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# PLGA-based particulate vaccine delivery systems for immunotherapy of cancer

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# Chapter 1

Introduction

# Abstract

Synthetic peptides hold great promise as well-defined antigens for immunotherapy of cancer. The exact sequence and length of synthetic long peptides (SLPs) derived from cancer antigens can be designed and SLPs are readily produced in quantities suited for therapeutic vaccination. However, the formulation of SLPs for *in vivo* administration still needs to be improved. So far, SLPs have been formulated in Montanide-based water-in-oil (w/o) emulsions in (pre-)clinical trials. However, the use of Montanide as an adjuvant has some important limitations, such as: non-biodegradability; significant local side effects; poor control of release rate; lack of specific dentritic cell (DC)-activating capacity; and the presence of organic solvents (needed to dissolve the peptides prior to mixing with the adjuvant) in the final formulation. Therefore, alternative formulations containing an effective delivery system for peptide-based cancer vaccines are highly needed.

Among the numerous vaccine delivery systems, poly(lactic-co-glycolic acid) (PLGA) biodegradable particulate delivery systems are particularly interesting because they are biocompatible; can protect soluble antigens from degradation and rapid clearance once administered; allow for co-encapsulation of (multiple) antigens and adjuvants; and mimic the size and structure of a pathogen, being more efficiently taken up by DCs than soluble antigen [1, 2].

This thesis describes fundamental studies on the design and applicability in a pre-clinical setting of PLGA-based particulate formulations for the delivery of SLP-based cancer vaccines.

# 1. Introduction

## 1.1. Vaccines

Vaccination is one of the greatest achievements in the history of medicine, being the most effective biomedical intervention against infectious diseases, since it was introduced by Edward Jenner in the 18th century, who showed that humans could be protected against smallpox after inoculation with material scraped from the blisters of cowpox infected cows. The mechanism of vaccination works by activating the immune system against a specific agent, in order to rapidly recognize and remove the threat on a later encounter. The concept was further developed by Louis Pasteur, who developed rabies and anthrax vaccines by using live attenuated strains or inactivated pathogens [3]. Since then, worldwide vaccination campaigns using whole inactivated or live attenuated microorganisms have led to the eradication of smallpox and a dramatic reduction of other diseases such as polio and measles.

Traditionally, vaccines were generated empirically, without deep insight into the nature of the pathogen or the immune response, usually based on live attenuated or chemically or heat inactivated bacteria or viruses. Though live attenuated or whole inactivated pathogens have proven very effective at eliciting both humoral and cellular immune responses [4], the possibility of adverse effects associated with these vaccines, such as disease symptoms resulting from the inability of immune-compromised or elderly patients to clear the vaccine [5], unwanted inflammation or the possibility of reversion of the microorganisms to virulence [6], has raised safety concerns.

The demand for safer alternatives led to the development of subunit vaccines, which instead of a whole organism contain only specific antigenic compounds derived from it. Based on this principle, numerous subunit vaccines have been developed based on purified proteins and capsular polysaccharides obtained from the respective pathogens. Since a couple of decades, vaccines have become available or are being developed based on substances that do not require the pathogen as a production source, such as recombinant proteins, synthetic peptides, and genetic vaccines containing DNA or RNA encoding the antigen(s) of interest [7]. Examples of subunit vaccines successfully applied for widespread vaccination include hepatitis B, tetanus, diphtheria, and human papillomavirus (HPV) vaccines.

The use of individual antigens rather than whole pathogens diminishes safety concerns. However, soluble antigens often are weakly immunogenic, as they are poorly taken up and processed by antigen-presenting cells (APCs), lack danger signals, and are rapidly cleared when administered *in vivo* [8]. The generation of strong immune responses upon vaccination requires the uptake of antigens by APCs, which process them intracellularly into small peptide fragments (epitopes) and present these on their surface bound to major histocompatibility complex (MHC) class I and II molecules [9]. The identification of dendritic cells (DCs) as the most important and potent APCs of the immune system, scavenging the environment for potential pathogens and directing the immune response, has made them the main target of antigen delivery [10-12]. Activated DCs are able to cross-present antigens to antigen-specific B cells, T helper (Th) cells and cytotoxic T lymphocytes (CTLs), which are able to attack virus-infected or tumor cells [11, 12].

