THESIS FOR THE DEGREE OF LICENTIATE OF ENGINEERING

Studying the influence of the physicochemical properties of lipid nanoparticles for mucosal vaccine delivery

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Cover picture: Schematic overview of the different lipid-based particles used in this work and an evil virus infecting a cell. Not to scale.

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Abstract

Lipid-based nanoparticles have attracted attention as promising pharmaceutical carriers. Reports of them having inherent adjuvant properties make them particularly interesting as vaccine vectors; however, the physicochemical profile of an ideal nanoparticle for mucosal vaccine delivery remains unknown. The aim of this thesis work is to contribute a better understanding of the connection between physicochemical properties of lipid nanoparticles used as vaccine carriers and the activation of the immune response at several different levels of complexity. As combined antigen and adjuvant, we used a novel fusion protein comprising the Cholera toxin A1 subunit, combined with either the M2e or Ealpha peptide and a dimer of the D subunit of *Staphylococcus aureus* protein A. This fusion protein was coupled to liposomes and lipodisks with systematically varied poly(ethylene glycol) (PEG) content, protein load, rigidity and size/shape. Firstly, a detailed characterization of the biological response *in vitro* and *in vivo*, in a mouse model, to two types of fusion protein-carrying lipid particles was performed. Compared with the free fusion protein, which is in itself already an effective vaccination compound, the result showed that the non-PEGylated liposomes more efficiently induce both cell- and antibody-mediated immune responses as well as protection against a lethal virus challenge than both free fusion protein and the PEGylated liposomes. Secondly, an *in vitro* study was performed, focusing on elucidating the effect of the physicochemical properties of the carrier particle on processing, in particular the antigen presentation in major histocompatibility complex class II (MHC II), by dendritic cells. Out of 6 different formulations, which varied with respect to PEGylation, fusion protein load, membrane rigidity, size and shape it was found that only the DSPC-based liposome formulation, the only liposome formulation in gel phase, was able to increase antigen presentation compared to free fusion protein. Additionally, this formulation lead to an increased amount of surface-bound MHC II, indicating that the liposomes themselves might have an immunostimulatory effect, making them a promising candidate for further evaluation as a vaccine carrier with inherent adjuvant properties.

Keywords: liposomes, lipodisks, nanoparticles, vaccine carriers, influenza, CTA1-DD, dendritic cells, antigen presentation, flow cytometry, TIRF microscopy

"I checked it very thoroughly and that quite definitely is the answer. I think the problem, to be quite honest with you, is that you've never actually known what the question is."

- Deep Thought
in The Hitchhikers Guide to the Galaxy
by Douglas Adams

Appended Papers

Paper I

A universal influenza vaccine based on a novel combined lipid nanoparticle adjuvant vector for broad protection against infection

Valentina Bernasconi, Karin Norling, Sabina Burazerovic, Ajibola Omokanye, Karin Schön, Dubravka Grdic Eliasson, Marta Bally, Fredrik Höök, Nils Y. Lycke

Manuscript in preparation.

My contribution: I performed a large part of the liposome production and characterization and wrote the corresponding parts of the manuscript with input from the other authors.

Paper II

DSPC-based vaccine-carrier liposomes increase antigen presentation by dendritic cells *in vitro*

Karin Norling, Valentina Bernasconi, Víctor Agmo Hernández, Nagma Parveen, Katarina Edwards, Nils Y. Lycke, Marta Bally, Fredrik Höök

Manuscript in preparation.

My contribution: I planned and performed the majority of the experimental work, the data analysis and wrote the manuscript with input from MB and FH

Publications not included in the thesis

Paper III

Mucosal Vaccine Development Based on Liposome Technology

Valentina Bernasconi*, Karin Norling*, Marta Bally, Fredrik Höök, Nils Y. Lycke

Journal of Immunology Research, vol. *2016, 16 pages, 2016*

Paper IV

Optical density and lipid content of extracellular vesicles revealed using optical waveguide scattering and fluorescence microscopy

Déborah L. M. Rupert, Mokhtar Mapar, Ganesh Vilas Shelke, Matthias Elmeskog, Karin Norling, Stephan Block, Björn Agnarsson, Jan Lötvall, Marta Bally, Fredrik Höök

Manuscript in preparation.

* These authors contributed equally to the work.

List of abbreviations

APC	Antigen Presenting Cell
CBQCA	3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde
Chol	Cholesterol
CLSM	Confocal Laser Scanning Microscopy
Cryo-TEM	Cryogenic Transmission Electron Microscopy
CTA1	Cholera Toxin A1-subunit
CTB	Cholera Toxin B-subunit
CTL	Cytotoxic T Lymphocyte
C-LR	C-type Lectin Receptor
DC	Dendritic Cell
DD	D-fragment dimer from *Staphylococcus aureus* protein A
DDA	dimethyldioctadecylammonium bromide
DMPG	dimyristoylphosphatidylglycerol
DIC	Differential Interference Contrast
DMTAP	1,2-dimyristoyl-trimethyl-ammonium propane
DOTAP	1,2-dioleyl-3-trimethylammonium propane
DPPC	Dipalmitoylphosphatidylcholine
DPPE	Palmitoylphosphatidyl-ethanolamine
DPPS	Dipalmitoylphosphatidylserine
DSPC	Distearoylphosphatidylcholine
Ea	Ealpha
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EPC	Egg Phosphatidylcholine
FAE	Follicle-Associated Epithelium
FRET	Förster Resonance Energy Transfer
FSC	Forward Scatter
FSDC	Fetal Skin Dendritic Cell
HA	Hyaluronic Acid
IL	Interleukin
IFN-γ	Interferon-γ
LDE	Laser Doppler Electrophoresis
LT	Heat-labile Toxin
NK cell	Natural Killer cell
NTA	Nanoparticle Tracking Analysis
M cell	Microfold cell
MHC I	Major Histocompatibility Complex class I
MHC II	Major Histocompatibility Complex class II
MPSPR	Multi-Parametric Surface Plasmon Resonance
M2e	Matrix protein 2 ectodomain from influenza A
NOD	Nucleotide-binding Oligomerization Domain

PA	Phosphatidic acid
PAMPs	Pathogen-Associated Molecular Patterns
PC	Phosphatidylcholine
PCA	Principal Component Analysis
PE	Phosphatidylethanolamine
PEG	Poly(ethylene glycol)
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PMT	Photomultiplyer Tube
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PS	Phosphatidylserine
PRR	Pattern Recognition Receptor
QCM-D	Quartz Crystal Microbalance with Dissipation monitoring
R18	octadecyl rhodamine B chloride
SSC	Side Scatter
TDB	Trehalose 6,6-dibehenate
TEM	Transmission Electron Microscopy
Th cell	T helper cell
TIRF	Total Internal Reflection Fluorescence
TLR	Toll-Like Receptor
TRSPC-FRET	Time Correlated Single Photon Counting Förster Resonance Energy Transfer
t-SNE	t-Stochastic Neighbor Embedding
VLP	Virus-Like Particle

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1
Introduction

Edward Jenner's trials with conferring protection against smallpox through controlled infection with the relatively harmless cowpox virus in the late 18th century is often cited as the birth of modern vaccinology.[1] Indeed, his work marked the initiation of the efforts that lead to eradication of smallpox in 1979 in what is arguably one of the biggest achievements of modern medicine.[2] Over these centuries, vaccine technology has developed from the early inoculations, where pus from lesions of infected persons was used; to safer, industrially produced vaccines containing inactivated whole viruses. Although such strategies have proven successful in the past, they have limitations that remain to be overcome and modern vaccination strategies aim at circumventing vaccination based on whole pathogens. This is motivated by the fact that using whole pathogens is not optimal from a safety perspective but also by the fact that such a strategy is not ideal for rapidly mutating organisms, such as influenza, since the vaccines need to be tailored to the specific strain causing the infection. As a consequence, vaccines based on whole pathogens can thus not be produced far in advance of the actual outbreak. Simultaneously, the growing prevalence of antibiotic resistance forces us to rely more and more on preventative measures, such as vaccines.[3] Additionally, our modern travel habits make us vulnerable to rapid regional and global spread of infectious diseases.[4, 5] Further, the situation is worsened by traditional means of vaccine distribution, which generally gathers large groups of people in limited spaces to have the vaccine administered systemically by medically trained personnel through means of injection. Taken together, this means that the response time for an unanticipated new strain of a pathogen remains long, while other treatment options are fewer and transmission is faster than ever. The demands on the next generation of vaccines are thus clear: they should be universal, effective against all strains of a particular pathogen; administration should be fast and easy, ideally not requiring trained personnel; and, of course, they should be safe and effective.

Universal vaccines can in principle be created by using evolutionarily conserved subcomponents rather than inactivated whole pathogens.[6] The composition and production of subunit vaccines are tightly controllable, usually making them safer with fewer manufacturing and regulatory concerns. They are however generally less immunogenic than formulations comprising whole pathogens. Ease of administration could be achieved by making mucosal vaccines, which are administered, for example, orally or intranasally.[7] Mucosal vaccines additionally have lower demands on purity compared to injected vaccines and

can achieve local immune activation, which cannot be achieved through systemic administration.[7] However, the environment at the mucosal interfaces naturally contains a high abundance of commensal ("healthy") bacteria and other potential triggers for the immune system, for example pollen and/or food proteins. As a consequence, the mucosal immune system is not easily triggered.[8] Thus, both current subunit and mucosal vaccine candidates often require large amounts of antigen and strong adjuvants, i.e. immunostimulatory agents that enhances the immune response, to be effective.

To improve the performance of mucosal subunit vaccines, particulate carrier systems have been proposed as a promising strategy. The advantage of such systems is that they can protect antigen from premature degradation while, at the same time, exhibit immunostimulatory effects in their own right.[9] Lipid-based delivery vehicles have been used with success for delivery of various drugs, macromolecules as well as diagnostic agents in the clinic and are promising candidates also as vaccine vectors.[10] Lipid-based particles are vastly customizable when it comes to their composition and physicochemical properties, which is one of their main advantages. However, this gives rise to questions about how to best design vaccine carriers to achieve and modulate the immune activation that follows administration. Indeed, it yet remains to be understood, in the context of vaccine vectors, how the physicochemical properties of the carrier affects the type of uptake mechanism that is employed, how the uptake process progresses and how it in turn affects the antigen presentation as well as modulation of the larger scale immune response that follows. As a consequence, being able to pinpoint which properties are decisive for efficient uptake and presentation by dendritic cells and further development of immunity and thus identify promising candidates at an early stage might help us rationalize the process of designing vaccine formulations.

The aim of this thesis work is to contribute a better understanding of the connection between physicochemical properties of lipid nanoparticles used as vaccine carriers and the activation of the immune response at several different levels of complexity. Firstly, a detailed characterization of the biological response *in vitro* and *in vivo*, in a mouse model, to two types of antigen-carrying lipid particles is described in Paper I. In a second step, we focused on an *in vitro* model to specifically quantify the antigen presentation (in Paper II), which enabled us to more rapidly identify a formulation with improved immunostimulatory properties. In addition, we have also started to explore single-cell imaging to investigate the uptake process of vaccine nanoparticles by antigen presenting cells, representing the very first step in activating the immune response (in additional experimental data).

The thesis has the following disposition: you are currently reading Chapter 1 and should already know what the thesis work is about. Chapter 2 provides a brief background to the immune system and the challenges and opportunities it provides for vaccination. Chapter 3 introduces the concept of lipids and lipid assemblies and how they can be used as carrier particles, while Chapter 4 focuses on the use of such particles as carriers in mucosal vaccines against infectious diseases. The techniques used for characterization of lipid particles as well as particle uptake and antigen presentation by cells are introduced in Chapter 5. In Chapter 6, the results of the appended papers are summarized and finally, Chapter 7 presents the future perspectives of this work.

2

Achieving immune system activation through mucosal vaccination

The immune system is a collection of mechanisms in place to recognize and defend the body from “non-self” elements, enabling us to fend off attacks from foreign entities such as bacteria and viruses but also damaged self-cells, such as cancer and virus-infected cells. The immune system consists of several layers of defense that interact through a complex interplay of molecular signaling and interactions.

The first line of defense is the innate immune system. It acts in an unspecific manner, meaning that it does not distinguish between different pathogens and does not, on its own, lead to protective immunity. The enzymes and acidic environment in the stomach, the complement system, natural killer (NK) cells and phagocytes (“eating cells”, for example dendritic cells and macrophages) are some of its constituents. Of particular relevance to mucosal vaccination is also the mucous membrane, which consists of a layer of connective tissue known as the lamina propria overlaid with tightly connected epithelial cells, which are not readily penetrable. On top there is a layer of mucus: a viscoelastic, negatively charged secretion containing, among other things, mucins and secreted antibodies (immunoglobulins)[11], as schematically illustrated in Figure 1 together with an overview of the activation of the adaptive immune response. Additionally, some of the epithelial cells are equipped with cilia: hair-like extensions that move in a coordinated fashion. The movement of the cilia combined with the viscoelastic properties of the mucus creates a directed outwards flow of the mucus; trapping and actively transporting foreign matter in what is known as mucociliary clearance.

The second line of defense is the adaptive, also known as the acquired, immune system; the body’s specific response that takes longer to be initiated but which, in contrast to the innate immune system, can discriminate between closely related pathogens. It involves lymphocytes: T cells and B cells, which recognize non-self components, often referred to as antigens. The antigen-specificity of T and B cells allows the adaptive immune system to retain a memory of past events, facilitating a faster and stronger defense upon subsequent exposures.[12] The purpose of vaccination is to engage the immunological memory in order to acquire protection without having to endure actual infection and the symptoms that entails.

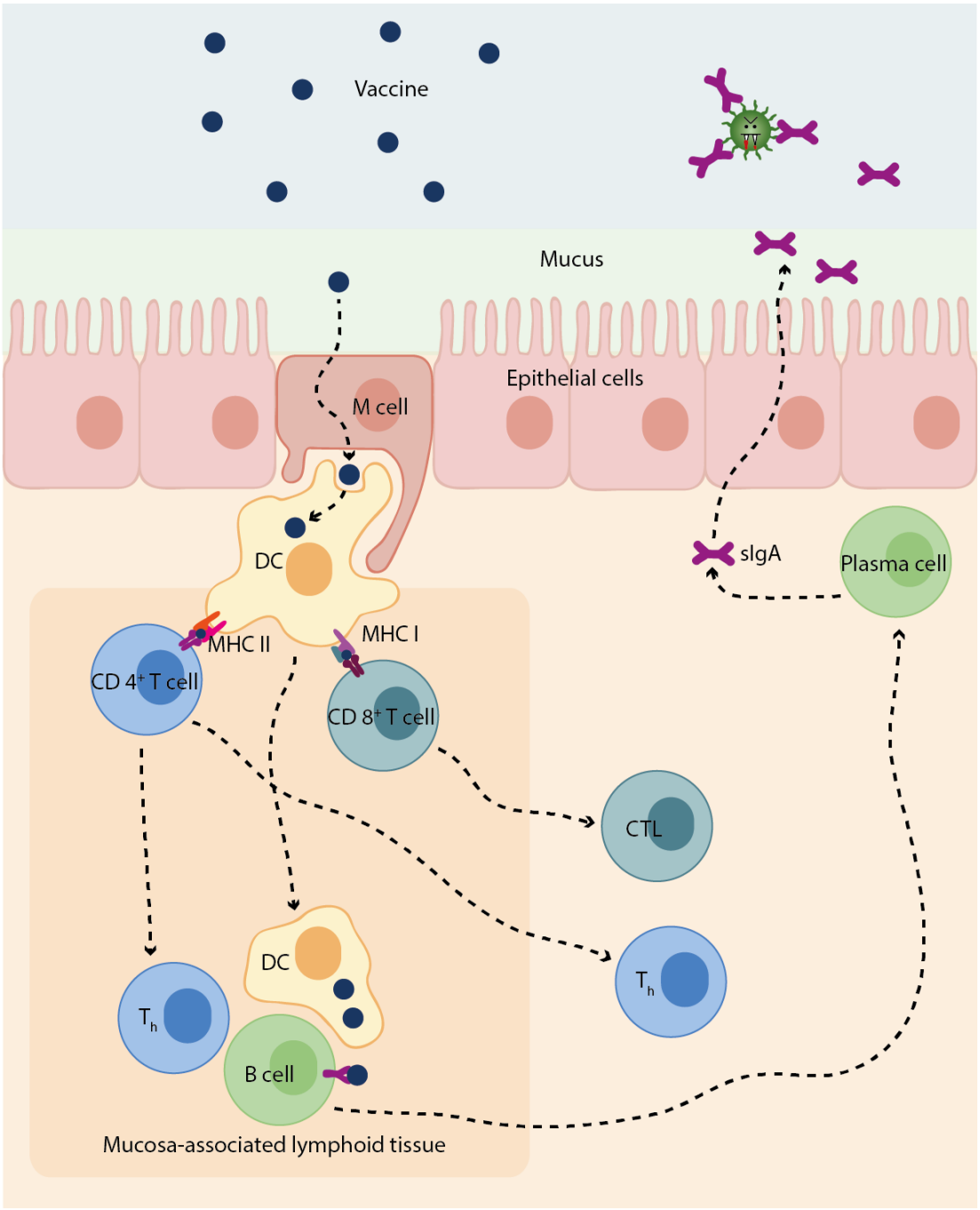

Figure 1. Example of induction of the adaptive immune response through mucosal vaccination. The vaccine needs to pass the mucosal barrier, for example by being taken up by an M cell, which transports it to the other side of the mucous membrane. There, it is available for uptake by DCs, a highly specialized APC, which take up, process and present antigen on their surface on MHC I and/or II. DCs migrate to nearby mucosa-associated lymphoid tissue where antigen presented on MHC I activates naïve CD8⁺ T cells, which differentiate into effector and memory CTLs. CTLs are cells specialized in killing damaged cells, such as those infected by viruses or bacteria. Antigen presented on MHC II activates naïve CD4⁺ T cells, which differentiate into effector and memory Th cells. Th cells modulate of the immune response through cytokine release. DCs additionally traffic antigen to the B cell zone. Naïve B cells are activated by the antigen and co-stimulation from Th cells, and differentiate into memory and effector (plasma) cells. Plasma cells release antibodies, for example sIgA, which carries out protective functions such as binding to surface proteins of pathogens.

