes are known as potent adjuvants for subunit vaccines. The purpose of this work was to study whether the content and the physicochemical properties of the positively charged compound affect the adjuvanticity of cationic liposomes. Cationic liposomes containing a cationic compound (DDA, DPTAP, DC-Chol, or eDPPC) and a neutral phospholipid (DPPC) were prepared by the film hydration-extrusion method and loaded with influenza hemagglutinin (HA) by adsorption. The liposomes were characterized (hydrodynamic diameter, zeta potential, membrane fluidity, HA loading) and their adjuvanticity was tested in mice. The formulations were administered twice subcutaneously and mouse sera were analyzed for HA-specific antibodies by ELISA and for HA-neutralizing antibodies by hemagglutination inhibition (HI) assay. First, the influence of cationic lipid concentration in the DC-Chol/DPPC liposomes (10 vs. 50 mol%) was investigated. The DC-Chol/DPPC (50:50) liposomes showed a higher zeta potential and HA loading, resulting in stronger immunogenicity of the HA/DC-Chol/DPPC (50:50) liposomes compared to the corresponding (10:90) liposomes. Next, we used liposomes composed of 50mol% cationic lipids to investigate the influence of the nature of the cationic compound on the adjuvant effect. Liposomes made of the four cationic compounds showed similar hydrodynamic diameters (between 100 and 170nm), zeta potentials (between +40 and +50mV), HA loading (between 55% and 76%) and melting temperatures (between 40 and 55°C), except for the DC-Chol liposomes, which did not show any phase transition. HA adjuvanted with the DC-Chol/DPPC (50:50) liposomes elicited significantly higher total IgG1 and IgG2a titers compared to the other liposomal HA formulations and non-adjuvanted HA. A similar trend was observed for the HI titers. These results show that the adjuvanticity of cationic liposomes depends on both the content and the physicochemical properties of the charged compound.

1. Introduction

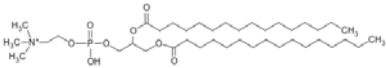
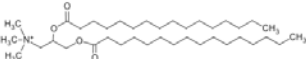
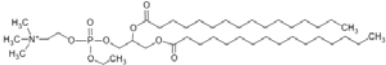
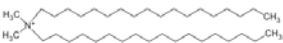
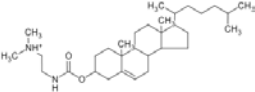
During the past few decades, new approaches for vaccination have been developed for the delivery of subunit vaccines based on (recombinant) purified proteins [1–3]. In general, subunit vaccines offer a better safety profile than live or inactivated vaccines, but are less immunogenic. The poor immunogenicity of subunit vaccines can be enhanced by the use of adjuvants such as colloidal aluminum salts (e.g., phosphate, hydroxide) or emulsions such as MF59 [4]. Depending on the nature of the adjuvant, it can enhance the immunogenicity of a vaccine via several mechanisms, for example, by creation of an antigen depot at the injection site, improvement of the antigen delivery to dendritic cells (DCs), or the induction of DC activation and the differentiation of T and B cells [5].

Liposomes have been studied as adjuvants for many decades [6–9]. From these previous studies, it emerges that the adjuvant effect of the liposomes depends on their physicochemical properties and may be related to prolonged release and protection of encapsulated antigen against the environment and enhanced uptake by DCs. It is generally accepted that cationic liposomes are more potent adjuvants compared to anionic and neutral liposomes [9]. Their adjuvant effect has been attributed to several mechanisms, such as non-specific cell damage (inducing inflammation) at the site of injection, formation of an antigen depot, and improved antigen uptake by antigen-presenting cells (APCs) through electrostatic interaction between the cationic liposomes and negatively charged groups on the surface of APCs. Cationic liposomes can both enhance and modulate the immune response. After antigen uptake by DCs, these cells mature, migrate and present the antigen on MHC II molecules to T helper (Th) cells, inducing two major subtypes: Th1 cells primarily involved in cellular immune responses and Th2 cells involved in humoral immune responses [10]. Interestingly, cationic liposomes have been shown to induce Th1-mediated responses, meaning potentially increased efficacy against intracellular pathogens [11] [12]. Many different amphiphilic cationic compounds have been tested for inclusion into liposomes and hold promise for vaccine delivery, such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [13], 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (eDOPC) [14], dimethyl dioctadecyl-ammonium (DDA) [15], and 3 β -[N-(N',N'-dimethylaminoethane)- carbamoyl] cholesterol (DC-Chol) [16]. It is very difficult to compare these vaccination studies as a wide range of conditions was used (e.g., different formulations, antigens routes of administration, immunization schemes, and read-out systems). The immunogenicity of different cationic liposomes has been compared systematically in only very few studies. For instance, Rosenkrands et al. [17] showed in a subcutaneous immunization study in mice that DDA liposomes mixed with an extract of lipids from the cell wall of *Bacillus Calmette-Guérin* (average hydrodynamic diameter = 738 nm) induced a strong Th1 response, whereas DC-Chol/BCG induced a Th2-biased response, and DOTAP/BCG liposomes only gave rise to a weak immune response. However, physicochemical

characteristics of the latter two formulations were not provided. Different results were obtained by Joseph et al. [18], who produced liposomes made of DC-Chol, DDA, DSTAP or DOTAP, mixed with DOPE (molar ratio of 1/1), and loaded with an influenza antigen. The relatively large hydrodynamic diameters ranged from 1 to 4 μm the zeta potentials from + 25 to + 83 mV. The results of an intranasal immunization study in mice showed that DC-Chol, DDA, and DSTAP liposomes induced a very low immune response, whereas DOTAP-based vaccines induced strong Th1 and Th2 responses [18]. Both the differences in liposome characteristics and the different routes of administration (subcutaneous vs. intranasal) could explain the different outcomes of these two studies. A recent study by Slütter et al. showed that the same cationic liposomes widely differ in immunogenicity depending of the route of administration [19]. The aim of this work was to study how the content and the physicochemical properties of the positively charged compound influence the adjuvant effect of cationic liposomes. To this end, we prepared cationic liposomes made of different cationic compounds but with similar physicochemical characteristics (particle size, zeta potential, bilayer rigidity), in association with an influenza HA antigen from a H3N2 A/Wisconsin strain. We selected three cationic compounds with a melting temperature (T_m) similar to that of DPPC [20]: DDA [21], DPTAP [18], and eDPPC [22] (see Table 1).

Furthermore, DC-Chol was studied as it also has shown immunogenic potential. Since no DC head group with a saturated lipid tail was available, it was included in our comparison, while recognizing that its characteristics will differ from the other cationic compounds with respect to both the head group and the hydrophobic tail. We aimed to prepare liposomes with a similar hydrodynamic diameter of about 200 nm, as it has been previously reported that nanoparticles below the size of 0.5 μm enhance the uptake by APCs [23] [24] or the INF γ secretion [25] better than larger particles (>500 nm).

Table 1: Phospholipids and cationic compounds used in this study.

Abbreviation	Name	Structure	T_m (°C)
DPPC (16:0)	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine		41 °C [20]
DPTAP (16:0)	1,2-dipalmitoyl-3-trimethylammonium-propane		45°C [22]
eDPPC (16:0)	1,2-diacyl- <i>sn</i> -glycero-3-ethylphosphocholine		42°C [23]
DDA (18:0)	Dimethyl dioctadecyl-ammonium		43°C [21]
DC-Chol	3 β -[N-(N',N'-Dimethylaminoethane)-carbamoyl] cholesterol		Not available

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-3-trimethylammonium-propane (DPTAP), 1,2- diacyl-sn-glycero-3-ethylphosphocholine (eDPPC), dimethyl dioctadecyl-ammonium bromide (DDA), and 3 β -[N-(N0,N0-dimethylaminoethane)- carbamoyl] cholesterol (DC-Chol) were obtained from Avanti Lipids (Alabaster, USA). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was kindly provided by Lipoid GmbH (Ludwigshafen, Germany). Influenza hemagglutinin (HA) antigen (H3N2 Wisconsin strain) was obtained from Solvay (Weesp, The Netherlands). Bovine serum albumin (BSA) was purchased from Merck (Darmstadt, Germany). ELISA plates were obtained from Greiner (Alphen a/d Rijn, The Netherlands). Horseradish peroxidase (HRP) conjugated goat anti-mouse G α g (c chain specific), IgG1 (c1 chain specific), and IgG2a (c2a chain specific) were ordered from Southern Biotech (Birmingham, USA). Chromogen 3,3',5,5'-tetramethylbenzidine (TMB) substrate buffer for ELISA, granulocyte macrophage colony stimulating factor (GM-CSF), and interleukin-4 (IL4) were provided by Biosource-Invitrogen (Breda, The Netherlands). Fetal bovine serum (FBS) and all culture media, including penicillin/streptomycin (PEST) and trypsin, were supplied from Gibco (Invitrogen, Carlsbad, CA). Nimatek® _ (100 mg/ml ketamine, Eurovet Animal Health B.V., Bladel, The Netherlands) and Rompun®_ (20 mg/ml xylazine, Bayer B.V., Mijdrecht, The Netherlands) were obtained from the pharmacy of Leiden University Medical Center. All other chemicals were acquired from Sigma-Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

2.2. Preparation of cationic liposomes

Liposomes were prepared by the film hydration method, followed by extrusion. Briefly, desired amounts of a cationic compound (DDA, DPTAP, eDPPC, or DC-Chol) and a neutral phospholipid (DPPC) were dissolved in chloroform, and mixed in a round-bottom flask of 50 ml. A thin lipid film was formed at the bottom of this flask using a rotary evaporator, under reduced pressure. The film was rehydrated in a HEPES sucrose buffer (20 mM HEPES, 10% (w/w) sucrose, pH 7.4) to obtain a final lipid (cationic compound + DPPC) concentration of 5 mg/ml. During the rehydration step, the temperature was maintained at 60 °C for 20 min, with continuous stirring at 300 rpm. The dispersion was extruded (LIPEX™ extruder, Northern Lipids Inc., Canada) 5 times through a polycarbonate filter (Nucleopore Track-Etched Membranes, Whatman, The Netherlands) with a pore size of 800 nm and 5 times through a filter with a pore size of 200 nm (Nucleopore Millipore, Amsterdam, The Netherlands). HA loaded liposomes (HA/liposomes) were prepared by adding the HA stock solution (453 μ g/ml) to the preformed liposomes (total lipid diluted to 2

mg/ml) to obtain a final HA concentration varying between of 2.5 and 10 µg/ml (corresponding to 0.5 and 2 µg/dose injected to the mice, respectively).

2.3. Characterization of the cationic liposomes

2.3.1. Hydrodynamic diameter and zeta potential

Particle's hydrodynamic diameter and polydispersity index (pdi) were determined by means of dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments). The zeta potential of the particles was measured by laser Doppler velocimetry on the same instrument, using a zeta dip cell (Malvern Instruments). Prior to analysis, samples were diluted 10-fold in 20 mM HEPES, pH 7.4.

2.3.2. Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were performed with a differential scanning calorimeter (TA Instruments Q2000) for determining the melting temperatures (T_m) of the liposomes. DSC analysis was done using sealed aluminum pans containing 10 µl of the undiluted samples or 10 µl buffer (20 mM HEPES, 10% (w/w) sucrose, pH 7.4) as reference. The measurements were done in an inert atmosphere within the temperature range of 20 °C to 80 °C, at 2 °C/min. T_m were determined from the onset of the endothermic peak.

2.3.3. HA loading

HA was labeled with IRDye 800CW (Licor Bioscience, The Netherlands) according to the manufacturer's instructions and the labeled HA (IR-HA) was used to estimate the extent of antigen adsorption to the surface of the cationic liposomes. The IR-HA was mixed with the cationic liposomes (2 µg IR-HA/400 µg total lipids). HA adsorption to the cationic liposome was measured via ion-exchange chromatography. More specifically, it was based on the recovery of labeled antigen after manual application to a cation-exchange column (Hi Trap CM FF, GE Healthcare, USA). The column was equilibrated at room temperature with 5 ml of starting buffer (50 mM HEPES, pH 10.5) after which 5 ml of elution buffer (starting buffer + 1 M NaCl), and finally, 5 ml of starting buffer were added. Then, 200 µl of the sample were applied and the column was washed with 10 ml of starting buffer at 1.2 ml/min, to elute the free IR-HA from the column. It was detected and quantified using a fluorescence plate reader (Infinite 1000, Tecan, Switzerland).

2.4. Immunogenicity study

2.4.1. Immunization of mice

Female C57-BL/6 mice, 8-week old at the start of the vaccination study, were purchased from Charles River and maintained under standardized conditions in the animal facility of the Leiden/ Amsterdam Center for Drug Research, Leiden University. The study was carried out under the guidelines compiled by the Animal Ethic Committee of The Netherlands. The mice received two subcutaneous injections of 200 μ l vaccine: a prime (day 1) and a boost (day 22). We used two different HA dosages: 0.5 μ g and 2 μ g HA/injection. The antigen was either injected alone or mixed with liposomes (400 μ g total lipid/ml). Blood samples were taken one day before prime and boost, and 3 weeks after the boost. IgG titers were determined by ELISA.

2.4.2. ELISA assay

The IgG subtype profile of influenza-specific antibodies was checked on day 20 and 42 by sandwich ELISA as previously described [26]. Briefly, ELISA plates (Greiner Bio-One B.V., The Netherlands) were coated overnight at 4 °C with 100 ng HA/well in coating buffer (0.05 M sodium carbonate/bicarbonate, pH 9.6). Plates were subsequently washed twice with PBS containing 0.05% Tween 20, pH 7.2 (PBST) and then blocked by incubation with 1% (w/v) BSA in PBST for 1 h at 37 °C. Thereafter, the plates were washed three times with PBST. Twofold serial dilutions of sera from individual mice were applied to the plates and incubated for 1.5 h at 37 °C. Plates were incubated with HRP-conjugated goat antibodies against either mouse IgG, IgG1, or IgG2a (Invitrogen, The Netherlands) for 1 h at 37 °C. After washing, plates were incubated with TMB and the reaction was stopped with sulfuric acid (2 M). The detection was done by measuring optical density at 450 nm. Antibody titers were expressed as the reciprocal of the sample dilution that corresponds to half of the maximum absorbance at 450 nm of a complete s-shaped absorbance-log dilution curve.

2.4.3. HI assay

Hemagglutination inhibition (HI) titers in serum were determined as described by Amorij et al. [27]. Briefly, serum was inactivated at 56 °C for 30 min. In order to reduce nonspecific hemagglutination, 25% kaolin suspension was added to inactivate sera. After centrifugation at 1200g, 50 μ l of the supernatant were transferred in duplicate to 96-well round-bottom plates (Greiner Bio-One B.V., The Netherlands) and serially diluted twofold in PBS. Then, four hemagglutination units of A/Wisconsin influenza inactivated virus were added to each well, and the plates were incubated for 40 min at room temperature. Finally, 50 μ l of 1% guinea pig red blood cells (obtained from Harlan Scientific, The Netherlands) diluted in PBS was added to each well, and the plates were then incubated for 2 h at room temperature. The highest dilution capable of preventing hemagglutination was scored as the HI titer.

2.5. In vitro dendritic cell maturation

Monocytes isolated from buffy coats (purchased from Sanquin, The Netherlands) were cultivated to differentiate into immature DCs, as described previously [28]. DCs were incubated for 48 h at 37 °C in RPMI 1640, containing 500 U/ml GM-CSF and 100 U/ml IL4, with a fixed volume of liposomes mixed with HA: 20, 50 or 100 µl of liposome suspension (containing 2 mg total lipid/ml and 10 µg HA/ml) in 1 ml cell culture medium, and LPS (100 ng/ml cell culture medium) was used as a positive control. Cells were washed 3 times with PBS containing 1% (w/v) BSA and 2% (v/v) FBS and incubated for 30 min with a mixture of 50_ diluted anti-HLADR-FITC, CD40-PE or anti-CD86-APC (Becton Dickinson) on ice, to measure the expression of MHCII, CD40 or CD86, respectively, on the surface of the DCs. Cells were washed and expression of the surface markers was quantified by using flow cytometry (FACS canto, Becton Dickinson). Live cells were gated based on forward and side scatter. The upregulation of the three surface markers by 100 ng/ml LPS was set as 100%. A minimum of 10,000 gated cells were analyzed in each experiment. The study was repeated using DCs from at least three different donors.