In order to strengthen the immune response against subunit vaccines, soluble antigens should be combined with adjuvants. An adjuvant (from the latin word adiuvare, to aid) can be any substance, such as an immunostimulatory molecule or a delivery system, that is used to enhance the efficacy of a vaccine. This can be attained by prolonging or boosting the otherwise poor immune response, or modulating it towards a specific cellular (Th1) or humoral (Th2) response. Although the clinical use of these types of adjuvants has been around for about 90 years, only a few have been approved for use in humans [13]. As prophylactic vaccines are widely administered to healthy populations, mostly young children, the safety of an adjuvant is of utmost importance [13]. The most commonly used adjuvants are aluminium salts (generally, Al(OH)3 and AlPO4), also known as alum [14]. Being the only FDA approved vaccine adjuvant for prophylactic vaccines for almost eight decades, alum has benefitted from unparalleled monopoly [13]. Alum's adjuvanticity relies mainly in depot formation from where the antigen is

slowly released in a sustained manner in particulate form; but other mechanisms have been recently discovered, such as facilitated antigen uptake by DCs and inflammasome activation through NOD-like receptor protein 3 [14-16]. Still, there are also drawbacks associated with alum. Alum promotes primarily a humoral response, i.e., antibody production, rather than a cellular response [13, 17]. Additionally, alum is known to cause local adverse effects, such as granuloma at the site of injection [13]. Over the past two decades, other adjuvants have been approved for use in the clinic, including nano-emulsions such as MF59 (Novartis), an oil-in-water emulsion containing squalene, polysorbate 80, and sorbitan trioleate used in the Fluad® influenza vaccine; AS03™ (GlaxoSmithKline (GSK)), another emulsion containing squalene, tocopherol, and polysorbate 80 used in the Pandemrix™ influenza vaccine; and AS04 (GSK) containing alum and monophosphoryl lipid A (MPLA) which is used in two licensed vaccines, Fendrix® for hepatitis B and Cervarix® for cervical cancer caused by HPV [13, 18-20]. Other vaccines such as Epaxal® or Inflexal® include virosomes [21, 22]. However, also these systems fail to stimulate strong cellular responses, required for therapeutic vaccines against intracellular pathogens or cancer.

The low immunogenicity associated with subunit vaccines makes them poorly effective against diseases that require a cellular immune response for protection, posing a challenge in designing new formulations. Incorporation of antigens into particulate systems, such as liposomes and polymeric particles, can potentially achieve a better immune response. The particulate form of these systems and their ability to co-encapsulate immune stimulatory molecules, often based on viral or bacterial structures, mimic a natural invading pathogen, being more likely taken up by DCs. These systems can be used to attain a long-lasting stimulation of DCs by sustained antigen release and to induce an adequate T-cell response. Furthermore, unlike traditional adjuvants like alum, which predominantly elicit humoral responses, particulate systems may elicit both humoral and cellular responses [23].

## 1.2. Dendritic cell-mediated immunity

The immune system is a network of cells that work together to protect the body from infection. These cells originate in the bone marrow and migrate to peripheral tissues to detect and eliminate potentially harmful agents [24]. The immune system can be divided into an innate and an adaptive branch, which mainly differ in response time and the level of specificity. The innate immune system is activated almost immediately after the detection of danger signals and involves the migration of phagocytic cells to the site of infection forming the first line of defense [1]. Innate immune cells recognize genetically conserved patterns, but lack antigen-specificity and immunological memory. In contrast, adaptive immunity is antigen-specific and retains memory of a previous infection. There are two branches of the adaptive immune response that contribute to the memory of a specific antigen: the humoral response, carried out by B cells and type 2 $CD4^+$ T cells; and the cellular response, which is performed by type 1 $CD4^+$ and $CD8^+$ T cells [1]. These cells differ from the innate immune cells by their antigen-specificity. APCs orchestrate the communication between the innate and adaptive

immune system [8].

APCs play an important role in inducing an adaptive immune response. APCs are a group of immune cells that are able to process and display both endogenous and exogenous antigens through the MHC molecules on their surface in a process known as antigen presentation. This process is responsible for the activation of T cells, which do not identify and respond directly to native antigens like B cells do, but can recognize processed antigen presented by MHC complexes through their T-cell receptors (TCRs). This allows detection of unexposed antigens, like those expressed by intracellular pathogens and mutated proteins in cancer cells [25].