2.1 Vaccine delivery across the mucosal barrier

There is a series of barriers in place that needs to be overcome before being able to successfully engage the adaptive immunes system to achieve protective immunity, particularly in the case of mucosal vaccines. The innate immune system has evolved to repel and degrade foreign matter to prevent infection. Accordingly, mucosal vaccines will, upon administration, immediately encounter the chemical and mechanical cleansing system that is in place at most mucosal surfaces.[8] A first hurdle when developing a vaccination strategy is therefore to prevent premature degradation of the antigen, which is particularly challenging in the case of oral immunization.[13] This, in turn, is the main motivation behind the efforts undertaken to improve the resistance of vaccine formulations to degradation in biological fluids, as summarized in section 4.3. Once the vaccine has survived the harsh environment encountered at the luminal side of the mucosa, the challenge to deliver the antigen across the mucosa remains. In order to do this, the mucociliary clearance needs to be avoided and the mucous membrane needs to be traversed.[8, 11] There are three main strategies to increase antigen transfer through this complex barrier. The first is to increase the mucopenetration of vaccine formulations, often by using neutrally charged and hydrophilic carrier particles in an attempt to avoid entrapment by the mucus (see section 4.6 for additional details). The second, opposite, strategy aims at increasing the mucoadhesion, in order to decrease the clearance rate, often by using particles of a positively charged and hydrophobic nature, as discussed in sections 4.1 and 4.6).[14] The choice between these two strategies depends on the properties of the target mucosa. For example, mucoadhesive carrier particles can be useful when targeting mucosa with a slow mucus turnover rate, while mucopenetrating particles can be used for traversing thick mucus layers.[15] The third strategy attempts to utilize the body's own system for transport across the mucosal barrier by targeting microfold cells (M cells) (see section 4.7). M cells are specialized in phagocytosis and transcytosis, i.e. transport through the interior of the cell, of macromolecules, particles and microorganisms across the follicle-associated epithelium (FAE) to the lymphoid tissues located in connection to the intestinal and nasal mucosa.[16] For this task, M cells have an intraepithelial pocket, where the antigens taken up from the luminal side are made available to cells on the other side of the mucous membrane.[16]

2.2 Activating the adaptive immune system: the role of antigen presenting cells

Once the vaccine formulation has crossed the barrier of the mucosa, it needs to be recognized by antigen presenting cells (APCs), considered the bridge between the innate and adaptive immune systems. APCs sample their environment using several different mechanisms of uptake, including endocytosis, phagocytosis and macropinocytosis.[17] They distinguish self from non-self by recognizing

evolutionarily conserved molecular structures exclusively found on pathogens and called pathogen-associated molecular patterns (PAMPs). Examples of PAMPs are bacterial cell-wall components, certain lipids, for example lipid A (seen in Figure 3), and different forms of microbial nucleic acids.[18-20] APCs use a group of receptors known as pattern recognition receptors (PRRs) for this discrimination process. These receptors include Toll-like receptors (TLRs), C-type lectin receptors (C-LRs) and nucleotide-binding oligomerization domain (NOD)-like receptors.[18-20] Ligand binding to PRRs signals danger and starts a signaling cascade whose end result is tuning of the immune response. PRR agonists are therefore often used to target vaccine delivery to APCs and as adjuvants to modulate the immune response (see section 4.7).

Once an APC has taken up antigen, the cell begins to mature, which leads to up-regulation of the antigen-processing machinery and migration to the lymphoid organs where T and B cells reside in spatially separate zones. After uptake, the antigen is processed, which involves the degradation of the foreign molecule into molecular fragments, often short peptides, in certain antigen-processing compartments, followed by mounting of the peptides on Major Histocompatibility Complex class II (MHC II). The peptide-MHC II complexes are then transported to the cell surface where they are displayed.[17] In some cases, the antigen is also presented on Major Histocompatibility Complex class I (MHC I), a process known as cross presentation, the importance of which is discussed further in section 2.3.

2.3 Inducing and tuning cell-mediated immunity

One of the main functions of APCs, once they reach the lymphoid tissue, is to initiate and influence the nature of the T cell response, which is referred to as cell-mediated immunity. In the context of inducing strong cell-mediated immunity, a type of APC called dendritic cell (DC) is a target of choice for vaccine delivery (see section 4.7).[21] Indeed, DCs are both exceptionally efficient at antigen uptake and processing and are key players in induction of the primary immune response (see Figure 1). The presentation of antigens by DCs is required for activation of naïve antigen-specific T cells, which can then differentiate into effector and memory T cells, being essential for long-term immunity. Which class of MHC the antigen is presented on is decisive for which subset of T cells the DC is able to activate.

In addition to association with antigen presented on MHC, T cells require interactions with costimulatory molecules, such as CD80 and CD86, found on the surface of mature DCs. Furthermore, DCs release cytokines, which are signaling molecules that together with the costimulatory molecules act on T cells in order to tune the type of response initiated. More specifically, recognition of MHC II-mounted antigens leads to the activation of $CD4^+$ T cells into T helper (Th) cells

whose main function is to release cytokines, thereby effectively tuning the immune response by regulating the activity of other cells. Often, Th cells are divided into two subsets: those promoting cell-mediated immunity (Th1) and those promoting antibody-mediated, also known as humoral, immunity (Th2, see section 2.4). On the other hand, cross-presentation of antigens onto MHC I leads to priming of naïve $CD8^+$ T cells, generating cytotoxic T lymphocytes (CTLs) (see Figure 1) that are important for the killing of, among other things, virus-infected cells.[12] Particulate delivery systems have been shown to increase the cross-presentation efficiency, making carrier particles particularly interesting from a vaccine development perspective.[8]

2.4 Inducing humoral immunity

The other branch of the adaptive immune system, which also needs to be stimulated by vaccination, is known as humoral immunity. This type of immune response is mediated by antibodies. Effector functions of antibodies include neutralization of toxins and microbes through binding to and blocking of their surface proteins, activation of the complement system and opsonization, a process by which a pathogen is marked for destruction by phagocytes.

Antibodies are large proteins able to bind to specific epitopes on antigens. Antibody-antigen recognition is highly specific due to the fact that the chemical properties of the amino acid sequence forming the antigen-binding site of the antibody geometrically and physicochemically match regions on the corresponding epitope on the antigen. This allows formation of a multitude of electrostatic and hydrophobic interactions and hydrogen bonds, the sum of which leads to an attraction.[12] Antibodies are classified into five different immunoglobulin classes according to their characteristics. Of relevance in the context of vaccination are IgG, the main serum antibodies, and secretory IgA which are generally secreted in the lamina propria in the mucous membrane to protect against infection at this interface.[12] Local secretion of IgA antibodies can only be triggered by local activation, highlighting the advantages of mucosal immunization in the context of vaccine development.

Antibodies are produced by B cells (see Figure 1) and exist in two forms: a free, secreted form and a cell-membrane bound form known as the B cell receptor. The B cell receptor is crucial to the activation and differentiation of naïve B cells into memory B cells, which remain in the body to provide prolonged protection, and into plasma cells, which are the main antibody-secretor cells. B cells can be stimulated by antigen binding to the B cell receptor alone and can, in fact, act as APCs. However, the trafficking of antigens by DCs to the B cell zones, known as follicles, in the lymphoid tissues greatly increases the likelihood that naïve B cells encounter their specific antigen. Additionally, most B cells require stimulation from activated Th cells to function optimally (see Figure 1).

2.5 Assessing the immune response

Historically in vaccine development, a large focus has been placed on achieving antibody-mediated immunity.[22] Indeed, antibodies are crucial for preventing infection since they can block the surface proteins of pathogens and thus prevent them from binding to and entering host cells. The cell mediated immunity is however necessary for attenuation and clearance of symptoms once infection has occurred. Therefore, a combination of the two is generally considered the most efficient and we thus aim to achieve engagement of both humoral and cell-mediated immune responses at both a local and systemic level.[22] The intensity and quality of an induced immune response is typically assessed by measuring a set of biological markers. Different types of antibodies and cytokines, such as interleukins (ILs), are commonly quantified and indicate activity of certain branches of the immune response. Some of the more commonly used markers and what they indicate in the context of vaccine development are summarized in Table 1.

Table 1. Commonly used markers of immune activation following vaccine administration.

Marker	*Type of molecule*	*Role*
IgG	Antibody	Indicates activation of systemic humoral immunity.
IgA	Antibody	Indicates activation of mucosal/local humoral immunity.
IL-4	Cytokine	Indicates Th cell-mediated activation of B cells. It induces the differentiation of B cells into plasma cells and stimulates proliferation of activated B and T cells. IL-4 induces differentiation of naïve helper T cells to Th2 cells, which upon activation by IL-4 produce additional IL-4 in a positive feedback loop. Additionally it up-regulates MHC II production while decreasing production of Th1 cells, macrophages and IFN-γ.
IFN-γ	Cytokine	Indicates activation of APCs. It is produced predominantly by NK and natural killer T cells as part of the innate immune response, and by Th1 cells and CTL once adaptive immunity develops. IFN-γ is an important activator of macrophages and inducer of MHC II expression.
IL-2	Cytokine	Is a signal for differentiation into memory and effector T cells. IL-2 also has roles in regulation of T cell activity.
IL-17	Cytokine	Indicates activity of T helper 17 cells, important for maintaining mucosal barriers and clearance. IL-17 has an important role in proinflammatory responses and induces the production of many other cytokines.

3
Lipids and lipid self-assemblies

Lipids are an important building block of many living organisms in that they are the main constituents of the cellular membranes. The membranes are thin, fluid films to which lipids provide a structure that allows for lateral movements of incorporated proteins and other biomolecules.[23] The membranes simultaneously form selectively permeable barriers that maintain appropriate intracellular concentrations of a vast number of molecules and ions, thus delineating organelles as well as the inside of a cell from the outside.[23] Among the variety of lipids found in living organisms, phospholipids are the ones most abundantly found in cell membranes. This type of lipids is also the main constituent of many man-made lipid structures, including vaccine vectors.[24, 25] They are made up of a hydrophobic tail consisting of two fatty acids linked by a glycerol backbone to a hydrophilic headgroup made up of phosphate and potentially another organic molecule (Figure 2A). Based on their headgroup, naturally occurring phospholipids can be sorted into 6 categories: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) or phosphatidic acid (PA). PS, PI, PG and PA are negatively charged while PC and PE are neutral but zwitterionic. The possibility to chemically modify both the head group and the tail region gives the possibility to synthesize phospholipids tailored to specific requirements. In this way, positively charged lipids have been created, for example 1,2-dioleyl-3-trimethylammonium propane (DOTAP), 1,2-dimyristoyl-trimethyl-ammonium propane (DMTAP) and dimethyldioctadecylammonium bromide (DDA). A common property for all phospholipids is that they are amphiphilic.

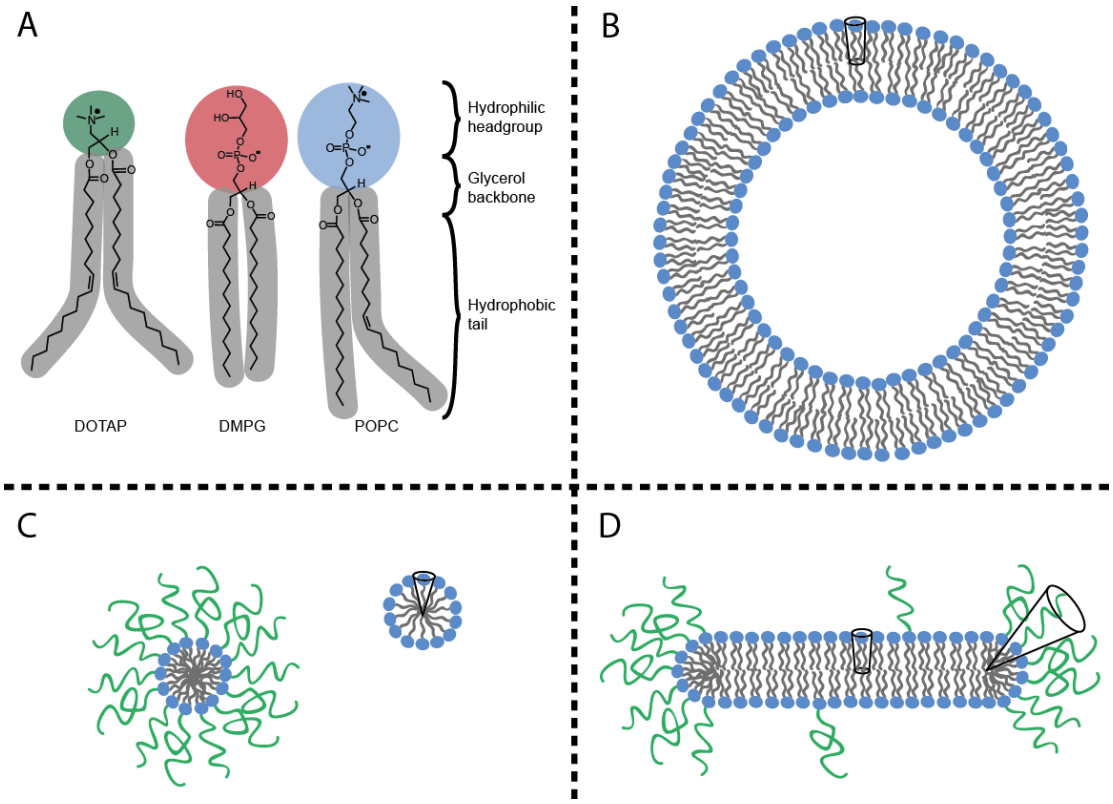

Figure 2. A: Examples of lipids with different properties: 1,2-dioleyl-3-trimethylammonium propane (DOTAP), with a positively charged headgroup and unsaturated alkyl chains; dimyristoylphosphatidylglycerol (DMPG), with a negatively charged headgroup and saturated alkyl chains and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), with a zwitterionic headgroup and one unsaturated and one saturated alkyl chain. B: A liposome is a spherical bilayer structure consisting of lipids with a cylindrical geometry. C: Spherical or elongated micellar structures consists of lipids with a conical geometry. D: Lipodisks, bilayer segments with edges stabilized by micelle-forming lipids, can be formed by mixing bilayer- and micelle-forming lipids at certain ratios.