2.6. Statistical analysis

All the data of the in vitro studies were analyzed with a one way analysis of variance (ANOVA) with Bonferroni's post-test. Regarding the in vivo data, Antibody titers were logarithmically transformed before statistical analysis. A one-way ANOVA with a Kruskal-Wallis post-test analysis was performed in order to demonstrate significant differences between the experimental groups, with the exception of the comparison between HA/DC-Chol₁₀ and HA/DC-Chol₅₀ liposomes (HA loaded liposomes containing 10 mol% and 50 mol% DC-Chol, respectively), which were analyzed using a Student's t-test. The statistical analysis was carried out using Prism (Graphpad, San Diego, USA), and a p value less than 0.05 was considered to be significant.

3. Results and discussion

3.1. Preformulation and immunogenicity testing of DC-Chol/DPPC liposomes

The stability of vaccine components is important for further development of experimental vaccines. Since it was not possible to produce stable 100% DC-Chol liposomes, we mixed the cationic compounds with a neutral phospholipid, DPPC, which has a similar T_m compared to the other cationic compounds selected (see Table 1). In order to study how the content of a cationic compound affects the liposome characteristics and the immune response, we first produced DPPC/DC-Chol liposomes with 10 and 50 mol% of the cationic compound, further referred to

as DC-Chol₁₀ and DCChol₅₀ liposomes, respectively. The two liposome formulations resulted in liposomes showed a similar average hydrodynamic diameter (ca. 160 nm) and polydispersity (Table 2). However, the surface charge of the two liposome formulations differed significantly: the zeta potential of the DC-Chol₁₀ and DC-Chol₅₀ liposomes was $+24.6 \pm 0.5$ mV and $+51.1 \pm 4.5$ mV (mean $\pm$ SD of $n = 3$), respectively. Based on a stability study (data not shown), we selected two relatively low HA/lipid ratios for the rest of our experiments: 1/ 800 and 1/200 (w/w), with an equivalent of 0.5 and 2 μ g HA/dose injected. The addition of both HA dosages to the empty DC-Chol liposomes resulted in four formulations: HA_{0.5}/DC-Chol₁₀, HA_{0.5}/ DC-Chol₅₀, HA₂/DC-Chol₁₀, and HA₂/DC-Chol₅₀ liposomes. After mixing the antigen with the liposomes, no detectable change in hydrodynamic diameter and zeta potential was observed for the HA_{0.5}/DC-Chol liposomes, whereas the HA₂/DC-Chol liposomes showed a slight increase in their hydrodynamic diameter and a decrease in their zeta potential (Table 2), suggesting adsorption of the negatively charged antigen to the surface of the cationic liposomes to occur. To estimate the amount of adsorbed HA to the liposomes, we used fluorescently labeled HA (IR-HA) and cation-exchange chromatography to separate the free IR-HA from the IR-HA/liposomes based on charge differences (IR-HA is negatively charged, whereas the IR-HA/liposomes are positively charged). There was a clear influence of the pH on the retention of free IR-HA to the cation-exchange column, and a pH of 10.5 was required in order to limit the retention of free IR-HA to 9%, whereas the liposomes were fully retained. When the HA/liposomes were applied to the column, any retention above 9% was assumed to be due to HA adsorption to the liposomes. While we recognize that the separation conditions necessary for optimum separation between free and liposome associated HA significantly deviate from physiological conditions, by using this method, we do obtain insight in the relative interaction of the different liposomes with the antigen. Separation of liposomes and HA by gel permeation chromatography was not successful because of the particulate nature of the free antigen (results not shown).

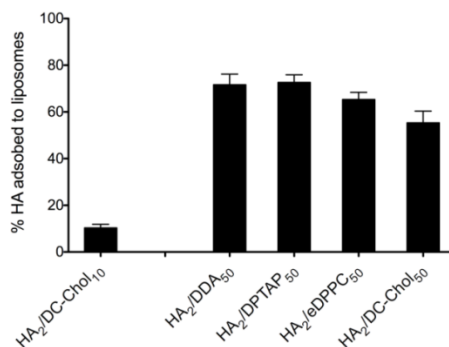


Fig. 1: Estimated amount of liposome-associated HA: The columns represent the percentage of fluorescently labeled HA retained on cation exchange column (compared to the total amount applied) after application of liposomal HA formulations. Error bars represent the standard deviation ($n=3$).

The method was applied to the IR-HA/DC-Chol liposomes. The results showed less retention of IR-HA with the IR-HA/DC-Chol₁₀ liposomes (11% retention) compared to IR-HA/DC-Chol₅₀ liposomes (55% retention) (Fig. 1). This proportional (fivefold) increase of both the amount of cationic compound and HA retained would imply that the higher positive charge density, resulting in a higher zeta potential (see results above and Table 2), led to a stronger interaction of the negatively charged antigen with the surface of the liposomes.

Next, we investigated the influence of the amount of the cationic compound in the liposomes on the immune response. In order to compare the immunogenicity of HA₂/DC-Chol₁₀ and HA₂/DC-Chol₅₀ liposomes, the liposome formulations were injected subcutaneously in mice, and HA-specific serum IgG1 and IgG2a were assessed after the first (prime) and the second (boost) immunization. The results show that HA₂/DC-Chol₅₀ liposomes induced significantly higher IgG1, IgG2a (Fig. 2), and HI titers (Fig. 5) than the HA₂/DC-Chol₁₀ liposomes (after both prime and boost). A high zeta potential (i.e., high density of cationic charge) and strong binding of HA to the liposomal surface seem to be a key to the induction of a strong immune response. These results correlate with recent studies which showed that increasing the density of cationic charge at the liposome surface induced stronger DCs maturation [29] and that cationic liposomes induced a stronger Th1 response than neutral ones [30]. Based on these results, we selected the 50% liposomes for the rest of our study (i.e., the comparison of the four cationic compounds).

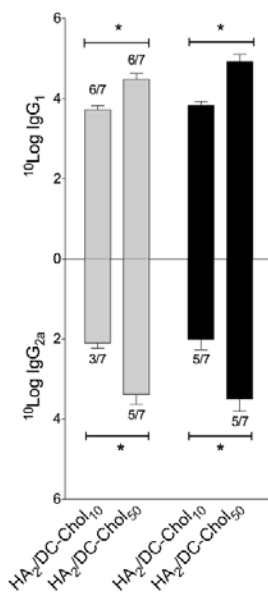


Fig. 2: Immune response in mice after subcutaneous injection with HA₂/DC-Chol₁₀ and HA₂/DC-Chol₅₀ liposomes: HA-specific serum IgG1 and IgG2a after prime (□) and boost (■). Each bar represents the average log titer of a group of mice + SEM (n=7). Significant differences between groups are indicated with * (p < 0.05).

3.2. Comparison of liposomes containing different cationic compounds

As previously detailed, the comparison of the four cationic compounds required the incorporation of a neutral phospholipid in the liposome formulation. Based on the pilot study, each formulation was based on HA mixed with empty liposomes composed of an equimolar amount of DPPC and one of the four cationic compounds: DDA, DPTAP, eDPPC, and DC-Chol. They are further referred to as: HA/DDA₅₀, HA/DPTAP₅₀, HA/ eDPPC₅₀, and HA/DC-Chol₅₀ liposomes. The liposomes were prepared, physicochemically characterized, and tested *in vivo*. As shown in Table 2, the DDA₅₀, eDPPC₅₀, and DC-Chol₅₀ liposomes showed similar average hydrodynamic diameters (Z ave between 148 and 174 nm) and zeta potentials (ca. 50 mV). However, the DPTAP₅₀ liposomes were smaller (Z ave 104 nm) and exhibited a slightly lower zeta potential (+41 mV). The size and the charge of the empty liposomes were found to be stable during storage at 4 °C for at least 56 days (data not shown). The melting temperature (*T_m*) of the liposomes as determined by DSC ranged from 40 °C (eDPPC₅₀ liposomes) to 55 °C (DPTAP₅₀ and DDA₅₀ liposomes) (Table 2). The higher *T_m* of the latter might be the result of concentrated and highly organized DDA or DPTAP regions with elevated phase transition temperatures, as observed previously [31]. Despite these differences in melting temperature, the liposomes will show similar rigidity at body temperature. The DC-Chol₅₀ liposomes did not show any observable melting temperature. The very similar structure of DC-Chol and cholesterol can relate these results to previous studies investigating the effect of cholesterol concentration into the liposome membrane on the bilayer organization. These studies showed that an amount of 10% cholesterol results in a slight decrease in the *T_m* (*T_m* DC-Chol₁₀ = 38 °C, as compared to 41 °C for pure DPPC liposomes), whereas above 30% cholesterol, a liquid-ordered phase is induced, where no *T_m* can be detected [32,33]. Each of the four types of liposomes was mixed with two different concentrations of HA (0.5 and 2 µg /dose), resulting in eight formulations, the characteristics of which are summarized in Table 2. The addition of 0.5 µg HA induced small changes in hydrodynamic diameters (from -2 to +29 nm), and a slight decrease (with 0.5–4 mV) in zeta potential. These changes were more pronounced with 2 µg HA: a rise in size (with 2–33 nm) and pdi (with 0.03– 0.1) was observed, along with a drop in zeta potential (with -2.3 to -9.3 mV). These results suggest the adsorption of the negatively charged antigen to the surface of the cationic liposomes.

Table 2. Physicochemical characteristics of cationic liposomes before and after mixing with HA*.

	Empty liposomes				HA _{0.5} /liposomes				HA ₂ /liposomes			
	Z _{ave} (nm)	pdi	ZP (mV)	Tm (°C)	Z _{ave} (nm)	pdi	ZP (mV)		Z _{ave} (nm)	pdi	ZP (mV)	
DC-Chol_{I0}	159 (±6)	0.05	+24.6 (±0.5)	38	156 (±5)	0.07	+25.4 (±0.7)		165 (±4)	0.11	+24.4 (±3.1)	
DC-Chol_{I50}	160 (±4)	0.07	+51.1 (±4.5)	ND	161 (±4)	0.07	+48.5 (±3.2)		165 (±5)	0.08	+41.8 (±9.5)	
DDA₅₀	174 (±18)	0.29	+47.5 (±1.1)	55	203 (±37)	0.34	+46.9 (±4.8)		207 (±11)	0.44	+44 (±3.3)	
DPTAP₅₀	104 (±3)	0.16	+40.7 (±5.5)	54	106 (±2)	0.16	+36.7 (±1.1)		123 (±7)	0.28	+34.5 (±4.4)	
eDPPC₅₀	148 (±2)	0.10	+50.1 (±0.8)	40	146 (±4)	0.10	+50.6 (±1.1)		150 (±5)	0.13	+47.8 (±1.4)	

* Abbreviations: HA_{0.5}: 0.5 µg HA /dose; HA₂: 2 µg HA /dose; Tm: melting temperature; Z_{ave}: Z-average hydrodynamic diameter; pdi: polydispersity index; ZP: zeta potential; ND: not detectable. Results are expressed as average ± standard deviation (n=3 independent batches).

The HA/liposome's hydrodynamic diameter and zeta potential were monitored for 56 days. No changes were observed for the zeta potential (data not shown). Regarding the size (Fig. 3) and the polydispersity (Supplementary Fig. S1), the HA/DC-Chol₅₀ and HA/eDPPC₅₀ liposomes were stable for at least 56 days, with both HA dosages. Similar results were obtained with HA_{0.5}/DDA₅₀ and HA_{0.5}/DPTAP₅₀ liposomes. However, with a higher amount of HA (HA₂/DDA₅₀ and HA₂/DPTAP₅₀), the liposomes showed a major increase in hydrodynamic diameter and pdi after 56 days. Besides, the retention rate of HA with the liposomes ranged from 55% to 76% (Fig. 1).

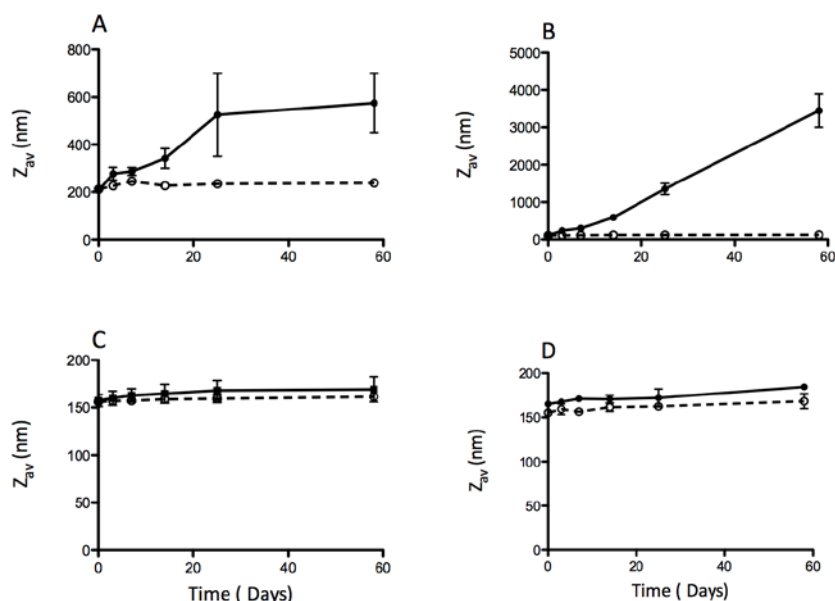


Fig. 3:

Colloidal stability of HA/DDA₅₀ liposomes (A), HA/DPTAP₅₀ liposomes (B), HA/eDPPC₅₀ liposomes (C) and HA/DC-Chol₅₀ liposomes (D) stored at 4 °C. Two different types of HA/liposomes have been tested: HA_{0.5}/liposomes (open symbols; dashed lines) and HA₂/liposomes (solid symbols and lines). Error bars represent the standard deviation (n=3).

These results confirm that there was a strong interaction between the liposomes and the antigen. The HA₂/DC-chol₅₀ liposomes showed somewhat lower adsorption rates compared to the three other formulations, which is likely due to the high pH used (10.5) during the cation-exchange chromatography, which was required to achieve separation of free and liposome-associated HA. At this pH, the tertiary amine group of DC-Chol becomes partially deprotonated, as reflected by a drop in zeta potential (not shown), whereas the other cationic compounds carry a quaternary ammonium group that remains charged independent of the pH. Therefore, at a physiological pH, the HA₂/DC-chol₅₀ liposomes may show a similar HA loading as the other formulations. In conclusion, we produced liposomes with

hydrodynamic diameters in the same order of magnitude and comparable positive zeta potentials. Despite the relatively unstable HA₂/DDA₅₀ and HA₂/DPTAP₅₀ formulations, the eight formulations were similar in size and zeta potential at the day of preparation and injection. This allowed us to study the influence of the liposome composition on the adjuvant effect. In order to compare the adjuvant effect of the liposomes containing any of the four cationic compounds, the liposome formulations (400 µg total lipid/dose) with two different doses of HA (0.5 and 2 µg HA/dose) were injected subcutaneously in mice. The HA-specific serum IgG, IgG1, and IgG2a titers were assessed after the first (prime) and the second (boost) immunization, and hemagglutination inhibition (HI) titers, as a measure for the level of functional antibodies, were measured after the boost. Since for all groups the HA-specific serum IgG titers were very comparable to the IgG1 titers, we present here only the IgG1 data. After immunization with 2 µg HA, the antigen alone (plain HA) showed fairly high levels of IgG1, with a small increase when it was associated to the cationic liposomes (Fig. 4A). However, this enhancement of the IgG1 response was only significant for the HA₂/DC-Chol₅₀ liposomes, after both prime and boost. With the lower dose of HA (0.5 µg), the plain HA induced lower IgG1 titers and we observed more differences between the groups (Fig. 4B). After the prime, the HA_{0.5}/DC-Chol₅₀ liposomes were significantly more immunogenic than plain HA and the HA_{0.5}/DPTAP₅₀ liposomes. After the boost, the HA_{0.5}/DC-Chol₅₀ liposomes enhanced the IgG1 titers compared to plain HA, the HA_{0.5}/DDA₅₀ liposomes, and the HA_{0.5}/eDPPC₅₀ liposomes. The other liposome formulations did not induce significantly higher IgG1 titers than plain HA. The IgG2a titers, although substantially lower than the IgG1 titers, showed a similar trend. With 2 µg HA, the HA₂/DC-Chol₅₀ liposomes enhanced significantly the IgG2a titers compared to the HA₂/DPTAP₅₀ and the HA₂/eDPPC₅₀ groups (Fig. 4A). Although HA₂/DC-Chol₅₀ showed superior titers compared to the non-adjuvanted group after the prime and the boost and for both HA dosages (0.5 and 2 µg), no statistical significance could be established as plain HA induced a response in only one or two mice (Fig. 4B). Similarly, the other formulations showed a trend toward increased IgG2a titers. Overall, the lower (0.5 µg) dose of HA resulted in lower IgG1 titers and enabled a better discrimination of the immunogenicity of the formulations as compared to the higher (2 µg) HA dose. Interestingly, the dose reduction had very little effect on the intensity of the IgG2a titers, but did reduce the number of responders, especially after the prime, both for IgG1 and IgG2a. Besides the intensity of the immune response, the type of immune response induced is an important criterion, and in order to induce a good antiviral protection, the Th1 pathway should preferably be induced [10]. A Th1 mediated immune response is notably characterized by high IgG2a antibody titers, and a Th2 type response by IgG1 titers. Thus, the high levels of IgG2a and IgG1 induced by the HA/DC-Chol₅₀ liposomes are in favor of a balanced Th1/Th2 response compared to the Th2-biased response induced by plain HA and the other cationic liposomes (Fig. 4).