APCs can be divided into two main categories: professional and non-professional ones. The so-called non-professional APCs include fibroblasts, thymic epithelial cells and vascular endothelial cells, and do not constitutively express the MHC class II molecules, though they can be induced to present antigens for short periods upon stimulation under specific circumstances [1, 25]. Professional APCs include DCs, which are specialized in priming of naïve T cells [24] and macrophages and B cells specialized in activating specific $CD4^+$ T cells. These cells express both MHC class I and class II molecules, being able to stimulate antigen-specific $CD4^+$ and $CD8^+$ T cells, which differentiate into Th cells and CTLs, respectively [11, 12]. CTLs are able to eliminate virus-infected or tumor cells, while Th cells promote B cell activation, macrophage function and maturation of other T cells [1].

Different APCs have different main functions, with the efficiency of antigen uptake, processing and presentation varying accordingly. B cells' main function is to produce antibodies against specific antigens; they have limited uptake capacity, as their membrane B cell receptors are restricted to a single specific antigen [25]. As for macrophages, their primary function is to clear tissues from pathogens, having extraordinary endocytosis and degradation capacity, though poorer antigen presentation ability [25]. Conversely, DCs' main specialized function is antigen presentation, thus efficiently uptaking, storing, processing and presenting antigens to initiate a T cell immune response [26].

Depending on the nature of the antigen, either endogenous or exogenous, it will be processed and presented through MHC class I or class II molecules, respectively. Intracellular antigens such as proteins produced by viruses or tumor cells are processed in the cytosol and presented by MHC class I molecules to $CD8^+$ T cells. Extracellular pathogens can be actively internalized by pinocytosis, phagocytosis or endocytosis, being processed in the endosome and presented by MHC class II molecules to $CD4^+$ T cells [25, 27]. However, DCs also have a superior ability to process exogenous antigens and present these peptides in the MHC class I pathway, leading to the activation of antigen-specific $CD8^+$ T cells, a phenomenon that is called cross-presentation [8, 25]. It is this ability that allows them to cross-present tumor antigens and generate tumor-specific CTL responses, reason why DCs have become the main target of cancer vaccines [1, 25].

DCs are the most professional APCs, scattered throughout the body at peripheral tissues, such as the skin and mucosal surfaces, the most probable entry sites for

pathogens, functioning as the sentinels of the immune system [12, 27].

Immunostimulatory molecules activate the immune system through their interaction with specific receptors of APCs, which can recognize certain evolutionary conserved molecular motifs associated with groups of pathogens, the pathogen-associated molecular patterns (PAMPs), via membrane-bound pattern recognition receptors (PRRs) [8]. PRRs are used by DCs to detect invading pathogens and increase the efficiency of uptake and include nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). As part of the innate immune system, TLRs recognize cell-wall components such as lipopolysaccharides, lipopeptides or lipoproteins associated with bacteria, or nucleic acids associated with bacteria and viruses such as double-stranded RNA fragments, acting as communicators between innate and adaptive immunity [8, 25]. These components are known as TLR ligands (TLRL). DCs use their PRRs to detect invading pathogens, and attachment to PRRs increases the efficiency of uptake. After internalization of pathogens, activated DCs migrate to the draining lymph nodes where they mature and come in contact with naïve or memory $CD4^+$ or $CD8^+$ T cells or B cells, presenting the antigen via MHC complexes [1, 25]. After recognition of the antigen-MHC class II complex by T cells, additional co-stimulatory signals are produced by DCs, leading to activation of the B and T cells [1, 25]. TLRs are also able to stimulate DC activation and antigen presentation to naïve T cells through MHC class I complex, triggering T cell maturation into Th1 through the secretion of pro-inflammatory cytokine release and expression of co-stimulatory molecules, stimulating CTL responses [8].