In an aqueous environment, amphiphilic molecules such as phospholipids self-assemble into different types of molecular assemblies where the hydrophobic parts face each other and form a protected compartment with the hydrophilic parts facing the aqueous solvent.[26] Such self-assembled amphiphilic structures exist in an equilibrium state, meaning that they are not definite and constant but often fluid in the sense that their size and shape is not sharply defined but rather a distribution.[27] The most energetically favorable, and therefore most likely, structures adopted by amphiphiles depend on concentration and physicochemical properties of the amphiphiles as well as on environmental factors such as the temperature, the ionic strength and the pressure, although the latter effect is generally small.[26] Among the properties of amphiphilic molecules the geometry is also of particular interest as it affects the molecular packing and thus the type of structures, or mesophases, that are formed. There are essentially two basic structures: micelles and bilayers. Micelles are spherical or cylindrical structures with a hydrocarbon core and a surface consisting of hydrophilic groups (Figure 2C). Amphiphiles with a conical geometry i.e. with large headgroup area, often due to a charged or bulky headgroup, and a

comparatively small hydrophobic tail tend to form micellar structures. Amphiphiles with a tail region that is large compared to the headgroup, for example when the hydrophobic tail consist of two alkyl chains, have a more cylindrical geometry, and will tend to form bilayer structures (Figure 2B).[27] So called lipid bilayers consist of two layers of amphiphiles assembled with the hydrocarbon chains facing each other, thereby protected from thermodynamically unfavorable interactions with the aqueous solution by the hydrophilic headgroups. Both bilayers and micelles can form large interconnected structures, crystalline phases, or remain as separate entities, thereby forming particles. Such particles are of interest for many types of drug delivery applications and for vaccine delivery purposes.[28]

One type of lipid-based particle commonly encountered is the vesicle, also called liposome. In this case, the energetically unfavorable outer edges of planar bilayer sheets are eliminated by forming a hollow sphere. The sphere consists of either a single or multiple phospholipid bilayers and liposomes are accordingly classified as either unilamellar or multilamellar. Liposomes where discovered in 1965 by Bangham et al. and in the 1970's they were for the first time explored for drug delivery purposes and as immunological adjuvants.[29-31] Liposomes have since been extensively explored as vaccine vectors, with several examples being in commercial use and clinical trials.[13, 32, 33] Particles which contain elements of both micelles and bilayers may form if conical and cylindrical lipids are mixed. One such example of direct relevance to this thesis, is the lipodisk, which was first described by Edwards et al. in 1997.[34] Lipodisks are flat, single bilayer disks, comprising bilayer-forming lipids with micelle-forming lipids eliminating the energetically unfavorable contribution from the high-curvature edges (Figure 2D). Lipodisks have been used as membrane mimics and for drug delivery purposes but until now they have, to the best of our knowledge, not been considered for use as vaccine vectors.[35-37] Other phases that have been considered for vaccine delivery include various types of emulsions and ISCOMs; immunostimulating complexes, comprising saponins from the *Quillaia saponaria* tree, cholesterol and phospholipids, which form hollow cage-like nanoparticles when mixed at a certain stoichiometry.[38-41] However, such formulations have so far not been considered within this work.

4
Lipid particles for mucosal vaccine delivery

Lipid particles have attracted considerable interest as carriers for mucosal vaccine delivery for a number of reasons. First of all, the membrane composition is easily adjustable and membrane constituents can be synthetic or sourced from vastly different organisms, which invites to biomimicry. Thus, attempts have been made to enhance the immunogenicity of a lipid formulation by choosing membrane components with archaeal, bacterial or viral origins.[42-48] Conversely, by choosing endogenous lipids, vectors can be made entirely innocuous: biodegradable, non-toxic and non-immunogenic.[25, 49] Oftentimes, highly immunogenic formulations are also toxic, and creating a formulation that is effective without unwanted side effects is a challenge. One may therefore attempt to systematically address which particular physicochemical properties are at the root of the immunogenic effect to be able to create formulations with the desired properties but without toxic constituents.

An advantage of lipid-based vaccine formulations is that the physicochemical properties of the lipid particles are vastly adjustable. The membrane properties can be tuned by altering the lipid composition: the surface charge of the particles is largely affected by the characteristics of the lipid headgroup, and the degree of hydrophilicity can be tuned by addition of polymers such as poly(ethylene glycol) (PEG).[10, 50] The headgroup charge, along with the length and degree of saturation of the alkyl chains of the tails, further influences the transition temperature of the lipids, which in turn determines whether a lipid membrane exists in gel- or fluid-phase at a certain temperature. Moreover, if a membrane consists of a mixture of lipids, phase separations can occur, resulting in heterogeneous distribution of different lipids. The stability of a membrane, i.e. its resistance to degradation, is affected by its fluidity and permeability as well as its bending rigidity, which are in turn influenced by the same lipid characteristics. Incorporation of cholesterol (Chol) is a common way to modulate the membrane permeability, fluidity and rigidity, which influence the liquid-to-gel phase transition temperature and stability.[28, 51] Liposome size and lamellarity can be tailored by altering the manufacturing method; for lipodisks, the size is affected by both method and composition.[51, 52]

An inherent property of lipid particles, which makes them useful as antigen carriers, is that they contain both hydrophobic and hydrophilic regions, allowing for a variety of coupling strategies. Hydrophobic peptides or proteins can be incorporated into the hydrocarbon center of bilayers or micelles, while

hydrophilic molecules can be coupled to the surface of lipid particles or encapsulated in the aqueous core of liposomes (see Figure 3C). Surface association can take place by covalent attachment or spontaneous association of the antigen to the surface through adsorption or electrostatic interaction or alternatively, the antigen can be attached to a hydrophobic anchor that inserts into the particle. In cases where the lipid particles are included in a vaccine formulation solely as an adjuvant, they may simply be co-administered with the antigen.

With the possibility to tailor both preparation method and composition, as well as ways of incorporating antigen in the formulation, there are virtually endless possibilities in the production of lipid structures with varying properties. However, it still remains to be understood why the immune response is modulated differently by different liposomal formulations, and which properties that are decisive for the outcome. This is a particularly challenging task as it is inherently difficult to isolate the contribution of different properties, as changing one property usually influences one or several others. For instance, when varying the surface charge by altering the lipid composition one may inherently affect other properties, such as membrane fluidity and rigidity, as well as their resistance to enzymatic degradation, etc. Hence, it may be difficult to directly assess the influence of changing different physicochemical properties of carriers on the immune response. Nevertheless, attempts have been made to systematically study the influence of physicochemical properties of liposomes used in mucosal vaccines against infectious diseases on immunogenicity, as summarized in further detail below, starting with *surface charge* and followed by sections addressing *size and lamellarity*, *resistance to degradation*, *rigidity*, *antigen localization* and *modifications to increase bioavailability.*

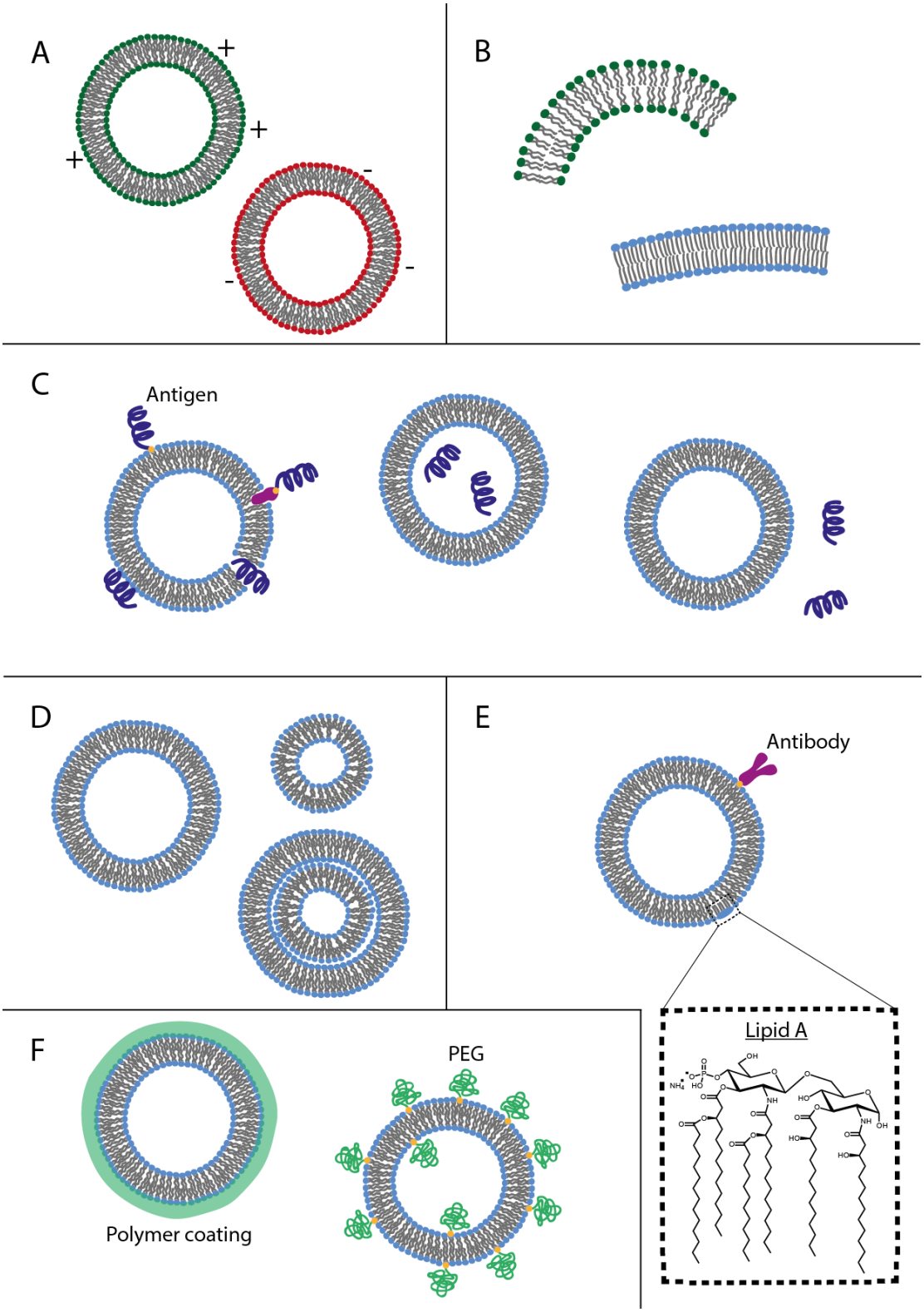

Figure 3. A: The effect of altered surface charge on liposome function has been extensively examined.[53, 54] B: The lipid composition is critically influencing the immune response.[55] C: Also the localization of the antigen, on or inside the liposome, plays an important role in shaping the immune response to the vaccine. There are several modes of antigen association to liposomes. Firstly, antigens may be encapsulated in the aqueous core or they could be linked to the surface via covalent attachment. Alternatively, a hydrophobic anchor can be used to attach the antigen to the surface via adsorption or through electrostatic interactions with lipids of opposite charge. For proteins with a hydrophobic region one may even successfully insert these in the liposome

membrane. The liposome may also be used as an immunoenhancer simply by admixing the antigen and the liposomes. D: Only few studies have addressed the impact of size or lamellarity.[43, 56] E: Modifications of liposomes to increase their immunoenhancing effect can be done through attaching PAMPs, such as lipid A (in inset, see section 2.2 for further information), or through specific targeting strategies using cell-specific antibodies (anti-CD103 or -DEC205).[46, 57] F: Other modifications, including addition of poly(ethylene glycol) (PEG) or different polymer coatings, that increase the liposome penetration of the mucosal barrier or to increase liposome resistance in biological fluids, have also been developed (see section4.6).[58]

4.1 Surface charge

One of the most commonly explored parameters in the context of mucosal vaccine delivery is the surface charge of the liposome (Figure 3A), which is generally assessed by the zeta potential, a measure of the electrostatic potential at the limit of what is called the diffuse electric double-layer that surrounds the particle (see section 5.4). The magnitude of the zeta potential depends on the concentration of ions within the double layer but also other factors, such as the ionic strength and pH of the dispersion medium. This must be kept in mind when comparing zeta potential values reported in different studies and under different conditions, as well as when considering the relevance of this parameter in *in vitro* and *in vivo* studies.

Because the cell surface as well as the mucus coating of the mucous membrane is negatively charged, positively charged liposomes will generally exhibit stronger interactions with the cell membrane as well as an increased mucoadhesion.[14, 25] The latter leads to reduced clearance rate, i.e. slower removal from the mucous membranes. This may be beneficial for antigen delivery as both increased interactions with the cell membrane and prolonged exposure time of the antigen at the mucosal surface, are thought to lead to increased cellular uptake of antigen and stronger immune responses.[59, 60] Indeed, cationic liposomes were found to effectively deliver antigen to both mucus and APCs as shown in an *in vitro* model of the airway epithelium with liposomes made with distearoylphosphatidylcholine (DSPC)/trehalose 6,6-dibehenate (TDB) (neutral) and DSPC/TDB/DDA (positive) with varying amounts of the positively charged DDA.[61] Moreover, cationic liposomes consisting of DOTAP/Chol, DMTAP/Chol or, most prominently, the polycationic sphingolipid ceramide carbamoyl-spermine (CCS) and Chol were shown to effectively stimulate systemic and mucosal humoral and cellular immune responses after intranasal immunizations in mice.[53] In contrast, neutral liposomes with dimyristoylphosphatidylcholine (DMPC) or anionic liposomes with DMPC/dimyristoylphosphatidylglycerol (DMPG) were comparably ineffective as immunogens.[53] While a positive charge appeared to increase the immunogenicity of liposomes in these cases, this may not always be true. In fact, there are scientific reports suggesting that negatively charged liposomes are more immunogenic than both zwitterionic and positively charged liposomes and it has even been postulated that anionic liposomes could exert an immunosuppressive effect on alveolar macrophages (a

type of macrophage found in the alveoli of the lungs, see section 2), and in this way promote an enhanced humoral immune response.[54, 62-65] Hence, it appears that several mechanisms can be modulated by the charge of the liposome. In particular, the influence of charge on immunogenicity may be highly dependent on the administration route, where different microenvironments with varying electrostatic properties may be encountered.

4.2 Size and lamellarity

A broad range of unilamellar and multilamellar liposomes with varying sizes (Figure 3D) has been proposed for vaccine delivery and the different particles have been found to induce different effects following mucosal immunization. However, the influence of these parameters on liposome immunogenicity has rarely been systematically investigated and unfortunately the degree of multilamellarity is not routinely reported. Lamellarity is typically assessed using techniques such as nuclear magnetic resonance spectroscopy, cryogenic transmission electron microscopy or small-angle X-ray scattering [51], which may still be considered specialized techniques that are not always readily available. The few studies reporting on the effects of lamellarity on immunogenicity show inconclusive results. Towards understanding the effect of size and lamellarity, a comparative study between unilamellar liposomes made from archaeal polar lipids (archaeosomes) with an average diameter of 100 nm and large multilamellar particulate aggregates clearly indicated better immunogenicity for the multilamellar aggregates.[43] However, it is noteworthy that not only the size, but also the lipid structure was different between the unilamellar and multilamellar constructs in this example. Another study reported that oral administration of a "double liposome", consisting of small (~250 nm) liposomes made from SoyPC, dipalmitoylphosphatidylcholine (DPPC), Chol and stearylamine encapsulated into a bigger (1 to 10 μm) outer liposome made from DMPC and DMPG, was found only marginally more immunogenic than small liposomes.[56] Additionally, a study using liposomes made from DPPC, DDA and Chol with sizes ranging from 70 to 1000 nm for intranasal immunization of mice similarly showed no significant effect of size on immunogenicity.[66]

Constructing homogeneous liposomes of controlled lamellarity is technically challenging and various degrees of multilamellar constructs may co-exist, making interpretations of experimental results difficult. Recent advances in the production of tightly size-controlled liposomes may allow for more accurate comparisons of the influence of size, lamellarity and overall structure in the future.[67]

4.3 Liposome resistance to degradation

The lipid composition (Figure 3B) is known to influence the stability, i.e. resistance to degradation, of the liposome; a more stable formulation might lead to a larger amount of bioavailable antigen and potentially also to a depot, i.e. slow release, effect. Han et al. made liposomes from various combinations of Chol, DPPC, Dipalmitoylphosphatidylserine (DPPS) and distearoylphosphatidylcholine (DSPC) and found that certain combinations decreased leakage of encapsulated carboxyfluorescein in different solutions simulating conditions in the gastrointestinal tract.[47] Liposomes with DSPC, having a higher transition temperature, were more stable *in vitro* and likely protected antigen better from degradation in the gastrointestinal tract.[47] As aforementioned, using archaeal lipids, liposomes can be made more immunogenic and archaeosomes were found considerably more potent than liposomes made with Egg phosphatidylcholine (EPC)/Chol at inducing antigen-specific IgG and IgA antibodies following oral administration in a mouse model.[42] This was attributed by the authors to an increased stability in the gastrointestinal tract and to the fact that the archaeosomes were better retained in the intestine. [42] However, the difference may also partly reflect the fact that the archaeosomes were negatively charged while the EPC/Chol-liposomes were neutral and, as discussed in section 4.1, negatively (or positively) charged liposomes are generally more immunogenic than neutral ones.

4.4 Liposome rigidity

Parameters that affect the stability to degradation of liposomal formulations generally also influence the membrane bending rigidity, or deformability. Experimentally quantifying membrane rigidity of nanoscopic lipid vesicles is however not trivial and the role of liposome rigidity in the context of mucosal vaccine delivery has not been expressly studied. However, this property has been ascribed an increased importance in cellular uptake.[68]

Theoretical modeling of cellular uptake has shown that the energy required to achieve full wrapping of the particle by the membrane is higher for softer particles and that they require higher adhesion energies to be successfully internalized compared to rigid particles.[69, 70] Soft particles are therefore more likely to remain partially wrapped, essentially trapped at the membrane surface.[69, 71] It has been confirmed experimentally that rigid particles are taken up to a larger extent than soft ones.[71, 72] Conversely, Allen et al. showed that by increasing the content of lipids hypothesized to increase the rigidity of the tested liposome formulations, the uptake by macrophages decreased.[73] These indications that membrane rigidity is a key factor governing liposome uptake prompted us to investigate if and how this parameter influences antigen processing by APCs (Paper II).