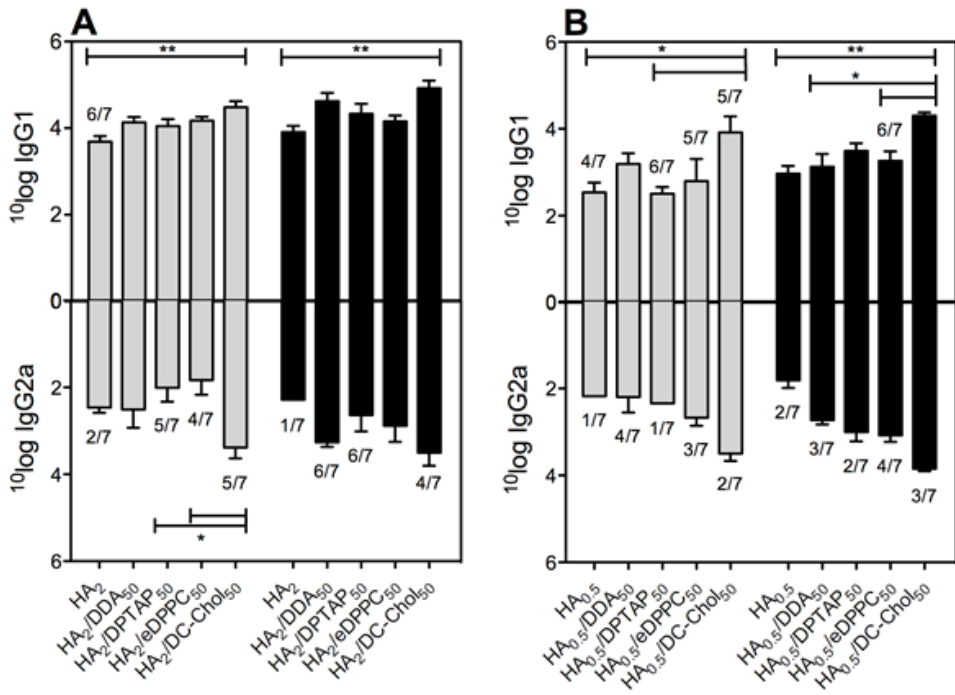


Fig. 4: Immune response in mice after subcutaneous injection with 2.0 µg (A) or 0.5 µg (B) of plain HA and liposomal HA: HA-specific serum IgG1 and IgG2a after prime (□) and boost (■). Each bar represents the average log titer + SEM of a group of 7 mice. For groups containing non-responders, the ratio of number of responding mice/total number of mice is indicated. Significant differences between groups are indicated with ** ($p < 0.01$), or * ($p < 0.05$).

These results correlate with the HI titers measured after boost immunization (Fig. 5). For both HA doses, the HA/DC-Chol₅₀ liposomes tended to induce the highest HI titers, with statistical significant differences observed with the HA_{0.5}/DC-Chol₅₀ liposomes. Unfortunately, we were unable to detect any significant increase in INF γ secretion by spleen cells (collected from spleens isolated at the end of the in vivo study) induced by our formulations, after restimulation with HA (data not shown). This is however in accordance with a study by McNeil et al., who showed that, unlike DSPC/Chol/DDA/TDB liposomes, DSPC/Chol/DDA liposomes (associated with the Ag85B-ESAT-6 antigen) failed to promote INF γ production from mice's isolated blood lymphocytes 3 weeks after immunization [34].

In summary, not only the intensity of the immune response was improved by the HA/DC-Chol₅₀ liposomes, but also the quality of the immune response, as reflected by a strong elevation of the IgG2a titers compared to plain HA. The other cationic liposome formulations (HA/DDA₅₀, HA/DPTAP₅₀, and HA/eDPPC₅₀) induced lower IgG2a titers (after boost), although they were superior to plain HA. Based on the

IgG2a levels induced after immunization with 2 μ g HA (Fig. 4A) and the HI titers, even though the latter did not show significant differences between each other, the potency of the formulations can be ranged in the following order: HA/DC-Chol₅₀ >> HA/DDA₅₀ > [HA/eDPPC₅₀ ~ HA/DPTAP₅₀] > HA.

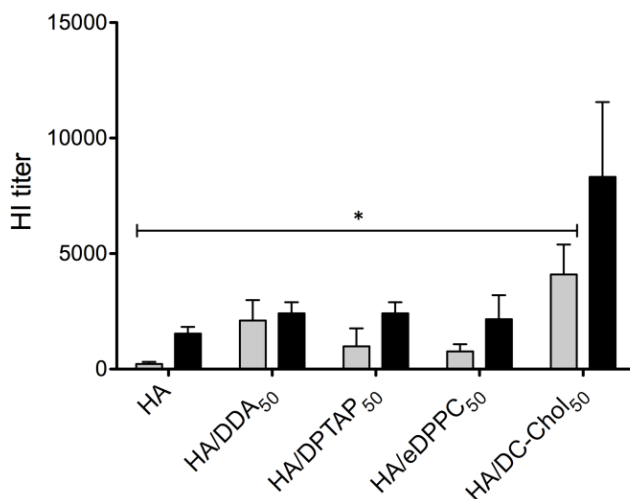


Fig. 5: HI titers after boost subcutaneous injection of mice with plain HA and HA/liposomes: 0.5 μ g HA/dose (□) and 2 μ g HA/dose (■). Each bar represents the average + SEM of a group of 7 mice. Significant differences between groups are indicated with * ($p < 0.05$).

In a recent study, Henriksen-Lacey et al. [30] compared the INF γ secretion by mice's peripheral blood mononuclear cells (PBMCs) induced by DDA, DC-Chol, and DOTAP liposomes mixed with trehalose dibehenate (TDB). The results showed that 400-nm DDA/TDB liposomes were more potent to induce a Th1 response compared to the 200-nm DC-Chol/TDB liposomes (no pdi value available) and 760-nm DOTAP/TDB liposomes (pdi = 0.32). Although the goal of that study was to investigate the adjuvant effect of several cationic lipids, the use of TDB, the heterogeneity of size, and zeta potential (DC-Chol/TDB liposome's zeta potential dropped from +45 to +20 mV after 56 days of storage) are other factors that could have influenced the immune response. The authors ascribed the superior adjuvant activity of DDA liposomes to a mechanism of longer-term retention and slower release of liposomes and antigen from the injection site, but it is still not clear which of the cationic lipids or what physicochemical characteristics are responsible for this effect. Indeed according to the literature, larger liposomes (0.5–2 μ m) remain at the site of injection and are taken up by DCs, whereas small liposomes (<200 nm) are drained to the lymph nodes [35,36], implying different mechanisms depending on the size of the liposomes. For comparison, the formulations tested in our study were very similar in size (hydrodynamic diameter between 100 and 200 nm), allowing us to compare the effect of different cationic compounds on the immunogenicity of the liposomes. Although a potential influence of small differences

in size on the antibody response cannot be totally excluded, we conclude that the DC-Chol-containing cationic liposomes have a stronger adjuvant effect than any of the other cationic compounds in liposomes with otherwise similar physicochemical characteristics.

3.3. Effect of HA/cationic liposomes on DC maturation in vitro

In order to obtain a better understanding of the superiority of DC-Chol liposomes over the other formulations tested, we studied the ability of DC-Chol liposomes compared to DDA liposomes (as a representative of the other liposome types) to enhance DC maturation. The up-regulation of the surface markers MHCII, CD40, and CD86 was determined after culturing immature DCs in the presence of the HA₂/liposomes. The HA₂/DDA₅₀ liposomes had no significant effect on the expression level of any of the surface markers tested (Fig. 6). However, after incubation with the HA₂/DC-Chol₅₀ liposomes, the DCs showed elevated MHCII expression compared to untreated cells. The levels of expression of MHCII proved to be concentration dependent, with statistical significant differences for the higher dosages, up to levels even higher than those induced with the positive control (LPS) (Fig. 6; see Supplementary Fig. S2 for representative histograms). A similar (but statistically not significant) trend was observed for the effect of HA₂/DC-Chol₅₀ liposomes on the expression of CD40, whereas only a marginal effect on the CD86 expression was observed. To rule out LPS contamination in our experiments, TLR-4 transfected HEK cells were exposed to the formulations and we found the LPS content to be below the detection limit (<0.1 ng/ml, data not shown). The concomitant increase in MHCII and CD40 expression is usually known as a sign of DC activation [37]. However, the low level of CD86 observed in this study is not in favor of such activation. The different levels of upregulation of the DC surface markers might be explained by the influence of the cholesterol backbone of the DC-Chol, as cholesterol has been shown to upregulate MHC-II expression [38]. The lack of DC activation by DDA liposomes is consistent with another study [39]. However, the upregulation of the DC surface markers induced by our DC-Chol liposomes differs from a previous investigation using a DC-Chol dispersion that did not result in the upregulation of DC surface markers [40].

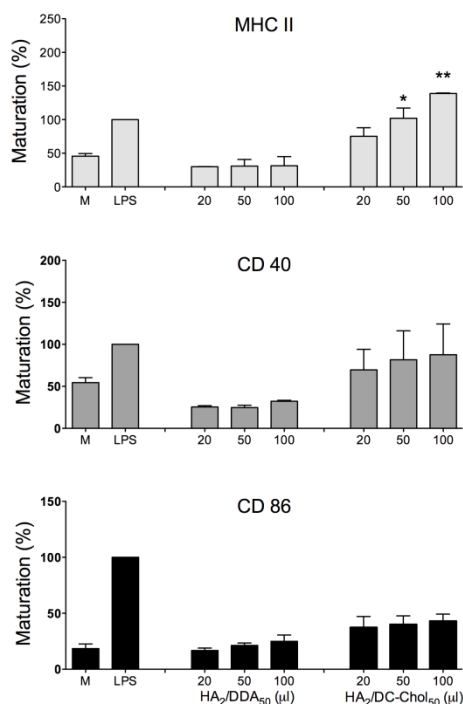


Fig. 6: Upregulation of DC maturation markers induced by raising amounts of HA₂/DDA₅₀ and HA₂/DC-Chol₅₀ liposome suspensions: 20, 50 or 100 µl (expressed as volume of liposome suspension, containing 2mg total lipid/ml and 10 µg HA/ml) added per 1 ml culture medium. M = culture medium, LPS = lipopolysaccharide. The values are expressed as mean fluorescence intensity (compared to that of the control group: 100 ng LPS /ml culture medium) after application of the liposomal HA formulations. Error bars represent the SEM (n=3). Significant differences between groups are indicated with ** (p < 0.01), or * (p < 0.05).

4. Conclusions and perspectives

In this study, we studied the influence of charge density, liposome composition, and chemistry of the cationic compound on the adjuvant effect of cationic liposomes, loaded with two different dosages of HA. Taken together the results of the immunogenicity studies, we noticed that (1) liposomes with a similar size, surface charge, and HA loading but different composition induced different immune responses, (2) DC-Chol liposomes containing a higher amount of cationic compound and showing a higher HA association were the most potent adjuvant for HA, (3) they induced a stronger immune response, and (4) they enhanced the expression of some DC maturation markers. This implies that, although the cationic charge is a critical factor for the adjuvant effect of cationic liposomes, the adjuvant effect is also influenced by the composition of the liposomes. The superiority of the HA/DC-Chol₅₀

liposomes raises a new question regarding the physicochemical properties responsible for the enhanced adjuvant effect induced by these cationic liposomes. The DC-Chol compound was the only one with a tertiary amine and also the only sterol-containing lipid included in our study. As detailed previously, the hydrodynamic diameters, the zeta potential and the HA loading of the HA/DCChol₅₀ liposomes were similar to those of the other liposomes tested; only the composition and the T_m differed. The absence of a detectable T_m in the HA/DC-Chol₅₀ liposomes can be related to the liquid organized state observed in lipid bilayers made of DPPC containing more than 30% DC-Chol. Whether this difference in bilayer organization or the chemistry of the head group of DC-Chol, or both, is responsible for the increased adjuvanticity of the HA/ DC-Chol₅₀ liposomes is subject of ongoing studies. However, as observed in the *in vitro* study, the interaction with the DCs seems to be one of the key point of the adjuvant mechanism of these formulations. In conclusion, the role of cationic liposomes as adjuvant for HA depends on their composition. The characteristics of both the hydrophilic head group and the hydrophobic tail may affect the immune response. Moreover, the differences observed between our results and other studies should make anyone cautious with generalizations about the adjuvant effect of cationic liposomes. In order to gain more insight into the influence of the physicochemical characteristics of the cationic liposomes on their adjuvant effect, studies comparing the effect of liposomes with different T_m, size, administered via different routes of administration [41] should be performed. Finally, the encapsulation of TLR ligands in the liposomes should be considered to further enhance the cellular response against HA.

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Supplementary information

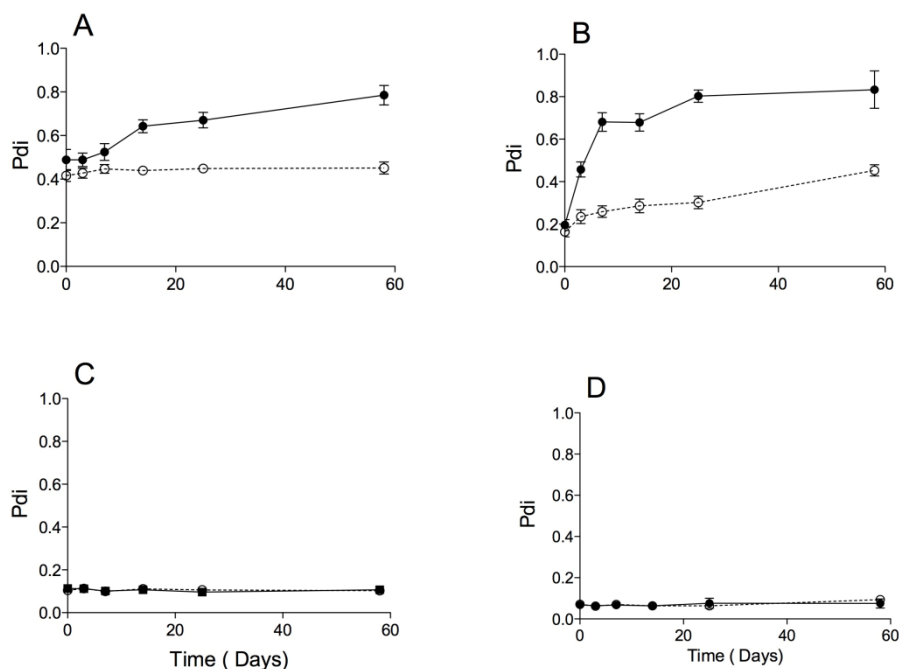


Fig. S1: Polydispersity of HA/DDA₅₀ liposomes (A), HA/DPTAP₅₀ liposomes (B), HA/eDPPC₅₀ liposomes (C) and HA/DC-Chol₅₀ liposomes (D) stored at 4 °C. Two different types of HA/liposomes have been tested: HA_{0.5}/liposomes (open symbols; dashed lines) and HA₂/liposomes (solid symbols and lines). Error bars represent the standard deviations (n=3).