A number of strategies have been developed to efficiently deliver antigen to DCs, such as emulsions, viral vectors, liposomes, and biodegradable polymeric particles [27]. Different formulations may be processed via different pathways, affecting their overall immunogenicity. It has been suggested that the antigen uptake mechanism is of importance in determining how antigen is processed and presented on MHC class I and II molecules [28]. Though the exact mechanism of cross-presentation pathways is poorly understood, they have been arranged into two distinct routes: the phagosomal/cytosolic pathway and the endosomal/vacuolar pathway [29]. Similarly to the classical endogenous antigen processing pathway, in the phagosomal/cytosolic route the internalized antigen is processed in the cytosol, after escape from the endosome, where it is transferred to the proteasome and degraded into smaller peptides, which are then transported to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), before being loaded on MHC class I molecules [25]. In contrast, the endosomal/vacuolar pathway is TAP independent, and the antigen is processed into smaller peptides in the endosome in a pH-dependent manner by active endolysosomal proteases. Some of these peptides bind to MHC class I molecules inside the endosome and migrate to the cell surface [25]. Soluble antigens appear to be mainly processed through the cytosolic route, whereas particulate antigens are processed predominantly via the endosome [30].

One requirement for effective T cell activation is the appropriate delivery of antigens to DCs, which is often poorly attained when using soluble antigens. Encapsulation of antigens in particulate systems facilitate the delivery process and uptake by DCs,

promoting long-lasting stimulation by sustained antigen release, which is critical to induce an adequate T-cell response [31, 32]. Though immunogenicity is noticeably enhanced by specifically targeting antigens to DCs, additional stimuli are required to properly activate DCs. By co-encapsulating TLR agonists together with the antigen within delivery systems, DCs can be targeted through their TLRs, improving antigen delivery and modulating the immune response in order to achieve a strong cellular response [33]. So, the design of a potent vaccine obviously is not only dependent on the nature of the antigens but also on the way they are formulated.

## 1.3. Immunotherapy of cancer

Cancer is one of the leading causes of death worldwide. The conventional treatments for cancer consist of surgery, radiotherapy and/or chemotherapy, which are unspecific and poorly effective against metastasized tumors, usually causing severe side effects and decreasing the patient's quality of life. For this reason, there has been a growing interest in new treatment strategies.

It is well known that the immune system is able to respond against tumors, allowing the development of novel therapeutic strategies aiming to activate the immune system against tumor cells [34]. The use of the specificity of the immune system to treat cancer is referred to as cancer immunotherapy. Several studies have been conducted in the area of immunotherapy of cancer, leading to the development of several cancer vaccine strategies that are now extensively studied in multiple clinical trials or have already been approved for standard therapy. Current approaches comprise cell-based, antibody and cytokine therapies [25, 28]. These include adoptive $CD8^+$ T cell transfer [28] and DC-based vaccines [35, 36], such as Provenge® (Dendreon) against prostate cancer [37, 38]; monoclonal antibodies (mAbs) such as rituximab (Rituxan®, Genentech) against B-type leukemias and lymphomas, and trastuzumab (Herceptin®, Genentech) against breast cancer; and the administration of interleukin 2 (IL-2) and interferon alpha (INF-$\alpha$) used in the treatment of various cancer types [39]. A therapeutic cancer vaccine called Oncophage® against renal cell carcinoma has been approved by the FDA, consisting of an autologous heat shock protein (HSP)–peptide complex produced from each patient's own tumor [25].

One of the most promising strategies in cancer immunotherapy is the use of immune checkpoints blockers [40]. These approaches use mAbs that target immune checkpoints in T cells rather than cancer cells, and have strongly contributed to making cancer immunotherapy the breakthrough of the year in 2013 according to Science magazine [41]. Ipilimumab (YERVOY®, Bristol Myers Squibb), is a mAb that works as an immune checkpoint blocker by targeting cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), a protein receptor on the surface of T cells that downregulates CTL responses when bound to an inhibitory signal [39-42]. By blocking this inhibitory mechanism, it activates cancer-specific CTLs. Ipilimumab has been approved by the FDA for the treatment of melanoma, significantly extending patients' lives. In addition to melanoma, ipilimumab is undergoing clinical trials for the treatment of prostate and lung cancer [40, 41].