4.5 Antigen localization

There are many ways of incorporating antigens into liposomes. This raises the question whether some strategies are more effective than others in the context of optimizing the immunogenicity of the liposome formulation. Antigens can be hosted in the aqueous core of the liposome, inserted into the hydrophobic part of the membrane or bound to the surface by covalent bonds or intermolecular forces (Figure 3C, Section 4). Hence, a plethora of combinations exist and those could be used, in combination with various lipid compositions, to enhance resistance against antigen degradation or to facilitate antigen uptake. Thus, the liposome formulation may be tailored for specific needs and purposes. If an oral vaccine is to be designed, one may hypothesize that encapsulating the antigen inside the liposomes is an effective strategy to prevent enzymatic degradation. However, by hiding the antigen in the liposome, the immunogenicity may be compromised because the antigen will not be immediately accessible for APCs. Therefore, choosing how to physically incorporate the antigen in the liposome may have critical consequences on the immune response. Unfortunately, such aspects have not been addressed in a systematic manner thus far. Studies report that when administered orally, encapsulated antigen may more effectively stimulate local IgA and serum IgG antibody responses compared to when soluble antigen is admixed with the liposomes.[74, 75] On the other hand, following intranasal administration, a mixture of antigen and liposomes has been quite effective even compared to liposome-encapsulated antigen.[53, 63] Interestingly, liposomes have been found to exert an immuno-enhancing effect even when administered 48 hours prior to the antigen.[63] Furthermore, surface-bound antigen has been found to be more immunogenic than encapsulated antigen following intranasal immunization.[64] These observations suggest that the intranasal route is less sensitive to antigen degradation compared to the oral route. Thus, depending on the route of administration, it seems clear that antigens may or may not be immunogenic when exposed, and for many formulations it may, in fact, be advantageous to have a combination of surface-bound and encapsulated antigens. This may also apply to molecular adjuvants; it was found that cholera toxin B-subunit (CTB) adjuvant bound to the surface of the liposome was more effective compared to when encapsulated in the liposome.[76] Furthermore, it has been observed that by altering the lipid-to-antigen ratio, the systemic and mucosal as well as the humoral and cellular immune responses can be differentially induced.[53, 77] Thus, it is likely that the immune response following liposome administration is susceptible not only to the choice of antigen and adjuvant but also to their relative proportions and localization in the liposome.

4.6 Modifications to increase bioavailability

The microenvironment at mucosal surfaces often promotes a high clearance rate of liposomes. Therefore, various strategies have been tested to enhance mucus

penetration or to increase antigen-carrying liposome-to-cell membrane adhesion in order to increase the bioavailability of the vaccine antigens (Figure 3F). Layer-by-layer deposition of polyelectrolytes onto the liposomes, for example, has been used as a liposome-stabilizing approach which resulted in higher specific IgA and IgG antibody levels as well as an increased T cell response.[78] Poly-vinyl alcohol or chitosan have been tested to enhance bioadhesive properties of the liposomes and it has been observed that chitosan-loaded liposomes, indeed, increased IgG antibody responses.[79] Chitosan is a positively charged polysaccharide that can form strong electrostatic interactions with cell surfaces and mucus and, therefore, increase retention time and facilitate interactions between the liposome and APCs in the mucous membrane.[80] Additionally, chitosan can transiently open tight junctions between epithelial cells to allow for transmucosal transport.[81, 82] Chitosan-modification of liposomes is accordingly a popular strategy for delivery of peptidic antigens.[58, 79, 83, 84] In fact, chitosan-coated liposomes have been shown to give better serum IgG antibody levels compared to other bioadhesive polymers, such as hyaluronic acid- or carbopol-coated liposomes, and host better immunogenicity than uncoated negative, neutral or positively charged liposomes.[84]

Considerable attention has been given to studying how liposomes are retained by and/or taken up across the mucous membranes. Liposome interactions with the intestinal mucosa have been studied *in vivo* and *ex vivo* as well as using various *in vitro* models.[42, 78, 85, 86] The latter models have addressed whether passage of liposomes through the tight junctions of epithelial cells can be achieved. Indeed, tight junctions were reported to be open when using PC/Chol-liposomes or liposomes coated with extract from *Tremella fuciformis*.[86] Enhanced immune responses was also observed with mucus-penetrating liposomes made with poly(ethylene glycol) (PEG) or the PEG-copolymer Pluronic.[58] Significantly higher specific IgA and IgG antibody levels were found with PEGylated than non-PEGylated liposomes. Modifications with PEG or Pluronic F127 also proved useful in preventing liposome aggregation through steric stabilization to obtain small (<200 nm) chitosan-coated liposomes. In fact, these shielded chitosan-coated and PEGylated liposomes yielded the highest functional serum antibody titers of all the formulations tested and the strongest IgA responses.[58]

4.7 **Cell-targeting modifications**

In the context of carriers for vaccine delivery, one of the most explored modifications is aimed at targeting the delivery of liposomes to subsets of cells that express a comparatively large amount of predefined receptors or binding sites. This is achieved by equipping the liposomes with various targeting elements to increase the amount of liposomes delivered to the target cell subset (Figure 3E). For example, targeting components may be added to enhance the

uptake by APCs or the penetration of the liposome through the mucous membrane. Additionally, the target receptor may be directly involved in immunological signaling and thereby enhancing the immunogenicity of the liposomes.

APCs in the mucosal tissues have a high density of surface GM1 and the strongly GM1-ganglioside-binding molecule CTB has been reported to enhance liposome immunogenicity.[46, 87, 88] DCs have similarly been targeted by use of mannosylated lipids or anti-CD40 antibody-coated liposomes, which promoted a stronger immune response.[89, 90] Another popular target on immune cells are TLRs, a type of pattern recognition receptor (PRR) used by phagocytes to recognize PAMPs, i.e. pathogen-associated structural motifs. For instance, when monophosphoryl lipid A, acting through the TLR4 receptors, was added to liposomes, their ability to stimulate the innate immune response was dramatically improved.[46, 47, 55] Other TLR agonists or *Escherichia coli* heat-labile toxin (LT) have also been used in combination with liposomes as adjuvants.[44] Furthermore, linking CpG, which acts through TLR9 signaling, or *Bordetella pertussis* filamentous haemagglutinin, whose effects include binding to macrophage integrins, to liposomes have been found to enhance immunogenicity.[91, 92] Targeting macrophages via C-type lectins by galactosylation of liposomes resulted in higher specific IgA and IgG antibody levels compared to unmodified liposomes.[93] Another strategy to target macrophages is to incorporate PS. PS is naturally exposed on the surface of cells undergoing apoptosis and in this way liposomes containing PS may trigger phagocytosis by macrophages. Accordingly, it has been found that liposomes containing DPPS induced stronger IgA responses compared to formulations without DPPS.[55] Combinations of both DPPC/DMPG and DPPC/PS have been found effective at targeting liposomes to macrophages, and DPPC/DMPG was the only formulation to induce a significant antibody response following oral immunization.[74]

Another strategy aims at making uptake through mucous membranes more effective by targeting M cells in the FAE, the thin epithelial cell layer that is responsible for antigen-uptake from the luminal side. Accordingly, the lectin Agglutinin I from *Ulex europaeus* was shown to improve M cell-mediated transport across the intestinal epithelium.[85, 94, 95] Similarly, liposomes functionalized with antibodies have been found to enhance binding to M cells, and as a result increased levels of IgG, Il-2 and IFN-γ were shown following intranasal immunization.[57]

Many strategies have been proposed to achieve cell-targeting of liposomes, with varying degrees of improved function. A plethora of possibilities can be explored when it comes to targeting liposomes to the cells of the mucosal immune system.

The combination of analytical tools for nanoparticle characterization with suitable *in vitro* and *in vivo* assays will greatly help identify the relative importance of liposome targeting and other properties discussed in this chapter and how they can influence the immune response.

4.8 **Concluding remarks**

To conclude this chapter, it is clear that the design of a lipid-based vaccine formulation is complex. Needless to say, it is important to consider all the properties of the formulation as well as the biological response. Thus, liposome size, lamellarity and surface charge as well as lipid composition and rigidity of the membrane can all influence the immune response following vaccination. Importantly, the choice of antigen, with its own inherent physicochemical properties, as well as the position of the antigen and any molecular adjuvant in the liposome critically affect the function of the formulation. Furthermore, the antigen/lipid ratio and properties of the added adjuvant are also important parameters that change the immunogenicity of the liposome.

Despite this complexity, it is clear that lipid carriers can be used to, in a fairly controlled manner, modulate the immune response in a wide variety of model systems. It remains, however, to be elucidated by which mechanism their immunomodulation takes place and therefore, how to tune their properties in order to alter their effect. What are then the ideal properties of a strong and effective lipid-based mucosal vaccine? This question is indeed difficult to answer, not only because the underlying mechanisms remain to be investigated, but also because there is currently no standardized procedure to assess the potential of lipid carriers in the context of vaccination. While specific aspects of the mode of action of liposomes are often studied, for example stability in simulated intestinal fluids, mucoadhesion and APC uptake, more systematic examinations of how different parameters influence different parts of the process remains to be seen. Especially the mechanism behind the immunostimulatory properties of liposomes, unrelated to their role as carriers, is poorly understood. However, the most fundamental step towards rational design of lipid-based vaccine particles would be to develop a systematic protocol for measuring vaccination outcome as well as for the physicochemical characterization of the particles themselves. Moreover, identifying the immune responses that elicit mucosal protection would aid the rational design of effective mucosal vaccines. One aim of this thesis work is to take one step in this direction.

5
Experimental techniques

The experimental techniques used in this work can be divided into two main categories: techniques used for physicochemical characterization of the lipid nanoparticles and techniques used for characterization of the elicited immune response, in particular uptake and processing by DCs. The former includes methods for quantification of protein content (Section 5.1), particle size determination (Section 5.2), examination of particle morphology (Section 5.3) and surface charge (Section 5.4). To characterize the elicited immune response, light microscopy is the technique of choice to follow particle uptake (Section 5.5) while flow cytometry has been used to quantify antigen presentation (Section 5.6).

5.1 Fluorometry for protein quantitation

Fluorometry relies on the concept of fluorescence, which is commonly visualized using a Jablonski diagram, as seen in Figure 4A.[96] In fluorescence, a molecule known as a fluorophore emits light when excited by incoming light of appropriate wavelength. Light of this wavelength consists of photons with an energy content that corresponds to the energy gap between the ground state (S0) and the next higher, excited, state (S1) of the fluorophore. When such a photon hits the fluorophore, the molecule is excited to the higher energy state. The higher energy state is not stable and hence the molecule rapidly returns to the ground state and in doing so light of a lower energy is emitted, as some energy is lost in thermal processes.[96] The energy, E, of a photon is given by:

$$E = \frac{hc}{\lambda}$$

where h is Planck's constant, c is the speed of light and λ is the wavelength. Hence, the emitted lower energy photon will have a longer wavelength. The difference between the peak excitation wavelength and the peak emission wavelength of a certain fluorophore is termed Stoke's shift.[97] A common method used to determine emission spectra for fluorophores and to generally quantify fluorescence is fluorometry. The basic components of a fluorometer are: a light source, a specimen chamber or sample holder and one or several photodectors such as photomultipliers and charge-coupled device cameras. Additionally, there is commonly monochromators or filters to select specific excitation and emission wavelengths.

Fluorometry can be used to determine protein content either by utilizing inherently fluorescent residues such as tryptophan or by the use of assays in

which a fluorescent tag is introduced. The CBQCA assay is one such assay in which the non-fluorescent molecule 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) reacts with primary amines in the presence of cyanide to form a highly fluorescent derivative (Figure 4B).[98] After acquiring a calibration curve, the magnitude of the emitted fluorescence can be used as a measure of total protein content of a sample.

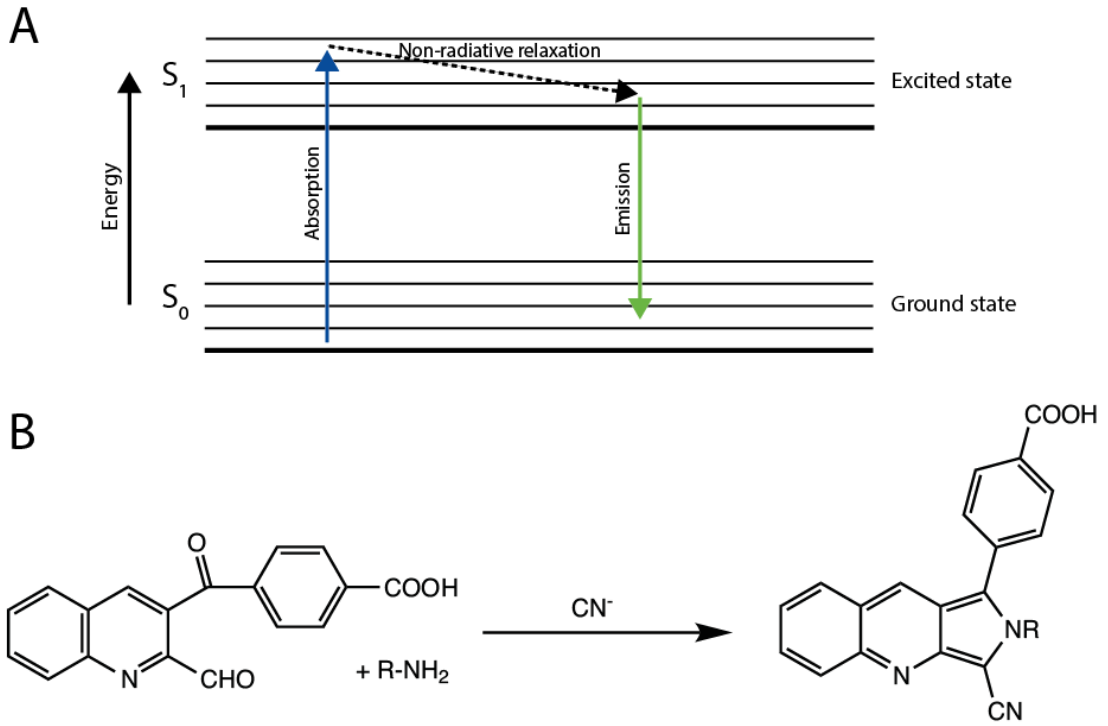

Figure 4. A: The principal of fluorescence illustrated with a Jablonski diagram. A fluorophore is excited from the ground state S_0 to the excited state S_1 through absorption of light. During relaxation back to the ground state, light of a lower energy is emitted. B: The reaction of the non-fluorescent molecule 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) with primary amines in the presence of cyanide to form a highly fluorescent derivative used to quantify total protein content.

5.2 Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) is a nanoparticle sizing and concentration determination technique that relies on visualizing particles in solution undergoing Brownian motion.[99] During a measurement, a laser beam is passed through the solution containing the nanoparticles, which are visualized through light scattering or fluorescence and whose diffusion is recorded using an optical microscope equipped with a camera (Figure 5). Particles in solution undergo Brownian motion in three dimensions. Under the assumption that the motion is uniform in all directions, the captured two-dimensional motions of the particles are tracked by the NTA software in order to determine their diffusion coefficients D from the relation:

$$D = \frac{{z_{2D}}^2}{4t}$$

where z_{2D}^2 is the mean square displacement in two dimensions during the time t.[100] Assuming spherical particles, the hydrodynamic radius r is given by the two-dimensional Stokes-Einstein equation:

$$r = \frac{k_B T}{3\pi\eta D}$$

where k_B is the Boltzmann constant, T is the temperature in Kelvin and η is the solvent viscosity.[99] The hydrodynamic radius, or Stokes radius, of a particle is not its actual physical size but the radius of a hard sphere with the same diffusion rate. This is important to keep in mind when working with non-spherical particles.