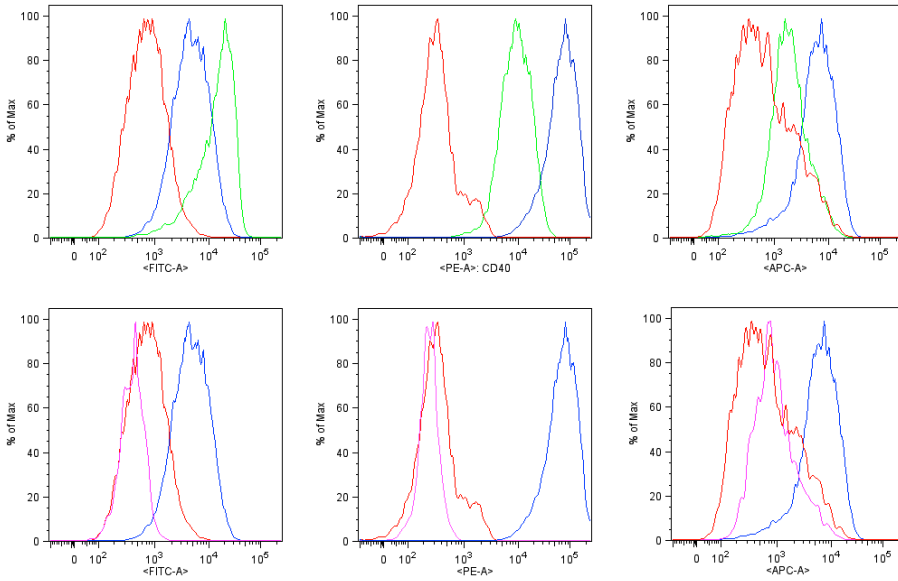


Fig. S2: Representative flow cytometry histograms of different DC maturation markers (FITC-MHCII, plots on the left; PE-CD40, plots in the middle; and APC-CB86, plots on the right) induced by: DC-Chol₅₀ liposomes (green histograms, upper plots), DDA₅₀ liposomes (pink histograms, lower plots), culture medium (red histograms) and 100 ng/ml LPS (blue histograms).

Chapter 6

Adjuvant Effect of Cationic Liposomes for Subunit Influenza Vaccine: Influence of Antigen Loading Method, Cholesterol and Immune Modulators

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Abstract

Cationic liposomes are potential adjuvants for influenza vaccines. In a previous study we reported that among a panel of cationic liposomes loaded with influenza hemagglutinin (HA), DC-Chol:DPPC (1:1 molar ratio) liposomes induced the strongest immune response. However, it is not clear whether the cholesterol (Chol) backbone or the tertiary amine head group of DC-Chol was responsible for this. Therefore, in the present work we studied the influence of Chol in the lipid bilayer of cationic liposomes. Moreover, we investigated the effect of the HA loading method (adsorption versus encapsulation) and the encapsulation of immune modulators in DC-Chol liposomes, on the immunogenicity of HA. Liposomes consisting of a neutral lipid (DPPC or Chol) and a cationic compound (DC-Chol, DDA, or eDPPC) were produced by film hydration-extrusion with/without an encapsulated immune modulator (CpG or imiquimod). The liposomes generally showed comparable size distribution, zeta potential and HA loading. In vitro studies with monocyte-derived human dendritic cells and immunization studies in C57Bl/6 mice showed that (1) liposome-adsorbed HA is more immunogenic than encapsulated HA, (2) the incorporation of Chol in the bilayer of cationic liposomes enhances their adjuvant effect, and (3) CpG loaded liposomes are more efficient at enhancing HA-specific humoral responses than plain liposomes or Alhydrogel.

1. Introduction

The main strategy against seasonal influenza outbreaks is vaccination. Subunit vaccines are known to be the safest influenza vaccines produced, but they are less immunogenic than whole virus and split vaccines. Some populations are less protected after vaccination, such as the elderly due to senescence of their immune system [1]. One way to enhance the immunogenicity of subunit vaccines is the use of adjuvants.

Cationic liposomes are known for their ability to enhance the potency of subunit vaccines and may serve to lower the dose and thereby, enable the increase of vaccine supply. There is a large number of publications investigating cationic liposomes as an adjuvant for diverse antigens (reviewed in [2–5]). It has been demonstrated that the surface charge of the liposomes influences the immune response: positively charged lipid vesicles are taken up more efficiently than negatively charged or neutral vesicles by macrophages and dendritic cells (DCs) [6,7]. However, the different formulations, immunization schedules and read-out models used in these studies hamper straight comparisons and make it very difficult to judge which cationic liposomes have the most favorable adjuvant effect.

The work presented here is a follow up of our previous study in which we investigated the adjuvant effect of cationic liposomes mixed with a subunit H3N2 influenza vaccine based on purified hemagglutinin (HA) [8]. In that study, we showed that the adjuvant effect of cationic liposomes not only depends on their charge, but also on the cationic compound selected and its amount in the liposomal bilayer. The best adjuvant effect was obtained with liposomes containing DC-Chol (compared to DDA, eDPPC and DPTAP). However, it is not clear whether the tertiary amine head group and/or the sterol backbone of the DC-Chol molecule was responsible for this.

The major aim of the present study was to get a better understanding of the superior adjuvant effect of DC-Chol liposomes. Ideally, we should compare liposomes made of DC-Chol with liposomes based on a cationic compound based on the same DC head group (a tertiary amine) but linked to a saturated carbon chain. Unfortunately, such a compound is not commercially available. Therefore, in the work presented here we focused on the influence of the Chol backbone in the cationic liposomal bilayer on HA immunogenicity by comparing the adjuvant effect of liposome formulations based on the same saturated cationic compounds mixed with either Chol or DPPC. Furthermore, we studied the effect of antigen loading method (encapsulation *versus* adsorption) and the encapsulation of immune modulators on the immune response against HA [9,10]. In general, co-delivery of an antigen with an immune modulator in one particulate system is an effective way to generate a strong immune response [11,12]. Moreover, the ability of cationic liposomes to enhance DC uptake could potentially help to target endosomal toll-like receptors (TLR), and for this reason we selected the following two TLR ligands:

bacterial cytosine phosphodiester guanine oligomer (CpG), an agonist of TLR-9 [13], imiquimod, a TLR-7 agonist [14,15]. The immunogenicity of the liposomal HA formulations was evaluated for APC maturation *in vitro* as well as in a mouse model and compared to that of HA formulated with aluminum hydroxide (Al(OH)₃), an adjuvant known to promote a Th2 type response.

2. Experimental Section

2.1. Materials

Cholesterol, 1,2-diacyl-*sn*-glycero-3-ethylphosphocholine (eDPPC), dimethyl dioctadecyl-ammonium bromide (DDA), 3 β -[*N*-(*N*',*N*'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) were obtained from Avanti Lipids (Alabaster, AL, USA). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was kindly provided by Lipoid GmbH (Ludwigshafen, Germany). Influenza hemagglutinin (HA) antigen (H3N2 Wisconsin strain) was obtained from Solvay (Weesp, The Netherlands). Bovine serum albumin (BSA) was purchased from Merck (Darmstadt, Germany). ELISA plates were obtained from Greiner (Alphen a/d Rijn, The Netherlands). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (γ chain specific), IgG1 (γ 1 chain specific) and IgG2a/c (γ 2a chain specific) were ordered from Southern Biotech (Birmingham, AL, USA). Chromogen 3,3',5,5'-tetramethylbenzidine (TMB) substrate buffer for ELISA, granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL4), were provided by Biosource-Invitrogen (Breda, The Netherlands). CpG (1826 and 2006) and imiquimod were purchased from InvivoGen (Toulouse, France). Alhydrogel was kindly provided by Brenntag (Frederikssund, Denmark). Fetal bovine serum (FBS) and all culture media, including penicillin/streptomycin and trypsin were supplied from Gibco (Invitrogen, Carlsbad, CA, USA). Nimetek® (100 mg/mL ketamine, Eurovet Animal Health B.V., Bladel, The Netherlands) and Rompun® (20 mg/mL xylazine, Bayer B.V., Mijdrecht, The Netherlands) were obtained from the pharmacy of Leiden University Medical Center.

2.2. Preparation of Cationic Liposomes

Liposomes were prepared by the film hydration method, followed by extrusion, as described previously [8]. Briefly, desired amounts of a cationic compound (DDA, eDPPC, or DC-Chol) were dissolved in a chloroform/methanol 9:1 (v/v) solution with a neutral phospholipid (DPPC) or with Chol, and mixed in a round bottom flask of 50 mL. A thin lipid film was formed at the bottom of this flask under reduced pressure by using a rotary evaporator. The film was hydrated in a HEPES sucrose buffer (20 mM HEPES, 10% (w/v) sucrose, pH 7.4) to obtain a final lipid concentration of 5 mg/mL. During the hydration step the temperature was

maintained at 60 °C for 20 min, with continuous stirring at 300 rpm. The dispersion was extruded (LIPEX™ extruder, Northern Lipids Inc., Burnaby, Canada) 5 times through a polycarbonate filter (Nucleopore Track-Etched Membranes, Whatman, 's-Hertogenbosch, The Netherlands) with a pore size of 800 nm and 5 times through a filter with a pore size of 200 nm (Nucleopore Millipore, Amsterdam, The Netherlands).

Loading of TLR ligands into the DC-Chol:DPPC liposomes was done in two different ways, depending on the TLR ligand: CpG was dissolved in the buffer used to hydrate the lipid film, while imiquimod was dissolved in a chloroform/methanol 9:1 (v/v) solution and mixed with the lipid solution before preparation of the lipid film. For both TLR ligands, a dose of 2 µg adjuvant/400 µg total lipids was used to obtain an adjuvant/antigen ratio of *ca.* 1:1 (w/w).

2.3. Preparation of HA Formulations

HA was adsorbed to liposomes (HA/liposomes) as described before [8]. Briefly, the antigen stock solution (453 µg/mL HA) was mixed with the preformed liposomes to obtain a final concentration of 10 µg/mL HA (corresponding to 2 µg HA per injected dose) and 2000 µg/mL lipid compounds. Al(OH)₃ formulations were prepared by diluting Alhydrogel with HEPES sucrose buffer. Subsequently, the antigen solution was added to an equal volume of adjuvant, to obtain a final concentration of 10 µg/mL HA and 600 µg/mL Al(OH)₃. For the encapsulation of HA in DC-Chol:DPPC liposomes, we adapted a method described by Babai *et al.* [16]. Briefly the HA/DC-Chol:DPPC liposomes, produced with the adsorption method described above, were freeze-dried overnight, followed by a stepwise rehydration with warm (40 °C) Milli-Q water.

2.4. Characterization of the Formulations

2.4.1. Hydrodynamic Diameter and Zeta Potential

Particles' hydrodynamic diameter and polydispersity index (pdi) were determined by means of dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments, Worcestershire, UK). The zeta potential (ZP) of the liposomes was measured by laser Doppler velocimetry on the same instrument by using a zeta dip cell (Malvern Instruments, Worcestershire, UK). Prior to analysis, samples were diluted 10 fold in 20 mM HEPES, pH 7.4. The measurements were performed at 25 °C and Malvern DTS software (version 6.10, Worcestershire, UK) was used for data acquisition and analysis.

2.4.2. HA Loading

HA was labeled with IRDye 800 CW (Licor Bioscience, The Netherlands) according to the manufacturer's instructions and the labeled HA (IR-HA) was used to estimate the extent of antigen adsorption to the cationic liposomes. The IR-HA was mixed with the cationic liposomes (2 μ g IR-HA/400 μ g total lipids). HA adsorption to the cationic liposome was measured via cation-exchange chromatography on a Hi Trap CM FF column (GE Healthcare, Pittsburgh, PA, USA) and measuring the fluorescence intensity of the unbound fraction, as described before [8].

2.4.3. Adjuvant Loading

The amount of CpG incorporated in the liposomes was indirectly determined by using FITC-labeled CpG (10% of total CpG). The free TLR ligand was separated from the liposomes by filtration using a Vivaspin 2 centrifugal concentrator (PES membrane, MWCO 300 kDa, Sartorius Stedim, Nieuwegein, The Netherlands) and quantified using a fluorescence plate reader (TECAN infinite M1000, Tecan Group Ltd., Männedorf, Switzerland). The same separation method was used for the imiquimod-containing liposomes and (unlabeled) imiquimod was quantified by its absorbance at 247 nm.

2.5. Immunogenicity Study

Female C57-BL/6 mice, 8 weeks old at the start of the vaccination study, were purchased from Charles River (Maastricht, The Netherlands) and maintained under standardized conditions in the animal facility of the Leiden Academic Centre for Drug Research at Leiden University. The study was done under the guidelines compiled by the Animal Ethic Committee of The Netherlands. The mice received two subcutaneous injections of 200 μ L vaccine containing 2 μ g HA: a prime (day 1) and a boost (day 22). Blood samples were taken one day before prime and boost, and 3 weeks after the boost. Hemagglutination inhibition (HI) titers in serum after boost were determined as described previously [8]. IgG isotype-specific analysis was performed by ELISA using the horseradish peroxidase-conjugated anti-mouse total IgG, IgG1 and IgG2a. C57BL/6 mice express the *Igh1-b* gene, which encodes the IgG2c isotype rather than IgG2a. However, here we used an anti-IgG2a isotype (which cross-reacts with IgG2c [17]) and titers are reported as IgG2a/c titers. Antibody titers were determined at the midpoint of the optical density-log dilution curves after subtraction of the naïve background, and non-responding mice were given an arbitrary titer of 10. Furthermore, mouse spleens were collected three weeks after the last immunization, and after homogenization the cells were re-stimulated *in vitro* with 5 μ g/mL of HA, while the release of interferon gamma (IFN- γ) was determined by ELISA.

2.6. In Vitro Uptake of HA by Dendritic Cells

HA was conjugated with FITC by using the FluoReporter® FITC Protein Labeling Kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Immature DCs were incubated for 4 h (at 4 °C and 37 °C) with to 2.5 µg/mL HA-FITC, free or adsorbed to 50 µL of a 2 mg/mL liposome suspension. The cells were washed three times with PBS containing 1% (w/v) bovine serum albumin and 2% (v/v) fetal bovine serum). Next, HA-FITC association with the DCs was quantified by flow cytometry (FACSCanto II, Becton Dickinson, San Jose, CA, USA). Living cells were gated based on forward and side scatter and HA-FITC association was expressed as the mean fluorescence intensity (MFI).

2.7. In Vitro Dendritic Cell Maturation

Monocytes isolated from buffy coats (purchased from Sanquin, Leiden, The Netherlands) were cultivated to differentiate into immature dendritic cells (DCs), as described previously [8]. Briefly, immature DCs were incubated for 48 h at 37 °C in 1 mL cell culture medium in presence of 10 µL of a 2 mg/mL liposome suspension. After being washed three times the cells were incubated for 30 min with a mixture of 50× diluted anti-MHCII-FITC or anti-CD86-APC, anti-CD40-PE (Becton Dickinson, Breda, The Netherlands) on ice. The expression of the surface markers was quantified by using flow cytometry (FACS canto, Becton Dickinson). Live cells were gated based on forward and side scatter. The up-regulation of the three surface markers by 100 ng/mL LPS (positive control) was set at 100%. At least 10,000 gated cells were analyzed in each experiment.

2.8. Statistical Analysis

Antibody and HI titers were logarithmically transformed before statistical analysis. All data were analyzed by a two-tailed Mann-Whitney test to demonstrate significant differences between the experimental groups, except for Figures 4 and 5 where the increase of the immune responses induced by DPPC:DC-Chol liposomes + immunomodulators was compared to the negative control (DPPC:DC-Chol liposomes alone), and thus we used a one-tailed Mann-Whitney test.

3. Results

3.1. DC-Chol:DPPC Liposomes with Adsorbed versus Encapsulated HA

We prepared DC-Chol:DPPC liposomes loaded with the antigen HA using two different methods: adsorption (HA ad./DC-Chol:DPPC) and encapsulation (HA enc./DC-Chol:DPPC). After preparation, the particle size, the zeta-potential and the HA loading of each formulation were determined.

Table 1. Physicochemical characteristics of DC-Chol:DPPC liposomes before and after hemagglutinin (HA) loading.

Formulation	Lipid molar ratio	HA/liposomes			
		Z _{ave} (nm)	pdi	ZP (mV)	LE HA (%)
DC-Chol:DPPC	1:1	160 (±4)	0.07 (±0.01)	+51.1 (±4.5)	not applicable
HA ad./DC-Chol:DPPC	1:1	165 (±5)	0.08 (±0.02)	+41.8 (±9.5)	60 (±4)
HA enc./DC-Chol:DPPC	1:1	155 (±18)	0.08 (±0.04)	+46.5 (±2.3)	63 (±5)

Results are expressed as average ± standard deviation ($n = 3$ independent batches).

The results (Table 1) showed that the size and pdi of the HA/liposomes were similar (also compared to the empty DC-Chol:DPPC liposomes). Additionally, the HA loading efficiency measured was very similar for the two HA loading methods (60% *versus* 63%). Finally, the positive ZP of the liposomes tended to become smaller with the encapsulation or adsorption of HA.