Another target for immunotherapy of cancer is programmed cell death protein 1 (PD-1), a cell surface receptor expressed on T cells and pro-B cells, which can bind to ligands PD-L1 and PD-L2 [43, 44]. Similarly to CTLA-4, PD-1 downregulates the immune system, promoting self-tolerance and reducing autoimmunity, by inducing apoptosis in antigen-specific T cells while simultaneously reducing apoptosis in regulatory T cells [43, 45]. PD-1/PD-L1 treatment strategies use mAbs that block PD-1 and PD-L1 interaction, thereby enhancing T-cell antitumor activity [41, 45]. Several mAbs targeting PD-1 receptors have shown promising results in clinical trials and are being developed as cancer treatments, such as nivolumab (Opdivo, Bristol Myers Squibb) for lung cancer, melanoma, and renal-cell cancer [46]. Pembrolizumab/lambrolizumab (Keytruda, Merck) has been approved by the FDA as a treatment against metastatic melanoma following treatment with ipilimumab [40, 41, 47]. Treatments combining PD-1 and CTLA-4 blockers have shown greater impact than when used alone, with very promising results [46]. Whereas ipilimumab treatment has been associated with severe immunological adverse effects due to T cell activation and proliferation, resulting in autoimmunity, PD-1 treatments seem to lead to milder side effects [41, 45]. These adverse effects should be taken into account during risk assessment when considering the treatment.

These early clinical successes with checkpoint-blocking antibodies unequivocally demonstrate that immunity to cancer exists but is however in a silenced state in a tumor environment. Therefore, the immune system can be successfully recruited towards tumors but requires adequate activation of the tumor-specific T cells, e.g., by specific vaccination.

### 1.3.1. Cancer vaccination

In recent years, in addition to the traditional prophylactic vaccines against infectious diseases, there has been an increased interest in broader application of vaccination for protection against other diseases, such as cancer [8, 27].

Currently only few prophylactic cancer vaccines have been approved: two that protect against the high-risk serotypes of HPV (Gardasil, Merck; Cervarix, GSK) and other two against the hepatitis B virus (HBV) (Recombivax HB, Merck; Engerix-B, GSK), which are the leading causes for cervical and liver cancer, respectively. However, prophylactic vaccines do not have therapeutic effects against pre-existing infections and do not prevent their development, as these vaccination strategies rely on antibody protection against the virus upon infection, whereas therapeutic vaccines must have the capability of inducing CTL responses to kill the infected cells of (pre-)malignant lesions [27].

Two main approaches have been used to target DCs in cancer immunotherapy: administration of *ex vivo* tumor antigen loaded DCs and *in vivo* delivery of tumor antigens. Though *ex vivo* loaded DC-based cancer vaccines have been extensively studied with promising therapeutic results, this approach requires each vaccine to be tailor made for each patient, involving extensive and time-consuming lab work as well

as logistics, thus making such vaccines extremely expensive [25]. These limitations may be overcome by delivering tumor associated antigens (TAAs) and adjuvant together to DCs *in vivo*.

Identification of TAAs for a variety of cancers has led to development of vaccines that target these antigens [25, 28]. Most prophylactic vaccines work by inducing an antibody response against specific viral or bacterial antigens. In contrast, therapeutic cancer vaccines require the induction of potent T cell responses against TAA-bearing cells [27, 48]. Cancer vaccines rely on the administration of TAA in order to induce the killing of tumor cells by cytotoxic T lymphocytes (CTLs) orchestrated by activated DCs, resulting in tumor regression. Quite a few cancer vaccines have been tested in the clinic, however, most of these trials have resulted in disappointing clinical results, due to insufficient generation of cell-mediated immunity [27, 28]. Several factors must be considered when developing a cancer vaccine, which in order to be effective must comprise the following three essential elements: an antigen, an immune potentiator, and a delivery system.

### 1.3.2. Peptide-based cancer vaccines

Several vaccination strategies against cancer have been able to the elicit tumor-specific T cell responses *in vivo*, including whole tumor cells or cell lysates, viral vectors or DNA encoding TAAs, as well as recombinant proteins or synthetic peptides, both of which have been the most popular form of therapeutic vaccines [49, 50].

The first step for developing subunit cancer vaccines is to select the target TAA(s), which can be (a) protein(s) or peptide(s) specific for tumor cells. In order to achieve a successful vaccine, these antigens must include 8-10 amino acid peptide sequences that can be processed and bind to the MHC class I molecules of DCs and will be recognized by T cells to elicit a strong tumor-specific CTL response [27, 28]. The cloning of the first gene reported to encode a CTL-defined human tumor antigen (MAGE-A1) paved the way to the discovery of more TAAs, allowing the production of vaccines for these targets and the study of vaccines against different types of cancer, such as melanoma and cervical cancer [28, 50-55].