NTA can be used for size determination of particles with diameters between approximately 30 to 1,000 nm, with the lower limit being determined by the refractive index of the particles.[101]

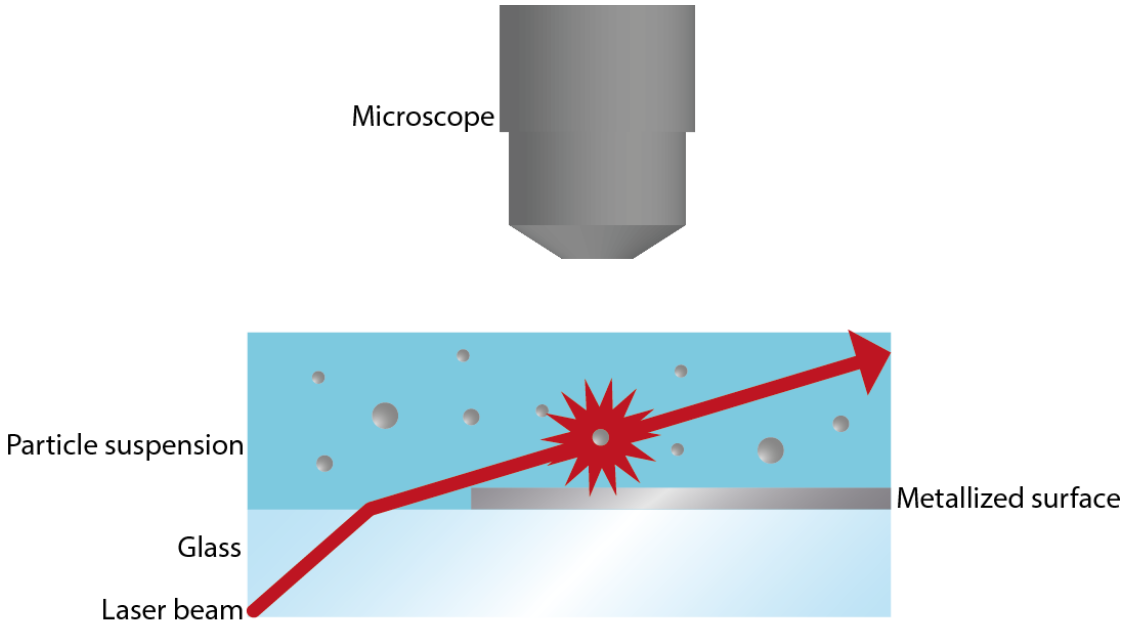

Figure 5. Schematic of a nanoparticle tracking analysis setup. Particles undergoing Brownian motion in solution are visualized through scattering of laser light. Their motions are tracked using specialized software in order to determine their diffusion constant and hydrodynamic diameter.

5.3 Cryogenic transmission electron microscopy

Transmission electron microscopy (TEM) is a high resolution imaging technique in which the image contrast is generated by the scattering of electrons when they encounter structures with high electron density within the specimen. The instrument consists of an electron source emitting an electron beam that is focused onto the specimen with electromagnetic lenses and a detector, commonly a CCD camera (Figure 6A).[102, 103] The imaging is by necessity performed in vacuum to avoid scattering of the electron beam by air. The analyzed samples are thin, 20-90 nm, and negative staining is often applied to increase the contrast. Negative stains generally consist of a salt of a heavy metal

with a high atomic number (42-92) that forms a thin glassy film on top of the specimen.[102] Conventional TEM on hydrated systems is however prone to artifacts due to staining and shrinking due to drying, which affects the perceived morphology of the specimen.[102]

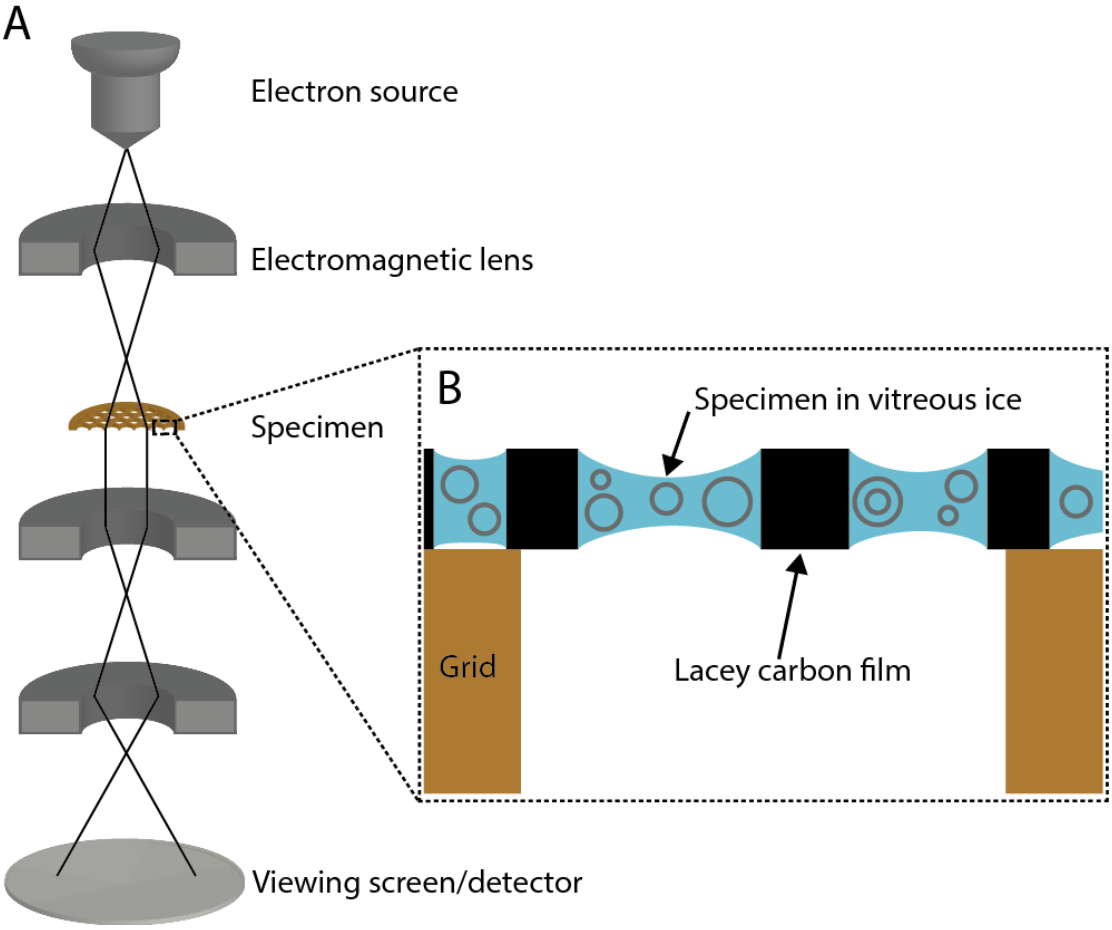

Figure 6. A: Schematic representation of cryogenic transmission electron microscopy; scattering of electrons is used to visualize structures of high electron density in a cryopreserved specimen. B: In a common type of cryopreservation the specimen, in solution, is applied to a lacey carbon film mounted on a copper grid. The supported specimen is plunged into a cryogen leading to formation of vitreous ice, preserving the morphology of fragile structures.

To overcome these limitations, cryogenic TEM (Cryo-TEM) relies on cryopreservation of the sample prior imaging to minimize artifacts. The sample preparation is aimed at creating vitrified specimens that maintains their structural integrity, generally through plunge freezing of thin films or cryo-sectioning of bulk samples.[102] Plunge freezing is a fast and fairly straightforward method in which a small amount of sample in liquid suspension form is added to a supporting substrate, often a lacey carbon film supported by a copper grid. The sample is blotted with filter paper so that only a thin film remains on the substrate and is then plunged into a cryogen with high heat capacity, such as liquid ethane at around -183 °C. Due to the very rapid freezing rate, the liquid suspension vitrifies instead of forming crystalline ice, thus keeping the structures within intact (Figure 6B). The vitrified samples are

transferred to the electron microscope's cryoholder under liquid nitrogen and are viewed at around -173 °C under vacuum.[102]

Due to the thinness of the film formed through this preparation method, it may be difficult to visualize larger structures. Furthermore, the elements comprising biomacromolecules and polymers, such as proteins and PEG, generally do not scatter enough to provide sufficient contrast to be readily visible with Cryo-TEM.[102] Cryo-TEM of protein- and/or polymer-conjugated lipid particles therefore gives information about the size and morphology of the lipid structures only, unless additional labeling is performed. It is important to note that since Cryo-TEM gives information about the physical size of particles, it can be misleading to directly compare sizes measured from Cryo-TEM images to data obtained with other sizing techniques that measures the hydrodynamic size, such as NTA or dynamic light scattering (DLS).

5.4 **Laser Doppler electrophoresis**

The charge of particles is commonly assessed by measuring their electrophoretic mobility, i.e. their velocity in an electric field.[26] This measure is independent of shape and size and can be assessed using laser Doppler electrophoresis (LDE). An LDE instrument generally consists of a laser providing collimated light that is split into two beams: the scattering beam and the reference beam (Figure 7A).[104] The scattering beam enters the scattering volume, an electrophoretic cell that contains the specimen, a suspension of particles, which scatters the incoming light. The movement of the particles undergoing electrophoresis causes a shift in the frequency Δf of the scattered light compared to the reference beam due to the Doppler effect according to:

$$\Delta f = 2v \cdot \frac{\sin(\theta/2)}{\lambda}$$

where v is the particle velocity, λ is the wavelength of light used and Θ is the scattering angle.[104] The light scattered at the angle Θ is combined with the reference beam before arriving at the detector. Analysis of the Doppler shift can be made using phase analysis light scattering in order to deduce the electrophoretic mobility of the particles.

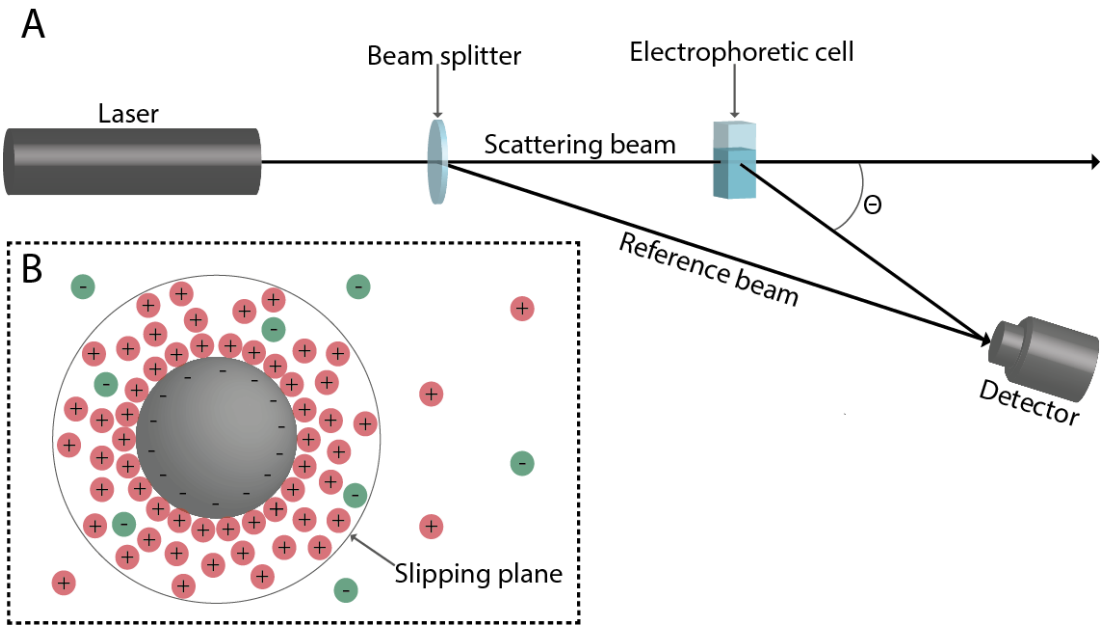

Figure 7. A: Schematic representation of a Laser Doppler electrophoresis instrument; laser light is split into a reference beam and a scattering beam, which is scattered by the particles undergoing microelectrophoresis. The frequency shift between the reference beam and the light scattered at angle θ, due to the Doppler shift is used to deduce the electrophoretic mobility of the particles which is used to determine the zeta potential. B: The zeta potential is the electric potential, not at the particle surface, but at the slipping plane at the edge of the diffuse electric double layer comprising ions loosely associated with the particle.

The oscillating electric field applied during LDE causes the particles to move at a velocity that is proportional, not to the charge directly at the particle surface, but rather to the zeta potential, which is the electrostatic potential at the slipping plane at the edge of the diffuse electric double-layer surrounding charged particles (Figure 7B). The double-layer consists of differently charged ions distributed in the near vicinity of the particle surface, thus shielding the surface charge. The thickness of the double layer is also known as the Debye length and is defined as the distance from the surface where the potential has fallen to 1/e of its value at the surface.[26] The zeta potential ζ can be calculated using the Henry equation:

$$\varsigma = \frac{3\mu\eta}{2\varepsilon_0 D} f(\kappa a)$$

where μ is the electrophoretic mobility, ε_0 is the permittivity of vacuum, η and D are, respectively, the viscosity and the dielectric constant of the dispersion medium and a is the particle radius.[105] f(κa) is Henry's function which depends on the Debye length κ^{-1}. The magnitude of both the Debye length and the zeta potential do not only depend on the particle charge, but also on the ionic strength and pH of the medium in which the particles are dispersed.[26] In cases where the particle radius is much larger than the Debye length (κa>>1), the Henry equation can be simplified using the Smoluchowski approximation of f(κa)=1.5, giving:

$$\varsigma = \frac{\mu\eta}{\varepsilon_0 D}$$

[105, 106] At physiological ionic strength, the Debye length is on the order of nanometers, so this approximation is thus often suitable.[26]

5.5 **Light microscopy**

Methods capable of identifying and quantifying cellular responses are useful when assessing how particle properties affect key events in the immune response. In the context of vaccination key events include antigen uptake by an APC. Uptake processes can be characterized and quantified using microscopy and current high-resolution imaging methods provide opportunities to do so with precision. In particular, live-cell imaging allows us to observe the dynamics of such processes in real-time. In this chapter, a brief overview of the basics of optical microscopy, some specialized microscopy techniques and examples of how they have been used to study cellular uptake of different types of particles in sizes ranging from tens to a few hundred nm will be given.

Convex lenses have been used for more than five hundred years to magnify objects beyond the human eye's ability to observe and the invention of the microscope took place in the 17th century.[107] Thanks to these advances, we have been able to visualize and understand microorganisms, our own cells and eventually also their constituents.

There are many microscopy subtypes and setups but the basic construction of an optical microscope is mostly the same in all cases. In essence, light from a light source such as a lamp, LED or laser passes through a condenser and then interacts with a specimen.[107] The light is collected by the objective, which contains one or several lenses and that helps focus the image of the specimen at the optical plane of an eyepiece or a camera.[107] In the simplest form of microscopy, brightfield, the light is simply transmitted through the specimen; all the light is collected and the contrast is given by the attenuation of the light due to the sample. However, many specimens have low contrast, making structures difficult to separate from the background. Therefore, there are variants of optical microscopy aimed at enhancing the contrast, such as darkfield, phase contrast, polarization microscopy and differential interference contrast (DIC).[108-111] In addition to the contrast, the resolution is a critical parameter for how well small objects are visualized using light microscopy. The resolution limit is a constraint that the wavelike properties of light place on conventional light microscopy. When light from a point source passes through a circular aperture, such as a lens, it is diffracted and will appear as a bright spot surrounded by a series of concentric circles (an Airy disk and Airy pattern).[112] When two objects come close together, their Airy patterns overlap and they cannot be visually separated. Ernst Abbe formulated the dependency of the resolution limit, d, on the wavelength of the light, λ, and the objective used for imaging:

$$d = \frac{\lambda}{2n \sin\theta} = \frac{\lambda}{2NA}$$

where n is the refractive index of the medium, θ is the half angle subtended by the objective and NA is the numerical aperture of the objective.[113] For modern instruments, this means approximately half the wavelength of the light, in practice.

An important component of many modern microscopy techniques is the use of fluorescent labels, such as antibodies conjugated to fluorophores (see section 5.1) or proteins which are expressed with the tag directly attached. This has been crucial not only to detect objects smaller than a couple hundred nanometers but also to visualize cellular structures and observe dynamic processes, such as uptake. Fluorescence has been instrumental in the development of the imaging field, both as a means to improve the contrast but additionally in allowing us to move beyond Abbe's limit using techniques such as confocal laser scanning microscopy and super-resolution imaging.