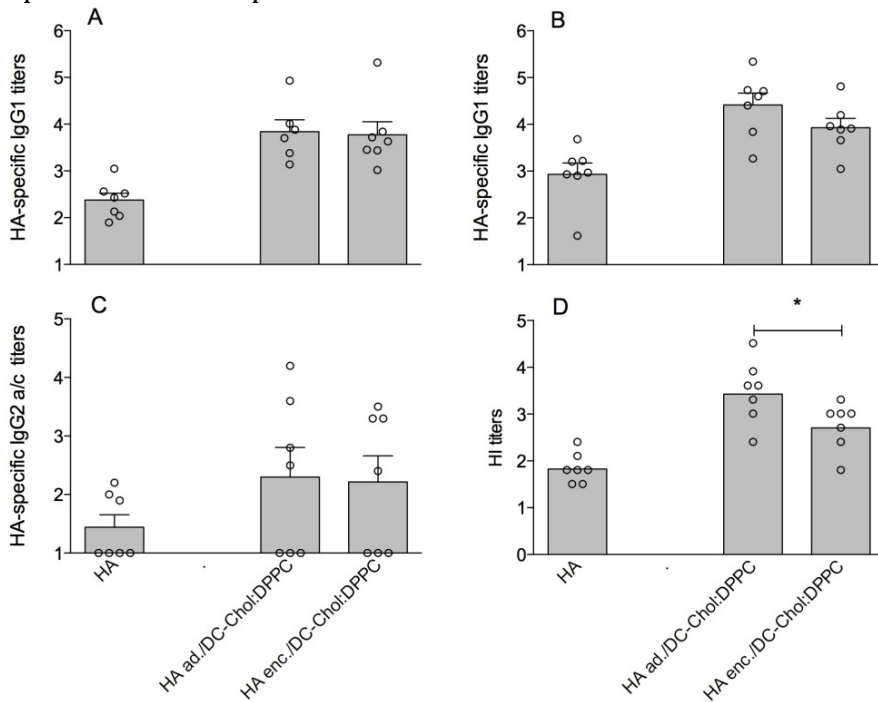


Fig. 1: Immune response in mice vaccinated with 2.0 µg of free HA *versus* HA adsorbed to DC-Chol:DPPC liposomes (HA ad./DC-Chol:DPPC) and HA encapsulated in DC-Chol:DPPC liposomes (HA enc./DC-Chol:DPPC): HA-specific serum IgG1 titer after prime (A) and boost (B); IgG2a/c titer after boost (C); and HI titer after boost (D). For panels A–C, each dot represents the log serum titer of an individual mouse (non-responding mice were given an arbitrary titer of 10) and bars represent average log titer + SEM. For panel D, each dot represents the log HI titer in serum of an individual mouse and bars represent the geometric mean. Significant differences between the groups treated with liposomal formulations are indicated with * ($p < 0.05$).

To assess the immunogenicity of the two liposomal HA formulations (HA ad./DC-Chol:DPPC and HA enc./DC-Chol:DPPC), these were injected subcutaneously in mice. HA-specific serum IgG1, and IgG2a/c titers were assessed after the first (prime) and the second (boost) immunization, and hemagglutination inhibition (HI) titers, as a measure for the level of functional antibodies, were measured after the boost. The results (Figure 1) show a superior HI titer induced by the HA adsorbed liposomes after the boost immunization compared to the HA encapsulated liposomes ($p < 0.05$) (Figure 1D). The IgG1 titers measured after the boost showed the same trend (Figure 1B), although the difference was not statistically significant. No difference was observed between the IgG2a/c titers induced by the two formulations (Figure 1C). In conclusion, HA ad./DC-Chol:DPPC liposomes were slightly more immunogenic and are easier to prepare than HA enc./DC-Chol:DPPC liposomes. Therefore, we followed the adsorption procedure in the follow-up studies described below.

3.2. Cationic Liposomes with Different Bilayer Compositions

A series of liposome formulations was prepared to investigate the influence of the Chol backbone on the adjuvant effect of cationic liposomes. As detailed earlier, we were not able to use the DC-Chol for this study as there are no commercially available compounds with the same head group linked to saturated carbon chains. Instead, we prepared cationic liposomes either composed of (50 mol% Chol + 50 mol% cationic compound) or (50 mol% neutral saturated phospholipid + 50 mol% cationic compound). For this study, two cationic compounds were selected: DDA and eDPPC (the two best cationic compounds after DC-Chol identified in our previous study [8]); and the neutral saturated phospholipid DPPC. The four resulting formulations enabled the comparison of cationic liposomes containing either 100% saturated chain liposomes or 50% Chol.

Using the HA adsorption method, the antigen was mixed with each type of liposomes and the physicochemical characteristics of the resulting formulations were determined, as summarized in Table 2. In line with our previous paper [8], the addition of HA to the four liposome formulations induced an increase in the liposome's hydrodynamic diameter (from 2–31 nm) and a slight decrease of their ZP (data not shown). These results indicate that the negatively charged antigen was successfully adsorbed to the surface of the cationic liposomes. The adsorption of HA to the liposomal surface was confirmed by measuring the retention of HA during cation exchange chromatography, showing HA loading between 68% and 79% (Table 2). Apart from the difference in pdi noticed for the DDA:DPPC formulation, likely due to some aggregation, the formulations were similar in size and ZP, and showed a similarly strong interaction with the antigen, irrespective of the presence of Chol. Furthermore, differential scanning calorimetry showed no sharp transition,

corresponding to the gel-to-liquid-crystalline phase transition temperature of the saturated lipid, for liposomes containing DC-Chol or Chol. In contrast, sharp transitions were detected at 40 °C and 55 °C for liposomes prepared from DDA:DPPC and eDPPC:DPPC, respectively (data not shown). These observations are consistent with our previous results [8].

Table 2. Physicochemical characteristics of cholesterol- and DPPC-based cationic liposomes after mixing with HA.

Formulation	Molar ratio	HA/liposomes			
		Z_{ave} (nm)	pdi	ZP (mV)	LE HA (%)
DDA:DPPC	1:1	207 (± 11)	0.44 (± 0.02)	+44 (± 3.3)	76 (± 2)
DDA:Chol	1:1	179 (± 2)	0.09 (± 0.01)	+46.5 (± 1.2)	78 (± 3)
eDPPC:DPPC	1:1	150 (± 5)	0.13 (± 0.02)	+47.8 (± 1.4)	68 (± 3)
eDPPC:Chol	1:1	166 (± 4)	0.10 (± 0.02)	+45.5 (± 0.9)	79 (± 1)

Results are expressed as average $\pm$ standard deviation ($n = 3$ independent batches).

We investigated if the cationic liposome composition influences the interaction between the antigen and monocyte derived DCs. For this purpose we produced a HA-FITC conjugate (referred to as HA*), which was adsorbed to different cationic liposomes formulations, and monitored the relative amount of DC associated HA* with flow cytometry. Figure 2 shows that after 4 h incubation at 37 °C, the DCs mixed with positively charged liposomes (HA*/DC-Chol:DPPC) showed a higher MFI compared to the groups incubated with either HA* alone ($p < 0.05$) or HA* mixed with a neutral liposome formulation (HA*/DPPC:Chol). Not only the liposome's charge, however, affected HA's interaction with the DCs, but also the presence of Chol in the cationic liposomes: incubation of DCs with HA*/eDPPC:Chol resulted in a higher MFI than DCs incubated with HA*/eDPPC:DPPC. Besides, for DDA liposomes, the MFI induced by HA*/DDA:Chol tended to exceed that induced by HA*/DDA:DPPC, but no statistical significance was demonstrated.

With the above incubation study we assessed HA* association with DCs (*i.e.*, the sum of adhesion and uptake). In order to study the extent of HA* uptake by DCs, the incubation study was also performed at 4 °C, where energy dependent uptake is inhibited but adhesion will still occur [18]. DCs incubated with the cationic HA*-containing liposomes at 4 °C showed a lower MFI compared to the 37 °C groups, indicating that the cell-associated fluorescence at 37 °C was mainly caused by HA* uptake rather than association with the cell membrane. In contrast, DCs incubated with HA* alone or mixed with neutral liposomes showed hardly any decrease in MFI compared to the 37 °C conditions, indicating that most of the (low) MFI intensity at 37 °C was due to adhered HA*.

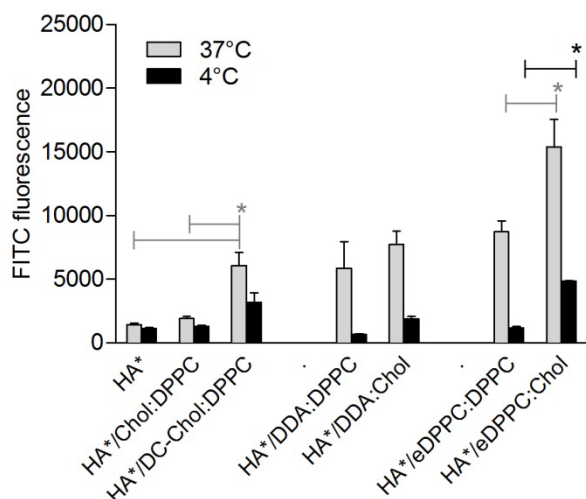


Fig. 2: Effect of formulations on HA-FITC (indicated as HA*) uptake by DCs. Bars represent the mean fluorescence intensity (+SEM) of three batches of DCs incubated with HA-FITC alone or mixed with liposomes for 4 h at 37 °C versus 4 °C. Significant differences between the formulations are indicated with * ($p < 0.05$). See Supplementary Material, Figure S1, for representative side scatter-forward scatter dot plots.

With the above incubation study we assessed HA* association with DCs (*i.e.*, the sum of adhesion and uptake). In order to study the extent of HA* uptake by DCs, the incubation study was also performed at 4 °C, where energy dependent uptake is inhibited but adhesion will still occur [18]. DCs incubated with the cationic HA*-containing liposomes at 4 °C showed a lower MFI compared to the 37 °C groups, indicating that the cell-associated fluorescence at 37 °C was mainly caused by HA* uptake rather than association with the cell membrane. In contrast, DCs incubated with HA* alone or mixed with neutral liposomes showed hardly any decrease in MFI compared to the 37 °C conditions, indicating that most of the (low) MFI intensity at 37 °C was due to adhered HA*.

The immunogenicity of the HA loaded cationic liposomes was assessed in mice. Figure 3 shows that the presence of Chol positively influenced the immune response against HA. In particular, eDPPC:Chol liposomes induced superior IgG1 titers after prime ($p < 0.05$) and boost ($p < 0.001$), as well as superior IgG2a/c and HI titers after boost ($p < 0.05$ and $p < 0.01$, respectively), compared to eDPPC:DPPC liposomes. The influence of Chol was not as clear for the DDA liposomes: DDA:Chol liposomes induced either a similar (IgG1 and HI titer) or a slightly but not significantly higher (IgG2a/c) immune response compared to liposomes without Chol (DDA:DPPC). These results are in line with the *in vitro* DC studies discussed above and suggest an overall positive effect of the presence of Chol in the liposomal bilayer on the adjuvanticity of the cationic eDPPC liposomes.

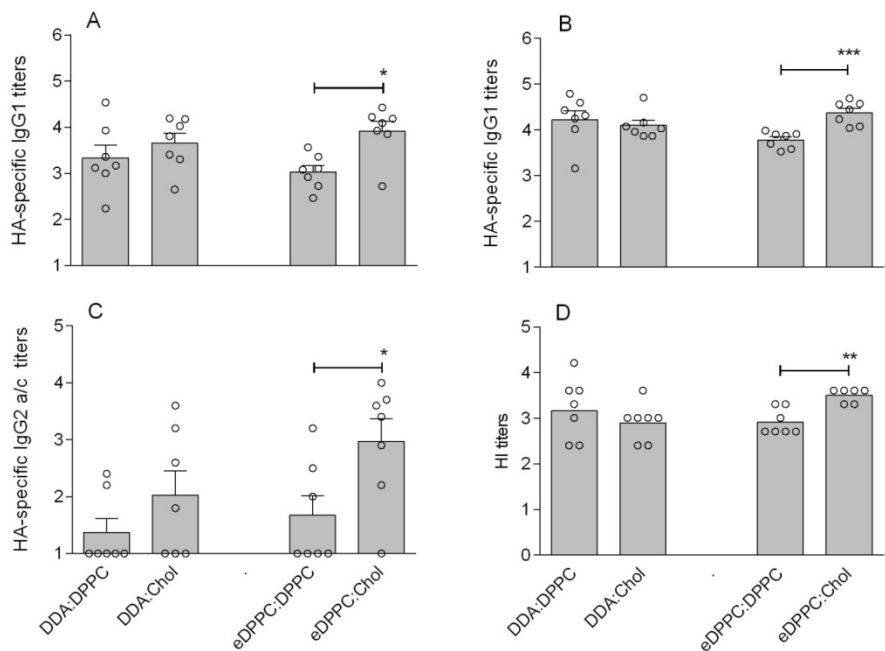


Fig. 3: Immune response in mice vaccinated with 2.0 µg HA, either free or mixed with liposomes with or without Chol: HA-specific serum IgG1 after prime (A) and boost (B); IgG2a/c after boost (C); and HI titer after boost (D). For panels A–C, each dot represents the log serum titer of an individual mouse (non-responding mice were given an arbitrary titer of 10) and bars represent average log titer + SEM. For panel D, each dot represents the log HI titer in serum of an individual mouse and bars represent the geometric mean. Significant differences between the groups treated with the liposomal formulations are indicated with * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$).

3.3. Encapsulation of Immune Modulators in DC-Chol:DPPC Liposomes

We prepared DC-Chol:DPPC liposomes with two different immune modulators: CpG and imiquimod (10 µg/mL). This resulted in three formulations with similar physicochemical characteristics and the addition of HA had little effect on their size, while a small drop of the ZP was observed (data not shown). The characteristics of the liposomes after mixing them with HA are summarized in Table 3. The average size of the adjuvanted HA/liposomes ranged between 165 and 177 nm and the average ZP between +43.4 and +47.2 mV. The incorporation of imiquimod in the DC-Chol:DPPC bilayer did not affect the HA loading efficiency. However, the encapsulation of CpG in the liposomes induced a drop of the HA loading efficiency from 60% (Table 1) to 30% (Table 3), probably due to competition between the antigen and the TLR ligand, both of which are negatively charged. The loading efficiency of CpG and imiquimod was practically 100%.

Table 3. Physicochemical characteristics of aluminium hydroxide and adjuvanted cationic liposomes after mixing with HA.

Formulation	Molar ratio	HA/liposomes (HA/alum)		
		Z _{ave} (nm)	pdi	ZP (mV)
Al(OH) ₃	1:1	5,624 (±543)	0.34 (±0.09)	+2.7 (±0.3)
DC-Chol:DPPC + CpG	1:1	177 (±4)	0.10 (±0.04)	+46.2 (±0.6)
DC-Chol:DPPC + Imiquimod	1:1	168 (±3)	0.09 (±0.02)	+43.6 (±2.6)

Results are expressed as average ± standard deviation ($n = 3$ independent batches).

In our previous study, DC-Chol:DPPC liposomes showed a relatively weak ability to activate DCs, in spite of their adjuvant effect demonstrated *in vivo* [8]. Since imiquimod and CpG have been described in the literature as potent immune modulators for DCs' activation [19,20], we investigated the potency of these two immune modulators incorporated in HA/DC-Chol:DPPC liposomes to enhance DC maturation. After 48 h of incubation, the DCs incubated with HA/liposome formulations containing immune modulators showed a significantly higher level ($p < 0.05$) of MHCII expression compared to DCs exposed to HA/DC-Chol:DPPC liposomes (Figure 4A). A comparable effect ($p < 0.05$) was observed for CD86 (Figure 4C), but not for CD40 (Figure 4B). These results indicate that encapsulating immune modulators in DC-Chol:DPPC liposomes enhances their adjuvanticity, as shown by the increased MHCII and CD86 expression indicative of DC activation [21].