5.5.1 Widefield Fluorescence Microscopy

The simplest type of fluorescence microscopy is widefield fluorescence microscopy, or epifluorescence microscopy. In this technique, multichromatic light is sent through an optical excitation filter that allows only light of wavelengths suitable for excitation of a particular fluorophore to pass; the light meets the main beam splitter, a component that selectively reflects or transmits light of different wavelengths. The beam splitter, or dichromatic mirror, reflects the short wavelength excitation light through the objective and onto the specimen. The light emitted by fluorophores in the sample is collected by the objective and meets the main beam splitter, which transmits this light that then passes through an emission filter that allows light of only certain wavelengths to pass to the eyepiece or camera (Figure 8).[114] The excitation and emission filters together with the dichromatic mirror are generally mounted into a filter cube. It is common to have multiple filter cubes suitable for imaging different fluorophores. One can then switch from one to the other and image different labeled structures in sequence.[115]

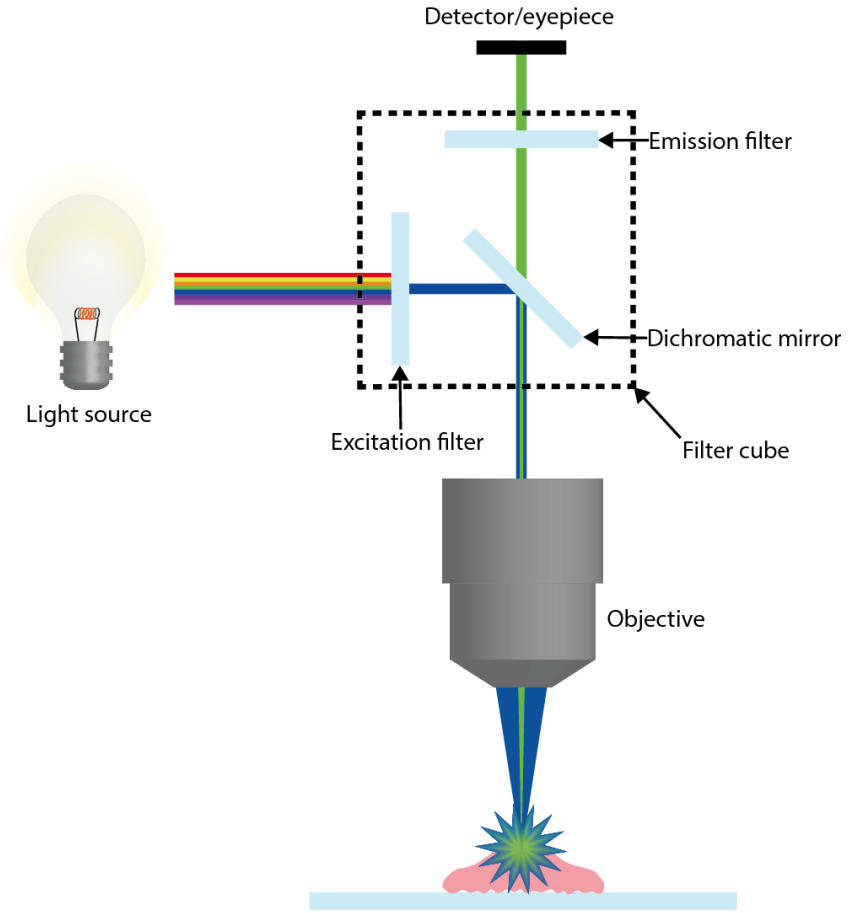

Figure 8. General setup of a widefield fluorescence microscope.

Widefield fluorescence microscopy has been applied to study cellular uptake of different particles of interest. Van der Schaar et al. used widefield microscopy to study cellular uptake of DiD-labelled Dengue virus.[116] They elucidated both the clathrin-mediated internalization process and the endocytic trafficking of the virus through fluorescent labeling of endocytic machinery components.[116] Using HIV-1 virus-like particles (VLPs), Endreß et al. showed two types of HIV-cell interactions: either the VLPs became immobilized upon contact or there was a very short-lived dynamic interaction (in the range of 20-50 ms) followed by dissociation.[117] de Bruin et al. used epidermal growth factor (EGF) to direct delivery of polyethylenimine polyplexes to cancer cells that overexpress EGF receptor (EGFR). Widefield microscopy revealed faster and more efficient internalization of EGFR-targeted compared to untargeted polyplexes.[118] Furthermore, Tian et al. studied cellular uptake and processing of exosomes.[119] Single particle tracking was used to study the movement of exosomes in medium, on the cell surface and intracellularly in endosomes and lysosomes, identifying distinct movement patterns distinguishing membrane-bound from freely diffusing exosomes. Trypan blue was used to distinguish between intra- and extracellular DiI-labelled exosomes. Octadecyl rhodamine B chloride (R18)-labelled exosomes were used to discern that exosomes were taken up through endocytosis and not fusion with the cell membrane.[119]

A drawback of widefield fluorescence microscopy is that since all emitted light within a wavelength range is collected, there is usually a high background from out-of-focus fluorophores. Total internal reflection fluorescence (TIRF) microscopy is one technique that has been developed in order to address this issue.

5.5.2 Total Internal Reflection Fluorescence Microscopy

TIRF microscopy is a surface sensitive technique that relies on selectively exciting fluorophores using an illumination depth restricted to the area closest to an interface between two materials of different refractive indices, n1 and, n2. The fluorophores that are outside of the illuminated area remain unexcited and do not contribute to the background fluorescence. The materials of the interface are generally a sample, with low refractive index, e.g. water, on top of a glass coverslip, with high refractive index, as seen in Figure 9.[120]

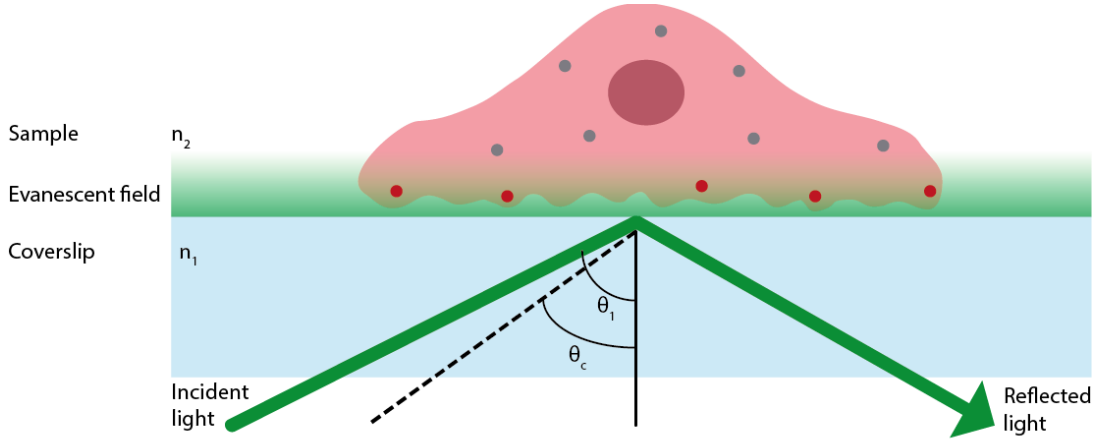

Figure 9. Schematic of TIRF microscopy. When incident light of angle θ1, greater than the critical angle θc, encounters an interface between a medium with high refractive index (n1, coverslip) and a mediim with low refractive index (n2, sample), the light is totally internally reflected. This leads to the formation of an evancescent field that extends a small distance into the sample. Thus, only the fluorophores whithin this field emit fluorescence while fluorophores that are further away from the interface remain unexcited.

How a light beam is refracted when passing through such an interface is described by Snell's law:

$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$

where θ_1 is the angle of incident light and θ_2 is the angle of refracted light. If the second material has a lower refractive index than the first ($n_1 > n_2$) and the angle of incidence is equal to what is termed the critical angle, θ_c, the refracted light will travel along the interface of the two materials. The critical angle is given by:

$$\theta_c = \sin^{-1}\frac{n_2}{n_1}$$

If the angle of incidence is larger than the critical angle ($\theta_1 > \theta_c$), the light is totally internally reflected at the interface, giving rise to an evanescent field that

extends a small distance into the second material.[120] The light intensity, I, decays exponentially with the distance from the interface, z, according to:

$$I(z) = I_0 e^{-z/d}$$

where I_0 is the intensity at the interface and d is the characteristic decay depth, defined as:

$$d = \frac{\lambda}{4\pi n_2}\left(\frac{\sin^2\theta}{\sin^2\theta_c} - 1\right)^{-1/2}$$

where λ is the wavelength of the incident light.[121] The surface-confined illumination eliminates background fluorescence, making TIRF microscopy a technique with high signal-to-noise ratio.

TIRF microscopy has been used to study cellular uptake for example by Schmidt et al., who studied pH-triggered fusion of vaccinia virions to HeLa cells using a microfluidic cell trap.[122] The viruses were labelled with a self-quenching concentration of R18 and the virus cores were GFP-tagged. A reduction of the pH was used to induce fusion, which caused dequenching of the R18 simultaneous to reduction of the GFP signal due to internalization of the virus core.[122] TIRF has also been used to visualize quantum dot diffusion on the surfaces of immune cells, with single particle tracking used to determine diffusion constants, followed by uptake quantified by measuring the overall decrease in fluorescence as the quantum dots disappeared from the field of view when taken up.[123] It was shown that the size and shape of the quantum dots influence both their final intracellular fate and their behavior on the cell membrane; more specifically, quantum dots with a high aspect ratio exhibited slower movement.[123] Furthermore, TIRF has been used to show the dynamics of cholesterol-modified Cy3-labelled siRNA internalization.[124]

5.6 **Flow cytometry**

An important step of the immune response is the antigen presentation by DCs, which is a crucial step for activation of T cells. The amount of antigen presented on the DC surface in response to changes in the physicochemical properties of vaccine vectors can be assessed using flow cytometry. Indeed, flow cytometry is often used for characterization and quantification of cells and cell constituents. It is a popular technique in different fields of research and has a set of uses ranging from, for example, determination of cell viability to quantification of phagocytosis.[125, 126] In recent years, flow cytometry has, in addition to being an invaluable research tool, become an important diagnostic and prognostic tool in the clinical treatment of cancer and immunological diseases.[127] Furthermore, the use of flow cytometry has stretched beyond the analysis of cells to also include biologically relevant micro- and nanoparticles, such as exosomes.[128]

The large variety of applications and their increasing complexity aside, the flow cytometry technique relies, in essence, on three basic building blocks: 1) the arranging of the cells into a single file through a flow cell, 2) the optical system comprising one or several lasers generating the illumination of the sensing volume through which the cells pass, as well as a set of lenses and filters to focus and direct the light which is scattered and/or emitted by the cells, and 3) the electronics used to convert this light to an electronic signal. Due to the different components comprising a flow cytometer, one might say that there is not a singular origin of the technique, but rather several advancing paths converging into the development of cell microfluorometry by van Dilla et al. in 1965.[129] This chapter will give a brief overview of the theory behind flow cytometry, describing a typical instrument and the physical phenomena it employs.

5.6.1 Principles and instrument design

As previously touched upon, a flow cytometer can be said to consist of three main components: a microfluidic system, an optical system and an electronic system that function in a synchronized manner (Figure 10).[130] In this section, each of these systems is presented in some detail.

5.6.1.1 Microfluidics

The role of the microfluidic system is to ensure that the cells pass in a controlled manner one by one through the sensing volume, or interrogation point as it is sometimes referred to. This is crucial to maintain uniform and reproducible illumination conditions. The arrangement of cells into "a single file" is achieved through hydrodynamic focusing by use of a sheath flow. A pressurized stream of sheath fluid is maintained into which the sample is injected at a higher pressure, causing a difference in flow speeds between the two fluids that maintains the cells in the central stream, the "core". A strategic narrowing of the flow cell causes a simultaneous increase of the flow speed and a decrease of the core cross-section to the point where cells generally pass through the sensing volume one by one.[131] Generally speaking, it is crucial to maintain laminar flow by avoiding e.g. too acute narrowing of the flow cell and sharp edges in the design as well as blockages and air bubbles during operation. The nature of the flow in a tube is determined by four parameters, whose relationship is often expressed by the dimensionless Reynolds number, Re:

$$Re = \frac{vd\rho}{\mu}$$

where v [m/s] is the average velocity throughout the cross-section of the tube, d [m] is the tube diameter, ρ [kg/m^3] and μ [kg/sm] is the fluid density and viscosity, respectively. At Re greater than 2,300 the laminar flow starts to break down in favour of turbulence.[132] The sensing volume is generally located in an area with "slug flow", characterized by constant flow speed across the diameter of the core, a result of the aforementioned narrowing of the flow cell. This is

advantageous as it minimizes the velocity differences between cells at different distances from the centre of the core compared to the parabolic flow profile of a laminar flow that arises from the no-slip boundary condition.[131]

5.6.1.2 Optics

When cells pass through the sensing volume, i.e. the laser beam, the light that is scattered, and emitted in the case of fluorescently labeled cells, gives information about their properties. Physically speaking, scattered light consists of light that has been diffracted, reflected, refracted, anomalously diffracted and Rayleigh scattered.[132] Maxwell's equations can be solved to describe the propagation of light after it has been scattered by an object. In the case of a cell, there are many intracellular objects with varying properties, and the morphological and biochemical complexity of a cell thus make the mathematical description of this process challenging even though it has been attempted.[133-137] Such modeling is however not standard in flow cytometry, where the light scattering properties of cells are measured and related to two main properties. Light that is deflected around the edges of the cells, parallel to the direction of the laser beam, is termed forward scatter (FSC) and gives information about the size of the cells. Light that is scattered perpendicularly to the laser beam is termed side scatter (SSC). Side-scattered light is primarily scattered from intracellular structures, therefore giving information about the internal complexity.[126] The intensity of the SSC is proportional to what is often termed cell granularity.

In addition to the information from the scattered light, which is obtained label-free, features of interest can be tagged with fluorescent labels (see section 5.1). Fluorescent probes are utilized in order to detect and quantify the amount of for example nucleic acids, proteins or to assess cell viability.[126] Since different fluorophores have different emission and excitation spectra, a cell can be stained with several probes with different fluorophores (fluorochromes). The most common fluorophores used for flow cytometry include fluorescein isothiocyanate (excitation/emission 495/520 nm), phycoerythrin (excitation/emission 565/578 nm) and allophycocyanin (excitation/emission 650/660 nm).[126] Once excited by lasers with light of suitable wavelengths, monochromatic mirrors and optical filters are used to guide emitted light from different fluorochromes in different "channels" to different detectors (see section 5.5.1).[131]

5.6.1.3 Electronics

The electronic system in a flow cytometer consists of a series of detectors that convert the scattered and emitted light into electrical currents that can be recorded and visualized. There are generally two types of detectors used: photodiodes and photomultiplier tubes. Photodiodes are typically used for the FSC, which has a high intensity, while photomultiplier tubes are used to amplify

and detect SSC and emitted light.[138] In both cases, the output signal is proportional to the incoming number of photons.[126]

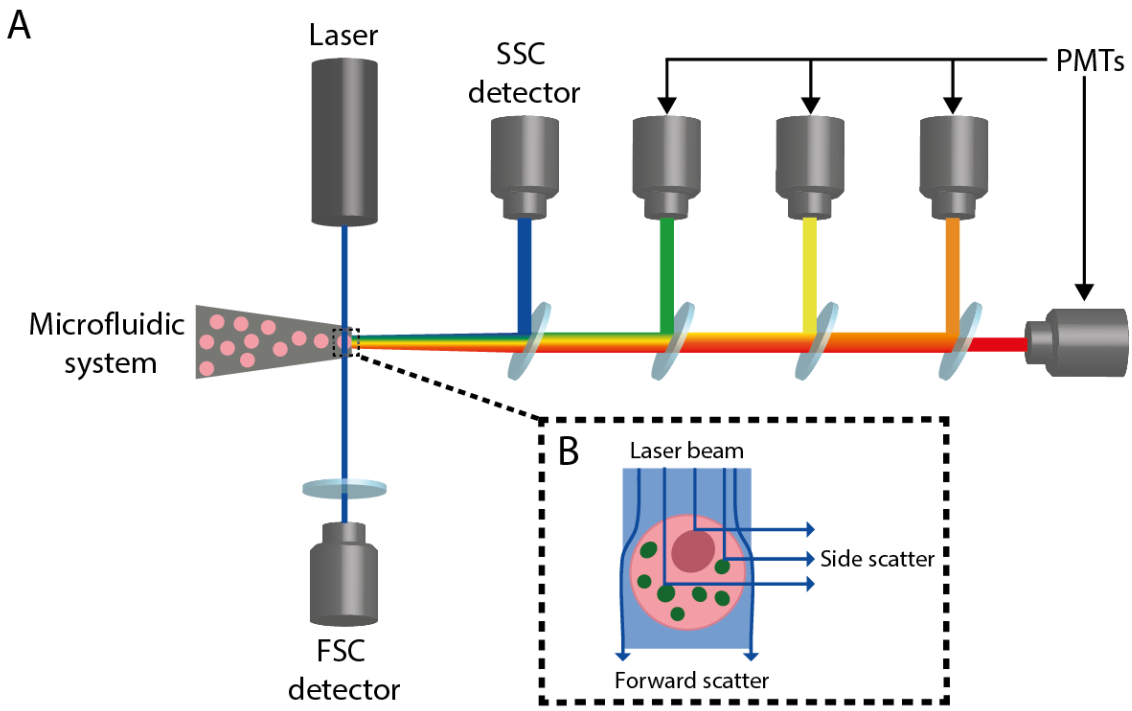

Figure 10. A: A schematic representation of a flow cytometer. The microfluidic system arranges the cells into a single file through the beam of one of several differently colored lasers, i.e. the sensing volume, B. The light that is scattered parallel to the laser beam, termed forward scatter (FSC), provides information about the size of the cells and the light that is scattered perpendicularly, termed side scatter (SSC), gives information about the internal complexity of the cells. Dichromatic mirrors and optical filters are used to guide the SSC and light emitted from fluorescent labels to different photomultiplier tubes (PMTs).

5.6.2 Data analysis

In flow cytometry many parameters are usually measured on numerous individual cells, which means that a large and important part of the method comprises the processing of the data. Common ways to display the data include dot plots, which visualize the magnitude of two parameters at the same time, and histograms, which visualize the number of cells and to which degree they are, for example, expressing a marker of interest.

5.6.2.1 Spectral compensation

Spectral (or colour) compensation is a standard procedure used when several fluorochromes are used in one measurement. Overlap of the emission spectra of the fluorophores means that light from several fluorochromes can pass through the emission filters and be detected in the same channel. Spectral compensation is thus needed to adjust for the light each fluorochrome contributes with in channels other than its own, and is done by measuring each fluorochrome alone and calculating how much of the total light each contribute with to the different

channels.[126] This information is then used to compensate for any overlap in the subsequent measurement.[139]

5.6.2.2 Gating

Gating is a process commonly used to eliminate results from debris and dead cells and to subsequently enumerate cells with certain properties.[126] Cells are visualized in a two-dimensional scatter plot in which the user defines one or several regions, restricting further analysis to certain subsets of cells. By in this manner iteratively visualizing and zooming in on cells with a certain set of markers, identification and quantification of cell subpopulations is performed.[140]

5.6.2.3 Computational flow cytometry

As progressively large and complex datasets are collected, the challenge in manually analyzing the data is increasing. It is impossible to fully visualize and manually see relationships between all parameters that modern flow cytometers are capable to measure at once. Therefore, dimensionality reduction techniques such as principal component analysis (PCA) and t-stochastic neighbor embedding (t-SNE) aimed at projecting high-dimensional data into two-dimensional representations provides useful overviews.[140] Automated clustering methods are useful to find and group similar objects together. Cells with similar profiles are assigned to clusters, which can then be interpreted as cell types.[140] Standardized and automated data analysis not only enables pattern-finding in high-dimensional data but has the further advantage of eliminating bias and increasing reproducibility in gating.[140]

6
Results

The main question addressed in this thesis is how the physicochemical properties of lipid nanoparticles used as mucosal vaccine carriers influence the activation of the immune response. Understanding which properties are decisive for development of protective immunity, and in extension the identification of promising candidates at an early stage, might help rationalizing the process of designing vaccine formulations. We have started addressing this challenge using antigen-carrying lipid particles, in model systems at several levels of complexity. Firstly, in Paper I, we performed a detailed characterization of the immunogenicity of two types of antigen-carrying liposomes both *in vitro* and in an *in vivo* mouse model. Secondly, in Paper II, we focused on an *in vitro* antigen presentation assay to both investigate the formulations introduced in Paper I in more detail, and to extend our search for immunogenic antigen carriers to both a broader, with respect to physicochemical properties, set of liposome formulations as well as a different type of lipid particle: the lipodisk. Additionally, presented in the additional experimental results, we have started to explore single-cell-and-particle imaging to investigate the interaction between nanoparticle vaccine carriers and the outer cell membrane of dendritic cells, representing the very first step in activating the immune response.

6.1 **Paper I**

In Paper I, we aimed at combining the universal influenza A vaccine candidate CTA1-3M2e-DD with liposomes into an effective mucosal vaccine formulation. CTA1-3M2e-DD is a fusion protein that combines the mucosal adjuvant CTA1-DD with the ectodomain of influenza matrix protein 2 (M2e), which is highly conserved in all human influenza A virus strains. In addition to three repeats of M2e, the fusion protein consists of the enzymatically active cholera toxin A1 subunit (CTA1), which has an immunomodulating effect, and a dimer of the D-fragment from *Staphylococcus aureus* protein A (DD): an effective DC targeting moiety. In this work, the fusion protein was formulated into two types of liposomes: PEGylated and non-PEGylated POPC-based liposomes with 10% Chol.

The liposomes had the fusion protein both encapsulated and covalently attached using the thiol-maleimide reaction either directly to the lipid headgroups (non-PEGylated) or to lipid-anchored PEG(2000) spacers (PEGylated). The liposomes were characterized with respect to size, protein load and surface charge. Both formulations were of a similar size: mean diameter approximately 150 nm, and both were slightly negatively charged. The main differences between the two

formulations, in terms of physicochemical characteristics, besides the PEGylation, were that the non-PEGylated formulation had approximately twice the negative charge (zeta potentials of -48 and -21 for non-PEGylated and PEGylated, respectively), while the PEGylated carried approximately 30-40% higher protein load. In all experiments however, the administered fusion protein dose was kept constant.

The immunogenicity of the free fusion protein as well as the two vectors was assessed using two *in vitro* assays. Firstly, B cells (used as APCs) were stimulated and their ability to activate M2e-specific $CD4^+$ T cells were assessed by measuring the T cell proliferation. It was found that, at low doses, particle-associated antigen more effectively induces T cell proliferation than free fusion protein. Secondly, an assay reporting the primary immune activation assessed by antigen presentation by DCs (further used in Paper II) was used. It was found that at short time scales, up to 1 hour, the nonPEGylated vector more effectively induced antigen presentation than both the free protein and the PEGylated vector.

In vivo immunogenicity assessment following intranasal immunizations in a mouse model, showed that the non-PEGylated vector more strongly induced an M2e-specific $CD4^+$ T cell-response than both the free fusion protein and the PEGylated vector. Furthermore, the non-PEGylated vector gave rise to higher levels of IFN-γ and serum IgG than the PEGylated, as well as higher local secretion of IgA in the lungs than the free protein. Additionally, we observed that intranasal immunization using the non-PEGylated vector lead to significant protection from a lethal challenge with a live heterosubtypic virus (a virus from a different strain than the M2e originated from), while naïve mice as well as mice immunized with free fusion protein or PEGylated vectors all succumbed to infection.

Taken together, the results from Paper I show that fusion protein packaged into non-PEGylated liposomes more efficiently induce both cell- and antibody-mediated immune responses as well as protection against a lethal virus challenge, than both PEGylated liposomes and free fusion protein, which is in itself already an effective vaccination compound. As previously mentioned, the PEGylated and non-PEGylated vectors differed with respect to protein load and charge, in addition to PEGylation. This thus raises the question: which of these properties was key to the improved immunogenicity of the non-PEGylated vector? We attempted to address this question, together with investigation of additional physicochemical alterations, in Paper II.

6.2 **Paper II**

In Paper II we focused our investigation on how the antigen presentation by DCs is affected by certain physicochemical properties of vaccine carriers. In order to do this, and simultaneously screen for promising carrier candidates we applied the *in vitro* antigen presentation assay introduced in Paper I. In order to observe the kinetics of the antigen presentation, the amount of functionally presented antigen on the DC surface was monitored at various time points up to 24 h after administration of the different formulations. The quantification of the antigen presentation was performed using flow cytometry and immunostaining with anti-MHC II and the YAe antibody, which recognizes the Ealpha (Ea) peptide when presented in the MHC II. Therefore, the antigen portion of the CTA1-DD-based fusion protein used in this study consisted of the Ea peptide. A more quantitative data analysis was performed compared to in Paper I, including quantification of the surface-bound MHC II as a measure of the level of DC activation. Informed by the results in Paper I, we attempted to systematically vary physicochemical properties of fusion protein-carrying lipid particles.

Firstly, PEGylated and non-PEGylated POPC-based liposomes (abbreviated POPC-PEG and POPC-PE) were formulated with fusion protein both encapsulated and surface-bound, using the same lipid composition as the liposomes used in Paper I. Similarly to in Paper I, the POPC-PEG had, compared to its non-PEGylated counterpart, higher protein load and zeta potential, likely due to a higher content of negatively charged lipids in the POPC-PE, combined with a charge shielding effect from the PEG in POPC-PEG. During the work presented in Paper I, it was observed that the non-PEGylated vector was the most promising candidate and additionally, it proved difficult to tightly control the portion of encapsulated fusion protein. Thus, secondly, the amount of surface-bound protein on non-PEGylated POPC-based liposomes was varied in an attempt to investigate the influence of protein load independently from PEGylation, percentage encapsulated protein and surface charge. Two different formulations with approximately three times difference in protein load were produced. Finally, to assess the influence of shape and size, DSPC-based lipodisks, i.e. flat bilayer circles stabilized by their high PEG content,[34] as well as liposomes of a similar composition (abbreviated DSPC-PEG) for direct comparison were produced. The lipodisks were 22 nm in diameter (determined using Cryo-TEM) while the DSPC-PEG were on average 127 nm in hydrodynamic diameter (determined using NTA). The DSPC-based formulations differ from the POPC-based ones in a significant aspect; DSPC is a gel phase lipid at physiological temperature, giving rise to particles with low fluidity and high rigidity. The POPC-based liposomes, on the other hand, are in the liquid disordered phase, which means that the membrane is fluid and the particles are easily deformable.[141]

We observed that all formulations gave rise to similar kinetics in the antigen presentation assay; a fast increase in antigen presentation in the first few hours, which leveled off at longer incubation times. There were overall little discernable differences among the POPC-based formulations. In Paper I, we observed an increased antigen presentation induced by non-PEGylated liposomes in the first hour, which was not observed with free fusion protein or PEGylated liposomes. In Paper II, we did not attempt to resolve such early differences but instead focused on the average antigen presentation over the whole time frame, in order to give a quantitative estimate of the immunogenicity of the formulations. Thus, it was observed that of all the formulations tested, only the DSPC-PEG liposomes lead to an overall increase compared to free fusion protein. This increase was substantial, reaching approximately 3 times more antigen presentation compared to free protein, as well as an increase in the total amount of surface-bound MHC II. This increase in MHC II indicates that the liposomes in the DSPC-PEG formulation have an independent adjuvant effect in themselves. The DSPC-PEG had comparable properties when it came to size, surface charge, protein load and level of PEGylation as POPC-PEG. The POPC-PEG formulation, however, actually lead to a slightly reduced antigen presentation compared to free fusion protein. Why does the DSPC-PEG carrier increase the immunogenicity of the vaccine formulation when POPC-PEG does not? Our hypothesis is that the answer lies in the phase of the lipid membrane; that the DSPC-PEG has a membrane in gel phase while the POPC-PEG membrane is in fluid phase at 37° C. We have formulated two hypotheses regarding the underlying mechanism.

Firstly, we hypothesize that this decrease in immunogenicity may be associated with an increased solubilization of lipid-fusion protein complexes from fluid phase membranes as compared to gel phase membranes, together with a reduced immunogenicity of the detached complexes. The partioning of a lipid into the aqueous phase is expected to increase if the hydrophilicity of the headgroup is increased, for example by a coupled fusion protein. Additionally, it is expected that lipid-fusion protein complexes would be more easily solubilized from a fluid phase bilayer than a gel phase one, due to the higher fluidity and less compact packing of the lipids compared to in gel phase membranes. The enzymatic activity of CTA1 is required for the adjuvant effect of the fusion protein.[142] Thus, the decreased immunogenicity of POPC-PEG compared to free (non-lipid coupled) fusion protein could be explained by a loss of enzymatic activity of the CTA1 moiety of the fusion proteins released from POPC-PEG liposomes. CTA1 has a hydrophobic binding site;[143] extraction of the lipid-fusion protein complex from the bilayer in a manner where the lipid tails might reside in the binding site could cause the formulation to exhibit decreased immunogenicity. In a system somewhat reminiscent of ours, Dubacheva et al. observed streptavidin, coupled via a single lipid moiety, releasing from a lipid membrane.[144] They hypothesized that this was possible due to

accommodation of the hydrophobic tail of the lipid in a hydrophobic binding pocket on streptavidin.[144] Thus, extraction of the lipid-fusion protein complex from the bilayer in a manner where the lipid tails may block the binding site of the adjuvanting moiety of the fusion protein, could cause the formulation to exhibit decreased immunogenicity. It also follows from this reasoning that if a considerable portion of the fusion protein is released from the POPC-based liposomes, the decreased antigen presentation induced by those formulations compared to free (non-lipid-coupled) fusion protein could be explained.

Secondly, rigidity has been suggested as an important factor in endocytic uptake of nanoparticles; theoretical modeling has shown that high bending and adhesion energies are required for deformable particles to be fully enveloped by the cell membrane, thus making soft particles more likely to remain trapped on the cell surface.[69-71] In addition, it has been confirmed experimentally that rigid particles are taken up to a larger extent than soft ones.[71, 72] Soft POPC-based liposomes and the small, flat lipodisks would require larger deformation of the cell membrane, making uptake more energetically costly than for of the more rigid DSPC-based liposomes. Since antigen uptake is a prerequisite for antigen presentation, more efficient endocytosis of the DSPC-PEG formulation might be an alternative explanation to why it performed well in the antigen presentation assay. Simultaneously, the lack of contrast between the different POPC-based liposomes may not necessarily mean that the physicochemical properties varied among them generally have no effect, but merely that the uptake is equally hampered by all the POPC-based liposomes, causing them to be trapped on the cell surface. Before concluding that e.g. protein load has no effect on processing by DCs it is important to assess what the underlying reason behind the observed differences, or lack thereof, is. With this latter hypothesis pertaining to nanoparticle rigidity and/or strength of antigen attachment to the cell membrane influencing cellular uptake in mind, we are now in the process of developing a method to characterize and quantify the initial interaction between vaccine carrier and DC.

6.3 Additional experimental results: TIRF microscopy-based study of cell-particle interactions

The first step in activation of the immune response is antigen uptake by APCs such as DCs, who can sample their environment using a variety of mechanisms. In the context of vaccine vectors it remains unclear how the physicochemical properties of the carrier affects the interactions with the cell membrane and how that influences the uptake mechanism, how that process progresses and how that in turn affects the downstream processing of the antigen. We aim to develop a method that allows us to visually, in detail, probe interactions between lipid vaccine particles and cells on a single-particle and single-cell level.

Visualizing movements of single particles requires a high signal-to-noise ratio and high spatial resolution. Therefore, TIRF microscopy was used. It is a surface sensitive technique, allowing for visualization of the movements of particles on the basal membrane alone and effectively minimizing the influence of background fluorescence. This allows for the use of SPT to analyze particle trajectories. The particles were tracked using a dedicated MATLAB script created within the division of Biological Physics, Chalmers University of Technology (available upon request). Additionally, we used a topographically patterned substrate with micropillars (Figure 11) to facilitate access to the basal membrane and uninhibited movement compared to flat glass. As a proof of concept a fetal skin dendritic cell line (FSDC), derived from mouse [145] was used, as robustness and ease of handling was a priority during the initial development.

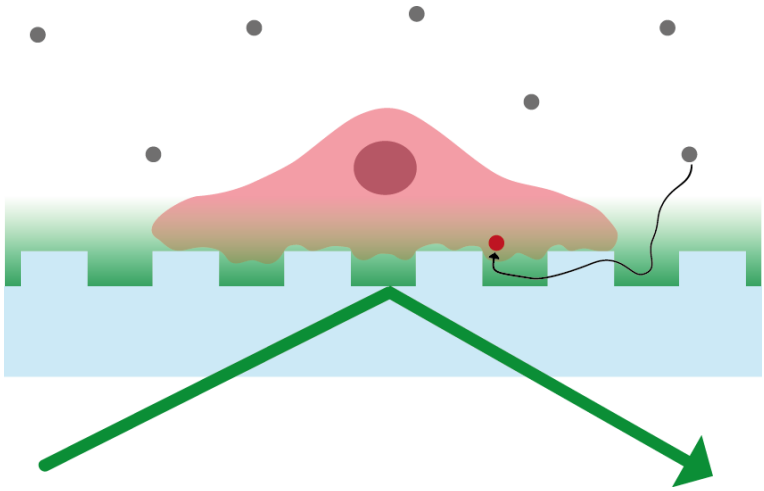

Figure 11. FSDCs are cultured on a micropatterned glass substrate with pillars, 400 nm high and with 1 µm diameter and spacing. This provides freedom of movement to lipid particles to interact with the basal cell membrane. The movements of the particles are observed using the surface sensitive technique TIRF microscopy, which selectively illuminates the volume closest to the glass substrate. SPT is used to analyze the particle trajectories.