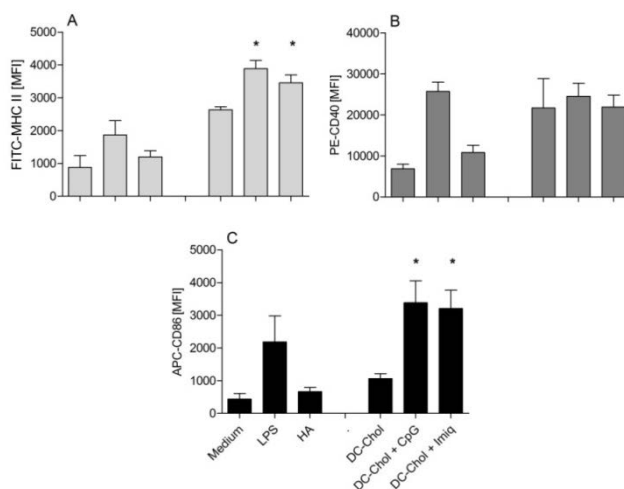


Figure 4. Upregulation of DC maturation markers induced by free HA *versus* HA mixed with liposomes: MHCII (A); CD40 (B); and CD86 (C). The values are expressed as percentage of mean fluorescence intensity (compared to a 100 ng/mL LPS control group, arbitrarily set as 100%). Error bars represent SEM ($n = 3$). Significant differences between the formulations and the DC-Chol:DPPC group are indicated with * ($p < 0.05$). See Supplementary Material, Figure S2, for representative side scatter-forward scatter dot plots.

The liposomal formulations were tested for their immunogenicity in mice in comparison with HA/DC-Chol:DPPC liposomes and HA adjuvanted with the licensed adjuvant: aluminum hydroxide (HA/Al(OH)₃) [22]. Our results showed CpG increased the immunogenicity in mice of the HA/liposomes, whereas imiquimod appeared to be ineffective (Figure 5). The incorporation of CpG in the HA/DC-Chol:DPPC liposomes resulted in a raise of the IgG2a/c titer and the number of responders compared to the HA/DC-Chol:DPPC liposomes alone ($p < 0.05$). Also the HI titer seemed to be increased, although not significantly ($p = 0.0548$). When compared to HA alone, Al(OH)₃ enhanced the anti-HA IgG1 titers (Figure 5A,B) and HI titers (Figure 5D), but not the IgG2a/c titers (Figure 5C). This is in line with aluminum salts known ability to enhance a Th2 type response [22]. The liposomal CpG formulation, however, not only induced significantly higher IgG1 and HI titers but also elicited higher IgG2a/c titers after the boost compared to HA/Al(OH)₃ (Figure 5B–D). In contrast, CpG alone (without liposomes) when mixed with HA did not induce a stronger immune response compared to HA/DC-Chol:DPPC liposomes or HA/Al(OH)₃ (unpublished results). This, together with the above-mentioned results, illustrates that encapsulation of CpG in cationic liposomes is advantageous for their adjuvant effect.

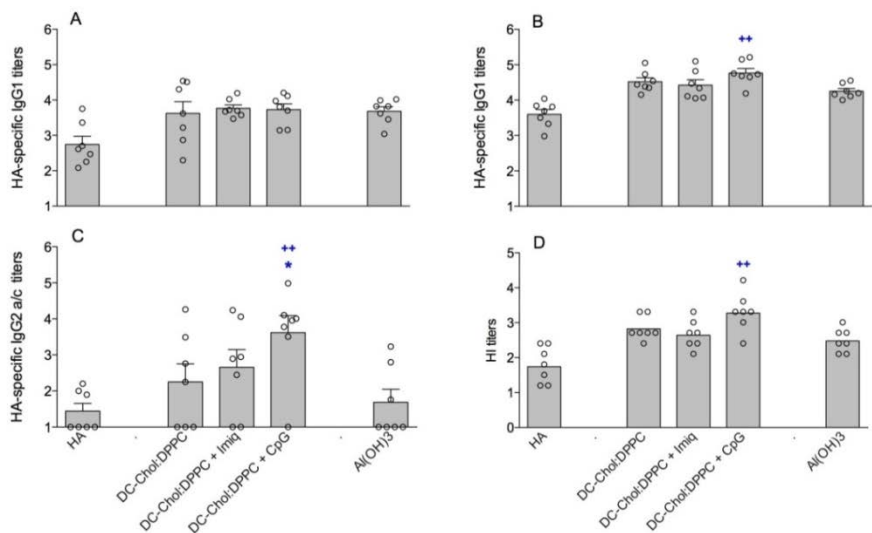


Fig. 5: Immune response in mice vaccinated with 2.0 μ g HA, free or mixed with Al(OH)₃ or with liposomes containing different immune modulators: HA-specific serum IgG1 after prime (A); and boost (B); IgG2a/c after boost (C); and HI titer after boost (D). For panels A–C, each dot represents the log serum titer of an individual mouse (non-responding mice were given an arbitrary titer of 10) and bars represent average log titer + SEM. For panel D, each dot represents the log HI titer in serum of an individual mouse and bars represent the geometric mean. Significant differences between the liposomal formulations and the DC-Chol:DPPC group are indicated with * ($p < 0.05$); significant differences between the liposomal formulations and the Al(OH)₃ group are indicated with ** ($p < 0.01$).

4. Discussion

In the present study, adsorption of HA to cationic DC-Chol:DPPC liposomes was shown to be a simple and effective method to enhance the immunogenicity of the antigen, as compared to liposomal encapsulation of the antigen. The higher HI titers induced by adsorbed HA compared to encapsulated HA might be explained by the presentation of the antigen at the outer surface of the liposomes, as previously detailed for a virosomal vaccine composed of influenza membrane fragments (including HA) mixed with phospholipids. It was shown that the positioning of the antigen in the virosomes influenced its processing and presentation pathway [23]. Although encapsulated and surface-associated liposomal antigens may induce T cell responses equivalently, several studies have shown increased antibody induction mediated by surface-associated antigen [24–26]. This may be because surface-associated antigen is available on the particle surface for antibody or B cell receptor recognition, whereas encapsulated antigen requires vesicle disruption to become accessible [27,28]. For surface-associated antigens, B cells may recognize intact liposomal antigen directly or via opsonized liposomes bound to Fc receptors or complement receptors on APCs [29]. Another hypothesis is that the way HA is associated with the liposomes could induce different processing of the antigen and a different kinetics of the anti-HA antibody response.

In our previous study, DC-Chol:DPPC liposomes were shown to be a stronger adjuvant for HA than cationic DPPC liposomes containing other cationic compounds (DDA, DPTAP, or eDPPC) [8]. However, it remained unclear whether the cationic tertiary amine (DC-) head group or the Chol backbone was responsible for the superior adjuvant effect. The results of our present study indicate that the presence of Chol contributes to the adjuvant effect of cationic liposomes. In particular, the HA/eDPPC:Chol liposomes enhanced both the antigen uptake by DCs *in vitro* and the immunogenicity of HA (HI titer, IgG2a/c and IgG1 antibody secretion) compared to the liposomes without Chol (HA/eDDPC:DPPC). A positive correlation between uptake by APCs and adjuvanticity *in vivo* has been described previously [30]. However, for the DDA-liposomes we noticed only a small influence of Chol, although there was a trend that the presence of Chol in DDA-liposomes increased HA uptake *in vitro* and enhanced the IgG2a/c response *in vivo*. The influence of Chol in liposomal formulations for vaccination has been investigated in some other studies. For instance, in a study about the relationship between the phospholipid composition and the immunogenicity of a liposomal tumor antigen, Bakouche *et al.* showed that optimal immunogenicity in rats was obtained with 20 mol% Chol in the liposomal bilayer [31]. Batenjany *et al.* [32] reported that the immunogenicity of a Muc1 mucin peptide in DPPC/Chol liposomes for immunotherapy of adenocarcinoma was optimal when the Chol content was above 30 mol%. Other studies also showed a beneficial effect of Chol on vaccination [33], but it is still not clear which mechanism is responsible for this effect. Considering that cell

membranes contain about 25 mol%–50 mol% of Chol, this lipid could play an important role in the interaction between cells and liposomes [28]. Furthermore, Chol is known to influence membrane fluidity and to enhance liposomal stability. For instance, the interaction of liposomes with plasma proteins has no negative effect on liposome stability when they are enriched in Chol [29], which is explained by its influence on the lipid packing in the liposomal membrane and its ability to prevent phospholipid loss due to uptake by high density lipoproteins [30].

Regarding the different antibody subtypes, we noticed a raise in the anti-HA IgG2a/c response after immunization with HA/eDPPC:Chol liposomes, which are known to modulate the immune response to a Th1 direction [34]. This effect might be explained not only by the improved ability of these liposomes to be taken up by DCs, but also by a more favorable environment for HA to interact with the cell membrane when associated with Chol-containing liposomes. Hemagglutinin in its natural environment (the influenza virus envelope) interacts with Chol rich membranes [35]. For virosomal HA it has been reported that, following endosomal uptake, acidification within the endosome induces HA-mediated fusion (resulting from a conformational change in HA), leading to release of the virosomes into the cytoplasm and a potential MHC class I presentation [36]. Therefore, even though we did not investigate those aspects in our study, it may be that HA itself enhances its own delivery into the cells, leading a potent immune response.

We focused our investigations on the influence of Chol in the lipid bilayer of different cationic liposomes. Christensen *et al.* [37] compared the immunogenicity of the antigen Ag85B-ESAT-6 combined with cationic liposomes prepared with either the saturated DDA (mixed with the immune modulator D-(+)-trehalose 6,6'-dibehenate [TDB]) or its unsaturated analog dimethyl dioleoyl ammonium bromide (DODA:TDB), which was also suggested as a comparison between rigid and fluid liposomes. The results showed that (gel-state) DDA liposomes were more retained at the injection site than (fluid-state) DODA liposomes, and were better at attracting APCs and inducing a Th1 response. Although we did not investigate the influence of liposome fluidity, the conclusion of Christensen *et al.* differs from what we observed in our work, as we concluded that liposomes based on a liquid organized state (50% Chol) were superior to rigid liposomes. However, it is very difficult to compare these two studies due to some major differences between the components used (e.g., the antigen, the immune modulator) and likely the physico-chemical characteristics (such as size and antigen loading).

In our last experiments, we used $\text{Al}(\text{OH})_3$ as a reference adjuvant. It is known that aluminum salts promote a Th2 response, and their adjuvant mechanisms is supposedly acting through antigen depot effect, enhancement of antigen uptake by antigen presenting cells (APCs) and the induction of inflammation (known to be activated through local release of uric acid and the triggering of the NALP3 inflammasome) [38,39]. In comparison, the encapsulation of immune modulators in the DC-Chol:DPPC liposomes not only enhanced the overall immune response, but also resulted in a raise of the anti-HA IgG2a/c response. This is in line with a raise of

INF- γ secretion by spleen cells (collected from spleens isolated after the *in vivo* study) induced by our formulations (see Supplementary Material, Figure S3). The encapsulation of CpG in nanoparticles has been shown in other studies to enhance the immune response against the co-encapsulated antigen toward a Th1 response [9,40,41,13]. For instance, Joseph *et al.* [42] succeeded to enhance the immunogenicity of a subunit influenza vaccines combined with CpG loaded DMPC:DMPG (dimyristoyl-phosphatidylcholine, dimyristoyl-phosphatidylglycerol) liposomes. Whereas they used 5 μ g CpG per dose, we used only 2 μ g. Dose reduction might be interesting with regard to the potential side effects of CpG. In contrast with liposomal CpG, free CpG administrated with HA was not effective in our study. This can be explained by the physicochemical properties of CpG for its delivery in soluble form to the intracellularly localized TLR-9 receptor.

Surprisingly, imiquimod, when encapsulated in HA/DC-Chol:DPPC liposomes, did not lead to a better immune response compared to plain HA/DC-Chol:DPPC liposomes. The beneficial activity of TLR-7 ligands was reported in by Geeraedts *et al.* [43], who explained the superior protection induced by H5N1 whole inactivated virus (WIV) compared with subunit or split virus by TLR-7 stimulation from the RNA contained in the WIV vaccine. Therefore, we expected a stronger impact of the TLR-7 agonist imiquimod on the immunogenicity of our influenza subunit vaccine. Weldon *et al.* [44] reported that skin delivery of influenza H1N1 subunit vaccine combined with imiquimod elicited higher levels of serum IgG2a antibody and HI titers as compared to unadjuvanted vaccine in Balb/c mice. They used a similar antigen/imiquimod ratio and imiquimod dose compared to our present study. Besides the different mouse model, the route of administration [45] could be a reason for the discrepancy between their and our results. Moreover, the imiquimod dose used in our study might have been too low. For instance, Rizwan *et al.* [14] succeeded to raise the humoral response against OVA after intramuscular injection in C57Bl/6 mice using liposomes adjuvanted with monophosphoryl lipid A combined with very high doses of imiquimod (150 μ g/immunization). Finally, the localization of the imiquimod in the DC-Chol:DPPC liposomal membrane might have limited its interaction with the TLR-7 receptor. Indeed, imiquimod is a hydrophobic molecule [46] and it was mixed with the lipid film before hydration. This might have led to entrapment deep in the lipid bilayer. It would be of interest in future studies to determine to which extent CpG and imiquimod are liberated from the liposomes.

As mentioned earlier, IgG1 and IgG2a/c antibody levels can be used as indicators of a Th2 and Th1 immune response, respectively. As demonstrate in this study, HA needs to be combined with adjuvants to efficiently stimulate both Th1 and Th2 responses, as has also been shown by others [16]. There is growing evidence that both (CD4+) T helper cells and (CD8+) cytotoxic T lymphocytes not only play an important role in controlling viral infection, but also may reduce the severity of disease and decrease mortality [47,48]. The use of cationic liposomes containing immune modulators such as CpG to increase the immunogenicity of the antigen and

modulate the immune response towards the Th1 direction is therefore a highly relevant approach.

5. Conclusions

Notwithstanding the clear advantage of using DC-Chol cationic liposomes as an adjuvant for HA, there is still a lack of knowledge regarding their adjuvant mechanism. The data presented here shed some more light on this. First, HA adsorption to the liposome surface was shown to be at least as effective, and an easier way to enhance the immunogenicity of HA, compared to HA encapsulation. Moreover, the presence of Chol in the cationic liposomes appeared to be beneficial for the immune response against HA, an effect that depended on the cationic compound selected. Furthermore, HA was taken up by DCs to a higher extent and was more immunogenic in mice when it was mixed with eDPPC:Chol liposomes compared to eDPPC:DPPC liposomes. Finally, co-delivery of CpG with HA/DC-Chol:DPPC liposomes was found to further promote the immunogenicity of the antigen. In conclusion, cationic liposomes have potential for influenza vaccination provided that the bilayer components and the immune modulator to be encapsulated in these liposomes are carefully selected and their adjuvant mechanism is further investigated.

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Supplementary Information

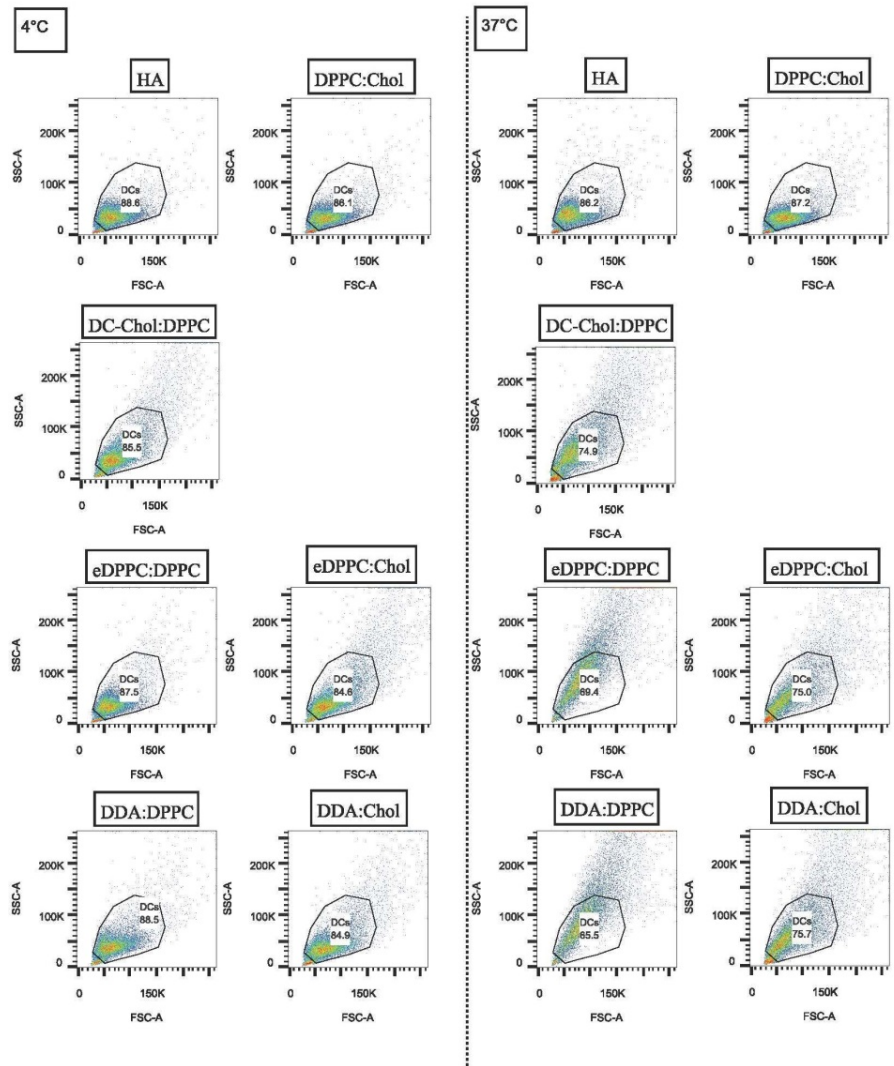


Fig. S1: Side scatter-forward scatter (SSC/FSC) dot plots of DCs after 4 h incubation at 4 °C and 37 °C with plain HA and HA/liposome formulations. The gate represents the DC population and excludes cellular debris. The percentages of cells included in the DC gate decreased by ca. 10% when the DCs were incubated with cationic liposomes, suggesting a slight toxic effect. No such a decrease was noticed with either HA or (neutral) DPPC:chol liposomes.