Initially, two different micropatterns were tried: the pillars were 400 nm high and their diameter and spacing were kept the same at either 1 µm or 2 µm. FSDCs were cultured on flat glass and the two kinds of topographically patterned substrates. The cells were fixed, permeabilized and stained with DAPI and rhodamine phallodin (nucleus and F-actin, a cytoskeleton marker, respectively). The morphology of the cells was inspected using epifluorescence microscopy. Since these cells are normally cultured on flat substrates the flat glass sample was considered the positive control (Figure 12A). Here, cells are seen to stretch out, forming thin filaments to outstretched attachment points. A similar morphology is seen on the 1µm pattern (Figure 12B), while on the 2 µm pattern cells appear more rounded and with fewer outreaching filaments (Figure 12C). Since the cells exhibited normal morphology on the 1 µm pattern, this was chosen for use in the assay.

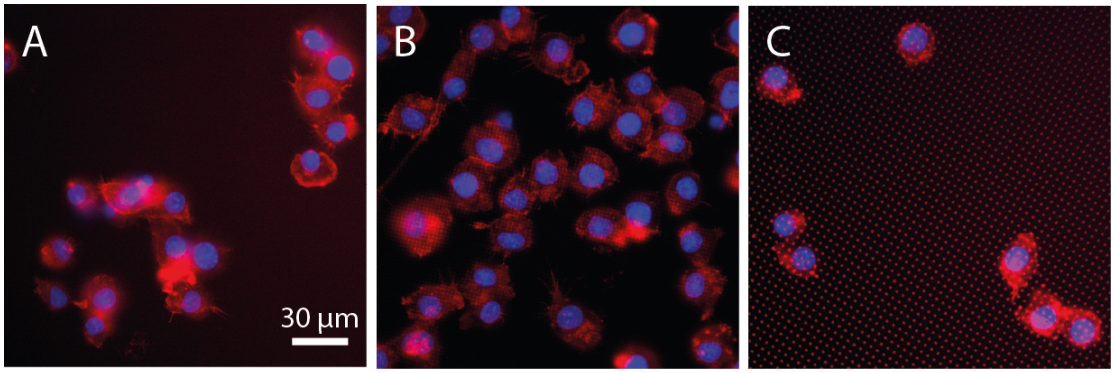

Figure 12. FSDCs stained with DAPI (nucleus, blue) and rhodamin phalloidin (F-actin, red), cultured on: A: flat glass, B: glass with 400 nm high pillars with 1 µm diameter and spacing, C: glass with 400 nm high pillars with 2 µm diameter and spacing.

In order to study the interaction between lipid particles and cells, rhodamine-labelled, CTA1-3M2e-DD-functionalized liposomes (Figure 13A) were added to FSDCs. The cell membrane was labeled with PKH67 in order to visualize the cells (Figure 13B). The imaging was performed at the level of the top of the pillars, by focusing on the thin layer of chromium deposited there in the manufacturing process, and the field of view was the same in Figure 13A and B. Thus, the movement of the individual particles tracked using SPT in (Figure 13C) is taking place on the cell surface. Time-lapses were recorded at a speed of 5 frames per second. No particles showed the random movement patterns associated with free diffusion. Over short time scales, particles exhibited confined movement (see inset in Figure 13C). Over time scales of several minutes, directed movement patterns could be observed (see main image in Figure 13C). It is however difficult to determine if these are decoupled from movement of the cell itself. Particles are occasionally observed to flicker in and out of the focal volume, which can be explained by the fact that the normal morphology of DCs is not smooth: they generally have a multitude of protrusions (dendrites) that range from being antennae-like to being more skirt-like depending on the level of activation.[146] Thus, it can be expected that particles exhibit considerable movement also in the z-direction.

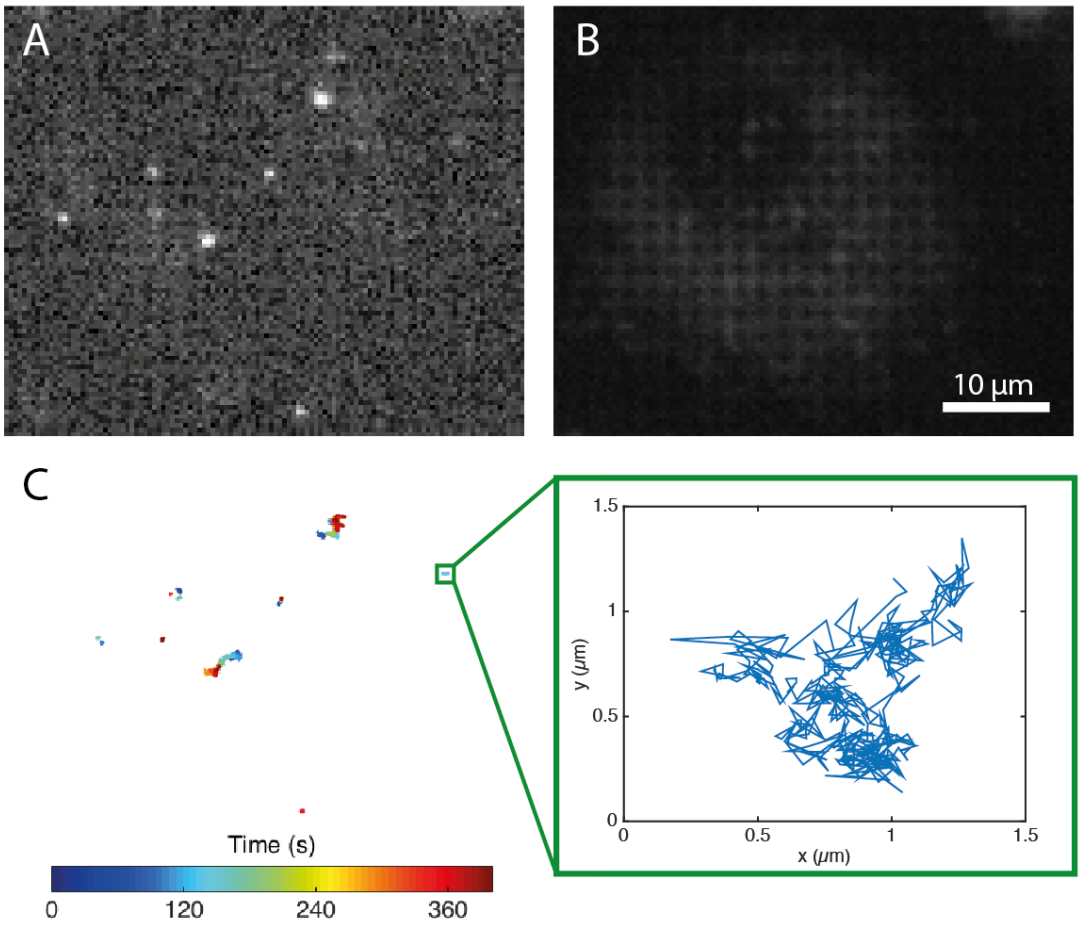

Figure 13. A: TIRF micrograph of rhodamine-labelled CTA1-3M2e-DD-functionalized liposomes attached to the surface of the PKH67-labelled FSDC cell seen in B. C: Trajectories over time of the liposomes in A. Inset shows detailed track.

In conclusion, we have developed a tool that enables study of the interaction between vaccine carriers and the cell surface, which is the first step in the uptake process. From the particle trajectories, it is possible to calculate diffusion rates. Using this type of information, we could characterize how changes to the particle properties influence the observed membrane interactions in a more quantitative fashion. Furthermore, by quenching the fluorescence of the extracellular particles, using for example Trypan Blue, the number of internalized particles could be quantified. This would additionally allow for use of this method to study the influence of particle properties on rate and degree of uptake.

7
Outlook

We set out to identify physicochemical characteristics of lipid-based vaccine carrier particles with the potential to improve the immunogenicity of fusion proteins based on the mucosal adjuvant CTA1-DD. It was observed that the liposome formulation DSPC-PEG dramatically increased the antigen presentation by DCs and we hypothesized that this was due to the gel-phase state of the membrane of the liposomes. These results gave rise to new questions such as: What is the mechanism(s) behind the observed improvement in immunogenicity obtained with DSPC-PEG? What is the nature of the immune response elicited by the DSPC-PEG formulation in a larger sense, and what is the mechanism of the immune response modulation? Can we further improve the performance of the DSPC-PEG formulation? These are questions that we intend to address in the continuation of this project, as described in this chapter.

7.1 Further immunogenicity assessment of DSPC-based liposomes

In Paper II it was observed, in an antigen presentation assay using DCs *in vitro*, that DSPC-based liposomes used as vaccine vectors improve the immunogenicity compared to POPC-based vectors, lipodisks and fusion protein alone. An increase in surface-bound MHC II was observed, but to further assess the immunogenic potential of the DSPC-PEG formulation it should be determined if this increase was due to upregulation of MHC II expression or merely reduced recycling from the surface. Additionally, it is important to further deepen the biological understanding of the effect of the DSPC-PEG on the DCs, in terms of cytokine secretion and expression of costimulatory molecules in addition to increased surface-bound MHC II. These three factors act in concert to impact the magnitude and quality of the T cell response, which is also an important biological response to characterize. To some extent this can be done *in vitro,* and by focusing on certain well-defined parts of the biology such assays are very useful tools for mechanistic studies and, as shown in Paper II, for performing wider range screening than what is practically feasible *in vivo*. However, as described in Chapter 2, achieving protective immunity through mucosal vaccination involves overcoming the mucosal barrier and engagement of the adaptive immune response, which in turn activates a vast network of inter- and intracellular signaling. This whole process, from vaccine administration to the modulation of the balance between cell- and antibody-mediated immunity, is difficult to mimic *in vitro*. *In vivo* trials are thus important, in their own right, as well as to validate the results obtained from the *in vitro* antigen presentation assay. Thus, in collaboration with Nils Lycke's group at the University of

Gothenburg, we plan to perform an *in vivo* immunogenicity study of the DSPC-PEG formulation; immunizing mice intranasally and quantifying serum IgG, local IgA, as well as the cytokines IFN-γ and IL-17 and characterizing the antigen-specific $CD4^+$ T cell response.

7.2 Further physicochemical characterization of lipid-based vaccine carriers

Paper II presents an attempt to identify which physicochemical properties of vaccine carriers that are important in order to achieve successful delivery to and activation of DCs, the cell type targeted with our fusion protein. We believe that the improved immunogenicity of the DSPC-PEG formulation observed is likely to have its basis in the higher membrane rigidity of that formulation compared to the POPC-based liposomes. However, the mechanism behind the improvement remains to be elucidated, which motivates further physicochemical characterization of the particles to understand how and why the DSPC-PEG liposomes are effective while the other formulations tested are not.

One possible reason is that the fluid phase particles do not facilitate uptake, but rather remain trapped on the cell surface due to their comparatively large deformability requiring high bending and adhesion energies for internalization, while the gel phase DSPC-PEG liposomes are more efficiently taken up. Quantifying the membrane rigidity of small vesicles is however not trivial, and the presence of proteins and PEG at the vesicle surface further complicate the matter. A technique that has previously been used for size determination of surface-bound vesicles with a complex composition is multi-parametric surface plasmon resonance (MPSPR).[147] The technique allows for quantification of the effective film thickness, which is translatable to the mean size of deformed surface-bound particles. By comparing the surface-bound size to the size in solution measured by NTA, the relative deformability of surface-bound DSPC-PEG and the POPC-based liposomes should be possible to establish also for protein- and PEG-decorated liposomes. Thus, it might be possible to determine whether a large deformation of the POPC liposomes upon binding to the cell membrane, and subsequent high bending energy required for internalization, offers a plausible explanation for their poor immunogenic effect. To provide a more realistic picture of how the particles interact with the cell surface, MPSPR could be combined with cell membrane-derived model surfaces that maintain the integrity and mobility of membrane proteins.[148] Studying the deformation and binding of lipid particles on cell membrane mimics can provide important structural information about the cell-particle interface but only limited insight about particle processing by DCs. In order to obtain information about the behavior of the particles at the cell surface and to quantify whether certain formulations are taken up to a larger extent than others, we intend to apply the TIRF-based assay described in section 6.3. Detailed information about which uptake mechanism is involved in the uptake as well as the intracellular fate of

the vectors can be investigated by fluorescently labeling cell constituents relevant to different uptake pathways and intracellular compartments, and observing colocalization with labeled vectors using TIRF and confocal microscopy.[149] There is reason to believe that the endocytic trafficking will be specifically influenced by carrier rigidity. For example, Hartmann et al. showed that rigid capsules, around 4 μm in diameter, were more slowly transported to the lysosomes than soft capsules.[150] We might therefore hypothesize that rigid particles may provide the antigen with a longer time frame in which it can bind to the MHC II, thus increasing the amount of presented antigen. The endosomal trafficking process for more or less rigid liposomes could be characterized using confocal microscopy to elucidate whether there is a correlation between endosomal processing time and antigen presentation. Additionally, it might be possible to probe the antigen-MHC II binding in real time using MHC II and Ea peptide labeled with a Förster resonance energy transfer (FRET) pair.

Another factor potentially contributing to the contrast between gel and fluid phase liposomes pertains to the possibility of increased solubilization of the lipid-anchored fusion proteins from the fluid phase particles compared to the DSPC-PEG formulation. It is possible that the hydrophobic portion of the lipid would obstruct the binding site of the CTA1 moiety of the fusion protein. In Paper I, it is shown that the enzymatic activity of the fusion protein is necessary for the immunostimulatory effect of the vaccine vectors. Thus, a decrease in the portion of enzymatically active fusion proteins in the fluid phase formulations could explain their poor performance in the antigen presentation assay (Paper II). In order to show whether there is indeed different partitioning of the proteins into aqueous medium from the fluid and gel phase formulations, time correlated single photon counting-Förster resonance energy transfer (TRSPC-FRET) and surface analytical tools, such as MPSPR and quartz crystal microbalance with dissipation monitoring (QCM-D) will be used.

7.3 Future directions in lipid-based vaccine vector design

Based on the current knowledge presented in this thesis, regardless of the reason(s) why DSPC-PEG demonstrates increased immunogenicity compared to the other formulations, future attempts at exploring the role of physicochemical properties will be done using the DSPC-PEG formulation. Since the DSPC-PEG gives a high signal in the antigen presentation assay it provides a good starting point for resolving differences that other physicochemical properties, besides rigidity, might cause. Similarly, the DSPC-PEG formulation will be used as a basis for further attempts to reach improved efficacy.

In Paper II, attempts were made to elucidate the combined effect of shape and size using lipodisks and liposomes. From the obtained results it is possible to say

that the lipodisks are not advantageous as vaccine carriers in our particular system. It is however not possible to conclusively say why: is it an effect of their shape, size, some other factor or a combination? For this reason, it is logical to attempt to investigate the size factor on its own using the well-functioning DSPC-PEG formulation. It is not possible to vary the shape of the DSPC-PEG liposomes while maintaining the composition, but the influence of size can be investigated. Since the size distribution of extruded vesicles is inherently broad, it may be that only a small size fraction is successfully taken up and processed, and that a considerable amount of material is without effect. Thus, the influence of vaccine carrier size is an interesting matter. Isolating this property for investigation can be done in a fairly crude manner by varying the pore size of the membrane used for extrusion or by separating different size subsets using size exclusion chromatography. Both of these methods will give rise to a narrower liposome size distribution, but a distribution of sizes it will remain. Alternatively, the method to create liposomes of a tightly controlled size using DNA templates, which has been proposed by Yang et al., may be interesting to pursue for a more clear-cut investigation of the influence of size on carrier immunogenicity.[67]

In Paper II, it was observed that changes to the carrier design could achieve considerable improvement in immune response to the vaccine formulation. It is however most likely that further improvements can be obtained by further tweaking the design. Targeting of vaccine formulations to certain cell types, such as DCs, is a core concept in many attempts to create successful mucosal vaccine formulations (see section 4.7), including ours. It is not well known how the DD-portion of the fusion proteins employed in Papers I and II targets DCs. Therefore there is currently an ongoing project in our close collaborator Nils Lycke's group aiming at replacing the DD-portion with a ligand of a known receptor, such as CD103, CD11c or Dec205, with the hope to improve targeting efficiency. It would be interesting to see what effect such changes might have on particle uptake efficiency. Changes to the fusion protein aside, the advantage of using a carrier is that other components can be incorporated in addition to the fusion protein. Lewis X oligosaccharides (sugar polymers) have been used to target DCs and it would be interesting to incorporate a lipid-anchored variant in the DSPC liposomes.[151] Similarly, TLR-ligands have been used to target DCs (see section 4.7) and it has been shown that using a combination of ligands for both cell surface- and endosomal TLRs can increase the CTL avidity, making them able kill target cells more rapidly and earlier in the infection process.[152] Additionally, an increased protection against viral challenge in mice was observed.[152] Thus, it would be interesting to use ligands, not only for cell targeting, but also for intentional modulation of the immune response in the future development of the vaccine carrier formulation.

8
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