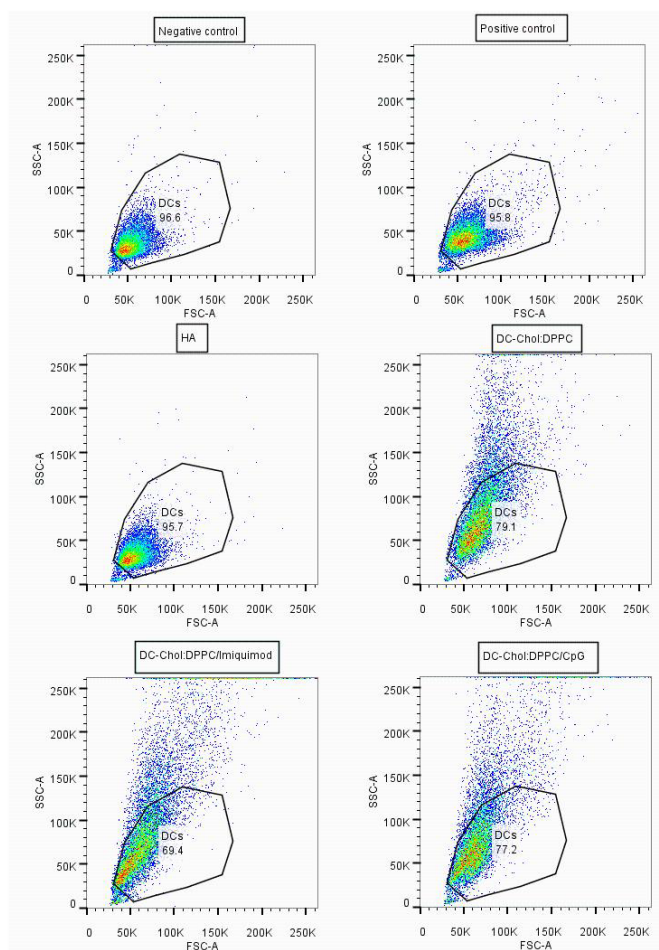


Fig. S2: Side scatter-forward scatter (SSC/FSC) dot plots of DCs after 48 h incubation at 37 °C with plain HA, plain HA/DC-Chol:DPPC liposomes and adjuvanted HA/DC-Chol:DPPC liposome formulations. The gate represents the DC population and excludes cellular debris. The percentages of cells included in the DC gate when the DCs were incubated with cationic liposomes were similar to those observed after 4 h (Figure S1).

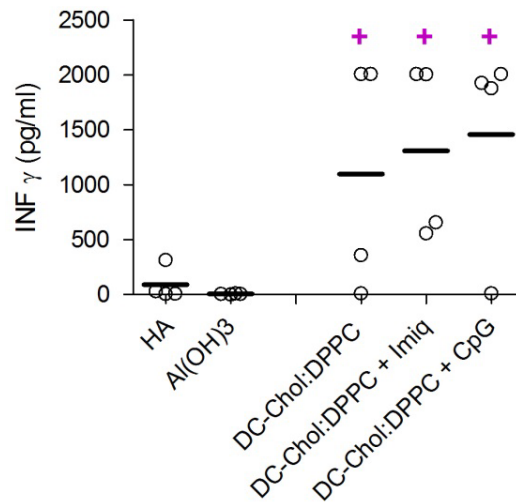


Fig. S3: IFN- γ levels secreted by spleen cells collected from mice immunized with 2.0 μ g HA, free or mixed with Al(OH)₃ or with liposomes containing different immune modulators. The spleens were collected three weeks after the boost immunization, after homogenization the cells were re-stimulated with 5 μ g/mL HA, and the release of IFN- γ was determined by ELISA. Significant differences between the liposomal formulations and the Al(OH)₃ group are indicated with + ($p < 0.05$).

Chapter 7

Summary and perspectives

7.1 – Summary

The best form of protection against influenza is vaccination, in terms of efficacy to protect individuals and reduction of the social impact of epidemics on our human societies. Chapter 1 of this thesis details the current influenza vaccines available and their lack of efficacy, and the current need for new adjuvanted influenza formulations. Pathogens are often particles and formulating antigens into nanoparticles (NP) results in systems that resemble the pathogens in terms of size, and notably can promote antigen uptake by dendritic cells (DC). The principal aim of the research in this thesis was to investigate how NP systems can act as an adjuvant for subunit influenza vaccine.

To achieve this aim two types of NP are described: peptide polymer NP and cationic liposomes, and the following three sub-aims were defined:

- to explore different nanoparticulate systems in order to modulate the immunogenicity of a subunit influenza vaccine;
- to study the impact of the composition, charge and preparation of these systems on their adjuvant effect;
- to investigate the co-delivery of HA antigen with immune-modulators, encapsulated into nanoparticulate systems.

First, the potential of polymer-peptide block copolymer NP as adjuvant for seasonal influenza vaccine was studied (**chapter 2- 4**). For this purpose we customized the constituents and the preparation method. The poly(γ benzyl L-glutamate)-E (PBLG-E) represents the first of a new class of peptides: polypeptide-b-peptides. These compounds are versatile regarding their chain length and functionality. Different methods are available to produce polymer-peptide based NP, but each of them has limitations and might not be suitable for vaccine delivery. Therefore, in **chapter 2** we have developed a new method for producing nanovesicles, also known as polymersomes, from polypeptide-b-peptides PBLG36-E, using a detergent removal method that has been used for many decades to produce liposomes. The method was adapted to be suitable for use with block copolymers, which have different assembly characteristics than lipids. The detergent aided polymersome preparation utilizes detergent molecules (sodium cholate) to molecularly disperse the block copolymer in aqueous solution, a role that is usually taken by organic solvents. The shielding effect of the detergent on the block copolymer is then reduced such that the intrinsic morphology of the block copolymer particles, i.e. polymersomes, emerges. This method has the advantage of not requiring organic solvent or a high energy input (e.g., sonication), known has factors which can denature biomolecules.

Chapter 3 presents a study of the adjuvant effect of polymersomes loaded with a seasonal influenza subunit vaccine (H3N2 A/Wisconsin strain). The polymersome's building material was PBLG₅₀-K block copolymer and the water-addition solvent-

evaporation method was used to produce the nanovesicles. The block copolymer was dissolved in tetrahydrofuran (THF) and the solution was quickly added to an aqueous phase, allowing the THF to evaporate in a couple of minutes. The PBLG₅₀-K was shown to be assembled into polymersomes with an average size of 250 nm and a negative zeta potential. Then different amounts of vaccine (the purified viral membrane protein hemagglutinin (HA)) were mixed with the NP, resulting in a raise of the average size and polydispersity. The physical association of HA and polymersomes in these aggregates was confirmed by transmission electron microscopy (TEM). The immune response induced by the polymersomes was investigated *in vivo* in a mouse model. The polymersomes succeeded to enhance significantly both total serum IgG and hemagglutination inhibition (HI) titers, compared to non-adjuvanted antigen. However, the polymersome formulation induced a high IgG1 response and a low IgG2a/c response, which is indicative of strong Th2 response, while the Th1 response was rather low.

To optimize the efficacy of the peptide polymer NP toward a Th1 response, two major changes were introduced, as described in **chapter 4**: firstly, a new peptide polymer copolymer (PBLG₃₀-TAT), based on the amino acid sequence derived from the cell-penetrating TAT peptide; second, an immune modulator (CpG) was encapsulated into the NP. After a comprehensive physicochemical characterization of these new systems, the immunogenicity was tested *in vitro* with human DC, and the formulations showed the ability to induce an upregulation of maturation markers (MHC-II and CD86) when codelivered with CpG (NP/HA+CpG). Furthermore, after intramuscular vaccination in mice, the NP/HA+CpG formulation elicited stronger HI titers compared to non-adjuvanted NP/HA and the Al(OH)₃/HA control. Besides, NP/HA+CpG provoked significantly higher levels of IgG2 a/c antibodies compared to all other formulations.

In **chapter 5 & 6**, cationic liposomes' adjuvant mechanism was investigated, using HA as a model antigen. The different immunological effects induced by cationic liposomes, when they are used for vaccine formulation, suggest that not only their cationic charges initiate their adjuvant abilities by increasing antigen protection and delivery, but also that possible specific effect of the lipids or liposomes exist. However, previous studies used liposomes that are not directly comparable (e.g., due to the use of variable antigens, different administration routes, etc.). Therefore, in **chapter 5** we studied how the content and the physicochemical properties of the positively charged compound influence the adjuvant effect of cationic liposomes. In order to enable the focus on the liposome content, we prepared cationic liposomes made of different cationic compounds (DDA, DPTAP, eDPPC) but with similar physicochemical characteristics (size, zeta potential, bilayer rigidity, etc.), loaded via adsorption with HA. We also included DC-Chol based liposomes in our comparison, while recognizing that their characteristics will differ from the other cationic compounds with respect to both the head group and the hydrophobic tail. In a mouse model, HA adjuvanted with the DC-Chol/DPPC

liposomes elicited significantly higher total anti-HA antibodies (IgG1 and IgG2a/c) and HI titers compared to the other liposomal HA formulations and non-adjuvanted HA. However, it was not clear whether the cholesterol backbone or the tertiary amine head group of DC-Chol was responsible for this.

Therefore, in **chapter 6** the influence of cholesterol in the lipid bilayer of cationic liposomes on the immunogenicity of adsorbed HA was studied. For this purpose, liposomes consisting of a neutral lipid (DPPC or cholesterol) and a cationic compound (DDA, or eDPPC) were produced and characterized. They generally showed comparable size distribution, zeta potential and HA loading. Furthermore, in vitro studies of the formulations with monocyte-derived human DC and immunization studies in C57BL/6 mice showed that the incorporation of cholesterol in the bilayer of cationic eDPPC liposomes enhances the cellular uptake and also their adjuvant effect in vivo. Moreover, to further improve the immunogenicity of HA-loaded DC-Chol liposomes, they were loaded with CpG or imiquimod. Whereas encapsulation of imiquimod did not seem to have any impact on the immune response, encapsulation of CpG in DC-Chol liposomes enhanced significantly the IgG2 a/c titers against adsorbed HA compared to HA adsorbed to non-adjuvanted DC-Chol liposomes or Alhydrogel, and showed increased IFN- γ production by restimulated splenocytes.

7.2 - Perspectives

This thesis has set out to study the impact of nanoparticulate adjuvants on the immune response against the antigen, HA. In the next sections several important aspects of adjuvanted influenza vaccine design are discussed and recommendations for future development are provided.

7.2.1 - Further investigation of the immune response induced by nanoparticulate adjuvanted influenza vaccines

Several aspects concerning nanoparticulate influenza vaccine formulations have been studied in this thesis, in particular the characterization of the formulations and their immunological effects (in vitro and in vivo). The results obtained showed that both cationic liposomes and peptide polymer NP have good adjuvant properties, enabling the raise of the immune response against HA. Importantly, IgG2a/c antibodies were significantly raised when HA was formulated in NP (chapter 4 & 6), which reflects the induction of a Th1 response (confirmed by the increase in INF γ secretion), and likely a CTL response which is more efficient at eliminating influenza infected cells. In order to evaluate the protective effect our best formulations, a challenge in mice (or ferrets) should be performed to determine if this increased

immunogenicity results in a better protection against influenza infection. Moreover, the dose-sparing ability of our adjuvant systems should be studied.

Subunit influenza vaccines might be less efficient at inducing cell-mediated immunity normally induced by natural infections. Subunit influenza vaccines are “clean vaccines”, purified and with a low content of influenza nucleoproteins, and rich in HA protein which is the most variable region of the virus. However, if the vaccines induce a humoral and a cell-mediated immune responses directed to conserved regions of influenza virus, they are more likely to induce protective immunity to a large variety of influenza viruses, including drift variants and viruses of novel subtypes. For instance, MF59 can both improve the antibody responsiveness to influenza and redirect the quality of the antibody response against influenza antigens. This oil-in-water emulsion induced more cross reactive responses when administered with split or subunit H5N1 vaccines than non-adjuvanted or aluminum-adjuvanted vaccines [1] [2]. Similarly, investigation of cross-reactive responses after immunization with our nanoparticulate influenza vaccines would be of high interest.

Also, the preliminary DC maturation studies conducted on human monocyte-derived DC suggest an immunostimulatory adjuvant effect of our formulations. Future experiments should be done to investigate whether the influence of these formulations on the immune response is the same in murine DC and in other animal models, along with toxicity studies (local and systemic). Additionally, more effort should be put to investigate the persistence of the immune response and to study the immunogenicity induced by other routes of immunization, especially the intradermal route. Intradermal vaccination, e.g. by using microneedle-mediated delivery, could also be an attractive alternative to intranasal immunization in the context of the potential induction of protective mucosal IgA. Nasal administration has been associated with adverse effects, such as the occurrence of Bell's palsy syndrome (facial nerve paralysis) induced by an adjuvanted virosomal vaccine after intranasal immunization in humans [3].

7.2.2 – Specific interaction between delivery systems with the influenza antigen

The core of each vaccine formulation is the antigen. It is therefore not surprising that the rational design of a vaccine should be based on the characteristics of the antigen. The model antigen in this thesis is (HA) a viral membrane protein, which is water soluble and negatively charged at physiological pH. As noticed in the different *in vivo* studies conducted in this thesis, HA is immunogenic when injected alone, but it induces mainly IgG1 antibodies and moderate HI titers. As demonstrated in chapter 4 & 5, HA interaction with cationic liposomes and peptide polymer NP is driven by electrostatic forces. Moreover, the antigen's lipophilic domains may profit from formulation in liposomes as these membrane proteins can be incorporated in

the liposomal bilayer, thereby mimicking more closely the natural way these antigens are presented to the immune system. Cryo-TEM characterization of the liposomes produced could help understand if the positioning of HA could contribute to the differences observed between our formulations. Interestingly, in Chapter 3 the interaction of HA with the peptide polymer NPs showed that HA/polymersome association was presumably a combination of both electrostatic and hydrophobic interactions, arising from the hydrophobic membrane-anchoring domain of the HA, the localized charge on the HA, and the charged corona of the polymersomes.

Moreover, the action of HA is not limited to its immunogenic properties. Following endosomal uptake, acidification within the endosomes induces HA-mediated fusion (resulting from a conformational change in HA), which likely leads to release of the liposomes or peptide polymer NP into the cytoplasm and a potential MHC class I presentation. Therefore, even though this was not investigated in this thesis, it may be that HA itself enhances its own delivery into the cells, leading a potent immune response.

7.2.3 – Need for a better understanding of the NP's adjuvant mechanisms

The immunogenicity of HA was notably improved by the use of DC-Chol liposomes. Although the presence of cholesterol does induce specific interaction of the liposome with the biological systems, it remains unknown whether the presence of the cholesterol backbone was also the reason for the superior immunogenicity of HA/DC-Chol:DPPC liposomes and, if so, by which mechanism. In Chapter 5, the use of DC-Chol liposomes loaded with HA in a mouse model increased the antibody responses, both the IgG1 and IgG2 a/c antibody responses, which is consistent with the results obtained in previous studies where DC-Chol lipids administered with hepatitis B surface antigen [6] or monovalent split inactivated influenza vaccine (H1N1) resulted in improved immune responses in animal models [7].

Studies to elucidate the mechanisms by which DC-Chol liposomes act as adjuvants have suggested a role for the chemokine CCL2, secreted by epithelial cells and involved in Langerhans cell recruitment [8] and complement activation [9]. Another mechanism may be the ability of DC-Chol liposomes to associate with antigen and initiate a depot-effect [10]. The raise of chemokine secretion probably leads to DC migration and local inflammation. It would be interesting to study the role of plain DC-Chol liposomes, by injecting separately the liposomes and the antigen at the same site of injection, which should preclude the antigen depot effect and enable the monitoring of the potential recruitment of the APCs and macrophages, and the activation of the NLRP3 inflammasome pathway. Finally, despite the concern about eventual cytotoxicity of cationic liposomes, DC-cholesterol has already been tested in the clinic with an HIV recombinant gp160 antigen [11]. The results showed a good tolerability of the vaccine after nasal or vaginal administration.

Following the work achieved in this thesis, the future of the peptide polymer NP as adjuvant system should

be addressed. Our studies showed some efficacy for their use in vaccine formulations with strong adjuvant and vaccine carrier abilities, allowing co-delivery of the antigen with a TLR-9 ligand (CpG). Insights into the liposome adjuvant mechanism could partially be extended to these new systems, which could notably be explained by a depot effect. However, more investigations should be carried out in order to get a better understanding of their adjuvant mechanism, and the influence of the formulation. Different immune stimulators could be either added or incorporated to further enhance the immunogenicity. Since vesicles composed of polypeptide-b-designed peptides can be easily functionalized, it is expected that these peptide-based NP will be able to act as delivery vehicles to specific targets in the body. Furthermore, their peptide sequence could be designed to modulate the immune response. For instance, the IC31® adjuvant [12], currently in phase II clinical trials, is based on the cationic peptide KLKL5KLK with successful results with the tuberculosis antigen Ag85B [13] [14].

Finally, an ideal vaccine should contain a sufficient amount of immune modulators for the activation of the innate immune response and alert the immune system, but at the same time without causing hyper-immunostimulation (which may result in anaphylactic shock or local tissue damage by excess of inflammatory mediators). The immune modulator(s) should also stay associated with the antigen until uptaken by APCs. The NPs seem to be the ideal system to ensure such co-delivery.

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Appendices

Nederlandse samenvatting

Abbreviations

List of publications

Curriculum vitae

Nederlandse samenvatting

De beste vorm van bescherming tegen griep is vaccinatie, zowel qua effectiviteit om individuen te beschermen als qua vermindering van de sociale gevolgen van een epidemie op onze samenleving. **Hoofdstuk 1** van dit proefschrift beschrijft de huidige verkrijgbare griepvaccins en hun gebrek aan doeltreffendheid, en de huidige vraag naar nieuwe op adjuvantia gebaseerde formuleringen voor griepvaccins. Ziekteverwekkers zoals griepvirussen kunnen beschouwd worden als deeltjes. Het formuleren van antigenen in nanodeeltjes resulteert in systemen die ziekteverwekkers nabootsen wat betreft grootte en die bovendien de opname van antigenen door dendritische cellen (DCs) kunnen bevorderen. Het voornaamste doel van het in dit proefschrift beschreven onderzoek was er achter te komen hoe nanodeeltjessystemen kunnen fungeren als een adjuvans voor subunit-griepvaccins (d.w.z. griepvaccins die gebaseerd zijn op onderdelen, subunits, van het griepvirus). Hiertoe werden twee soorten nanodeeltjes onderzocht: op polypeptide gebaseerde nanodeeltjes en kationische liposomen. Er werden drie sub-doelen gedefinieerd:

- het ontwerpen van verschillende nanodeeltjessystemen om de immunogeniciteit van een subunit-griepvaccin te moduleren;
- het bestuderen van de gevolgen van de samenstelling, lading en bereidingsmethode van deze systemen op hun adjuvanseffect;
- het onderzoeken van de gecombineerde toediening van het gezuiverde virale membraaneiwit hemagglutinine (HA) en immuunmodulatoren, verpakt in nanodeeltjessystemen.

Als eerste werd de potentie van op polypeptide gebaseerde nanodeeltjes als adjuvans voor een vaccin tegen seizoensgebonden griep bestudeerd (**hoofdstuk 2-4**). Hiertoe werd gebruikgemaakt van poly(-benzyl L-glutamaat)-E (PBLG-E), de eerste van een nieuwe klasse van peptiden: de polypeptide-*blok*-peptiden. Deze verbindingen zijn veelzijdig wat betreft hun ketenlengte en functionaliteit. Er zijn verschillende methoden beschikbaar om nanodeeltjes die op polypeptiden gebaseerd zijn te produceren, maar elk van hen heeft beperkingen en is mogelijk niet geschikt voor de toediening van vaccins. Daarom hebben we een nieuwe methode ontwikkeld voor het produceren van nanoblaasjes (ook bekend als polymeersomen) uit polypeptide-*blok*-peptiden PBLG₃₆-E (waarbij het getal 36 het gemiddeld aantal benzyl L-glutamaat-residuen per PBLG-E molecuul weergeeft), gebruikmakend van een detergens- verwijderingsmethode die al vele tientallen jaren gebruikt wordt om liposomen te produceren (**hoofdstuk 2**). De methode werd aangepast voor PBLG-E, dat andere assemblage-eigenschappen heeft dan lipiden. De op detergens gebaseerde polymeersoombereiding maakt gebruik van detergensmoleculen (natriumcholaat) die het PBLG-E solubiliseren in een waterige oplossing – waar normaal gezien een organisch oplosmiddel voor wordt gebruikt.

Tijdens de verwijdering van het detergens wordt het solubilisatie-effect verminderd waardoor de PBLG-E moleculen zich organiseren in polymeersomen. Deze methode heeft als voordeel dat er geen organisch oplosmiddel of hoge energie (zoals bij sonicatie) aan te pas komt – factoren waarvan bekend is dat ze biomoleculen kunnen denatureren.

Hoofdstuk 3 beschrijft een studie van het adjuvanseffect van polymeersomen beladen met een subunitvaccin tegen seizoensgebonden griep (HA afkomstig van de H3N2 A/Wisconsin-stam). Het bouw materiaal voor de polymeersomen was het PBLG₅₀-K polypeptide-*blok*-peptide en de watertoevoegings-oplosmiddelverdampingsmethode werd gebruikt om de polymeersomen te produceren. Het PBLG₅₀-K werd opgelost in tetrahydrofuraan (THF) en de oplossing werd snel toegevoegd aan een waterige oplossing, zodat de THF binnen enkele minuten verdampte en polymeersomen met een gemiddelde diameter van 250 nm en een negatieve zeta-potentiaal gevormd werden. Vervolgens werden verschillende hoeveelheden van het antigeen (HA) gemengd met de nanodeeltjes, resulterend in een verhoging van de gemiddelde grootte en polydispersiteit. De fysieke associatie van HA en polymeersomen werd bevestigd door transmissie-elektronenmicroscopie (TEM). De door de polymeersomen geïnduceerde immuunrespons werd onderzocht in een muismodel. De polymeersomen slaagden erin om zowel de totale serum IgG- als de hemagglutinatie-inhibitie (HI)-titers significant te verhogen ten opzichte van antigeen zonder adjuvans. De polymeersoomformulering induceerde echter een hoge IgG1-respons en een lage IgG2a/c-respons, wat op een sterke Th2-respons duidt, terwijl de Th1-respons vrij laag was.

Om de werkzaamheid van de op polypeptiden gebaseerde nanodeeltjes richting een Th1-respons te optimaliseren werden twee belangrijke veranderingen doorgevoerd, zoals beschreven in **hoofdstuk 4**: ten eerste werd een nieuw polypeptide-*blok*-peptide (PBLG₃₀-TAT) ontwikkeld, gebaseerd op de aminozuurvolgorde afgeleid van het celpenetrerende TAT-peptide; ten tweede werd een immuunmodulator (CpG) verpakt in de nanodeeltjes. Na een uitgebreide fysisch-chemische karakterisering van deze nieuwe systemen werd de immunogeniciteit *in vitro* getest in menselijke DCs, waaruit bleek dat de formuleringen een verhoogde afgifte van maturatiemarkers (MHC-II en CD86) konden induceren wanneer ze samen met CpG werden toegediend (nanodeeltjes/HA+CpG). Verder induceerde de nanodeeltjes/HA+CpG formulering na intramusculaire toediening aan muizen sterkere HI-titers in vergelijking met nanodeeltjes/HA zonder adjuvans en HA geadjuveerd met Alhydrogel [Al(OH)₃]. Daarnaast wekte de nanodeeltjes/HA+CpG formulering aanzienlijk meer IgG2a/c-antilichamen op dan alle andere formuleringen, hetgeen wijst op een verhoogde Th1-respons.

Hoofdstukken 5 & 6 beschrijven het onderzoek naar het adjuvansmechanisme van kationische liposomen, waarbij HA als modelantigeen werd gebruikt. De verschillende door kationische liposomen geïnduceerde immunologische effecten suggereren dat niet alleen hun lading, maar ook een specifiek effect van de lipiden

ten grondslag ligt aan hun werking als adjuvans. In eerdere studies werden echter liposomen gebruikt die onderling niet direct vergelijkbaar waren (door gebruik van verschillende antigenen, andere toedieningsroutes, etc.). Daarom bestudeerden we hoe de samenstelling en fysisch-chemische eigenschappen van de positief geladen component het adjuvanseffect van kationische liposomen beïnvloedt (**hoofdstuk 5**). Om de invloed van de liposoomsamenstelling te onderzoeken werden kationische liposomen bereid met verschillende kationische componenten (DDA, DPTAP, eDPPC) maar met vergelijkbare fysisch-chemische eigenschappen (grootte, zeta-potentiaal, vloeibaarheid van de bilaag). De liposomen werden beladen met HA door middel van adsorptie. We namen op DC-Chol gebaseerde liposomen ook op in onze vergelijking, erkennende dat de eigenschappen van DC-Chol verschillen van die van de andere kationische componenten wat betreft zowel de hydrofiele kopgroep als de waterafstotende steroidstructuur. HA met de DC-Chol/DPPC-liposomen als adjuvans wekte significant hogere totale anti-HA-antilichamen- (IgG1 en IgG2a/c) en HI-titers op in een muismodel dan de andere liposomale HA-formuleringen en HA zonder adjuvans. Het was echter niet duidelijk of de waterafstotende steroidstructuur of de tertiaire amine-kopgroep van DC-Chol hiervoor verantwoordelijk was.

Daarom werd de invloed van cholesterol in de bilaag van kationische liposomen op de immunogeniciteit van geadsorbeerd HA bestudeerd (**hoofdstuk 6**). Hiertoe werden liposomen bestaande uit neutrale lipiden (DPPC of cholesterol) en kationische componenten (DDA of eDPPC) bereid en gekarakteriseerd. Over het algemeen lieten ze een vergelijkbare grootteverdeling, zeta-potentiaal en HA-belading zien. Verder lieten *in vitro* studies met monocyt-afgeleide menselijke DCs en immunisatiestudies in C57BL/6-muizen zien dat de incorporatie van cholesterol in de bilaag van kationische eDPPC-liposomen de cellulaire opname en ook hun adjuvanseffect *in vivo* verhoogt. Om de immunogeniciteit van HA-beladen DC-Chol-liposomen verder te verbeteren, werden ze bovendien beladen met CpG of imiquimod. Waar belading met imiquimod geen invloed op de immuunrespons leek te hebben, verbeterde de verpakking van CpG in DC-Chol-liposomen aanzienlijk de IgG2a/c-titers tegen geadsorbeerd HA in vergelijking met HA geadsorbeerd aan DC-Chol-liposomen zonder adjuvans of HA geformuleerd met Alhydrogel. Bovendien liet het een verhoogde IFN- γ productie door geherstimuleerde splenocyten zien.

Abbreviations

^1H -NMR, ^1H Nuclear magnetic resonance
ANOVA, Analysis of variance
APC, Antigen-presenting cell
BCA, Bicinchoninic acid protein assay
BSA, Bovine serum albumin
Chol, Cholesterol
CMC, Critical micelle concentration
Con A, Concanavalin A
CpG, Cytosine phosphodiester guanine oligomer
CTL, Cytotoxic T lymphocyte
DC, Dendritic cell
DC-Chol, Dimethylaminoethane-carbomyl cholesterol
DCM, Dichloromethane
DDA Dimethyldioctadecylammonium bromide
Dh, Hydrodynamic diameter
DLS, Dynamic light scattering
DMEM, Dulbecco's minimum essential medium
DMF, Dimethylformamide
DMF, N,N Dimethylformamide
DMPG, Dimyristoyl-phosphoglycerol
DMSO, Dimethyl sulfoxide
DOPC, Dioleoyl- glycerophosphatidylcholine
DOTAP, Dioleoyl-trimethylammonium-propane
DPPC, Dipalmitoyl-glycerophosphatidylcholine
DPTAP, Dipalmitoyl-trimethylammonium-propane
DSC, Differential scanning calorimetry
E, Glutamic acid
eDOPC, Dioleoyl-glycerophosphocholine
eDPPC, Dipalmitoyl-glycerophosphocholine
ELISA, Enzyme-linked immunosorbent assay
EM, Electron microscopy
FACS, Fluorescence-activated cell sorting
FCS, Fetal calf serum
FITC, Fluorescein isothiocyanate
Fmoc, Fluorenylmethoxycarbonyl
G, Glycine
GM-CSF, Granulocyte-macrophage colony-stimulating factor
GPC, Gel permeation chromatography
HA, Hemagglutinin
HBSS, Hank's balanced salt solution

HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
 HI, Hemagglutination inhibition
 HPLC, High-performance liquid chromatography
 HRP, Horseradish peroxidase
 I, Isoleucine
 IgG, Immunoglobulin G
 IL, Interleukin
 INF γ , Interferon gamma
 K, Lysine
 L, Leucine
 LC-MS, Liquid chromatography-mass spectrometry
 LPS, Lipopolysaccharide
 MALDI-TOF, Matrix-assisted laser desorption-ionization time-of-flight
 MHC I/II, Major histocompatibility complex class I/II
 MPL, Monophosphoryl lipid A
 MS, Mass spectrometry
 MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
 NA, Neuraminidase
 NCA, N-carboxyanhydride
 NMP, Nitroxide-mediated polymerization
 NP, Nanoparticle
 O/W, Oil-in-water (emulsion)
 OVA, Ovalbumin
 PAMP, Pathogen associated molecular pattern
 PBLG, poly(γ -benzyl L-glutamate)
 PbP-A, polypeptide-b-peptide amphiphile
 PBS, Phosphate buffered saline
 PC, Phosphatidyl choline
 PDI, Polydispersity index
 PEG, Poly(ethylene glycol)
 PRR, Pattern recognition receptor
 PTA, Phosphotungstic acid
 R, Arginine
 RNA, Ribonucleic acid
 ROP, Ring-opening polymerization
 RP-HPLC, Reversed-phase high-pressure liquid chromatography
 S, Serine
 s.c, Subcutaneous
 SEM, Scanning electron microscopy
 TCR, T cell receptor
 TDB, Trehalose 6,6'-dibehenate
 TEM, Transmission electron microscopy
 TFA, Trifluoroacetic acid

Tg, Glass transition temperature

Th, T helper cell

THF, Tetrahydrofuran

TLR, Toll like receptor

Tm, Melting temperature

TMB, Tetramethylbenzidine

UV, Ultraviolet

VIS, Visible

WIV, Whole inactivated virus

ZP, Zeta potential

List of publications

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Curriculum vitae

Christophe Barnier-Quer was born on the tenth of March 1974 in the city of Hyeres (France), which is located in the Provence. He studied medicine at the University of Paris VII, France. In addition, he obtained a Master of Science degree in pharmaceutical technology and biopharmacy at the University Paris South, France. During this period he completed a research project “Liposome encapsulation of a cyclin-dependent kinase Inhibitor (Roscovitine): effect on tumor growth” at the French National Centre for Scientific Research (CNRS), under the supervision of Dr. Michel Lenoir.

In 2007 he started his PhD project on “Adjuvanted nanoparticulate seasonal influenza vaccines” led by Prof. Wim Jiskoot and Dr. Alexander Kros, at the Division of Drug Delivery Technology of the Leiden/Amsterdam Center for Drug Research (LACDR), Leiden University, the Netherlands. This project was performed within the framework of Top Institute Pharma project number T4-214.

Since April 2012 he works as a formulation scientist at the Vaccine Formulation Laboratory, University of Lausanne, Switzerland, where he is responsible for formulations and pre-clinical activities.