Vaccination with TAA-derived synthetic short peptides (SSPs) covering exact MHC class I epitopes initially appeared to be a promising approach for immunotherapy of cancer, as T cells recognize target antigens as peptide fragments presented by MHC class I molecules at the cell surface [27]. However, this approach showed major drawbacks [51, 56], such as peptide-specific immunological tolerance, associated with enhanced tumor growth instead of protection [57, 58], as well as short-lived and suboptimal CTL responses, caused by the lack of proper co-activation of $CD4^+$ T cells and subsequent induction of memory $CD8^+$ T cells [59, 60].

Tolerance was mainly induced because of the exogenous binding of these short peptides to MHC class I molecules that are present not only on professional APCs

like DCs, but also on all somatic cells which generally lack the ability of expressing the surface co-stimulatory molecules that are required to the generation of effector T cells [27, 56-63]. In a direct comparison, vaccination with a SLP bearing both CTL and Th epitopes in Montanide ISA 51 elicited much stronger CTL responses after vaccination than vaccination with a SSP covering an exact HPV16E7 MHC I binding epitope. The former approach resulted in effective eradication of established tumors in a mouse model of HPV16-induced tumors, owing to the activation of both $CD4^+$ and $CD8^+$ T cells [64]. Therefore, the use of longer peptides, such as SLPs, covering both CTL and Th epitopes has been launched as a strategy to overcome the problem of suboptimal CTL responses [27, 65]. These peptides cannot bind to MHC I molecules directly, but have to be taken up and processed by DCs before being presented to T-cells, inducing a more effective immune response [57, 63, 64]. It has been suggested that such SLPs considerably facilitate MHC class I presentation and induce stronger CTL responses in comparison to proteins because SLPs are more efficiently internalized, processed, and cross-presented by DCs [33, 64].

Another disadvantage about the use of SSPs is that the polymorphic nature of MHC molecules (human leukocyte antigen, HLA) in genetically diverse populations restricts their broad use as there is a risk of missing important epitopes [27]. Due to the multiple HLA class I and II molecules, it is unlikely to identify immunogenic CTL and Th epitopes that would cover all individuals, requiring patient selection according to their HLA type. The use of vaccines consisting of overlapping SLPs covering the whole sequence of highly immunogenic regions of TAA proteins may overcome the HLA-dependency problem, allowing the *in vivo* epitope selection according to each HLA-profile after uptake by DCs [27]. Following this concept, numerous SLP vaccines have been studied in clinical trials against different types of cancer and other diseases, one of the most prominent example being HPV-induced cancers [27, 42].

HPV-induced cancers often have viral sequences integrated into the cellular DNA. Some of the HPV “early” genes, such as E6 and E7, are known to act as oncogenes that promote tumor growth and malignant transformation. Because the majority of HPV-16 derived cancers express E6 and E7 oncoproteins, they are attractive candidates as target antigens for immunotherapy of HPV-induced cancer [66-72].

Several protein and peptide vaccines against HPV E6 and/or E7 have been successfully tested in preclinical and clinical models. A cancer vaccine consisting of 13 overlapping SLPs covering the entire sequence of the E6/E7 oncogenic proteins of high-risk HPV16 has been investigated in several (pre-)clinical trials [65]. Therapeutic vaccination with a HPV16 SLP vaccine emulsified in the mineral oil adjuvant Montanide ISA 51 has shown to be able to increase both HPV16-specific $CD4^+$ and $CD8^+$ T cells in murine models of cervical cancer [33, 66, 73]. In clinical trials, this vaccine has shown robust immunogenicity in end-stage cervical cancer patients, and caused complete regression of premalignant HPV16-induced I lesions and eradication of virus in 9 of 20 women with high-grade vulvar intraepithelial neoplasia [52, 65].

Despite these promising results, there is still room for improvement, especially regarding the SLP vaccine formulation. Montanide has been used as the “gold-standard” adjuvant

for protein- and SLP-based cancer vaccines [65, 74, 75]. Montanide is a clinical-grade version of incomplete Freund's adjuvant (IFA) with similar properties. The w/o emulsion is composed of a mineral oil, a surfactant and an aqueous phase, slowly releasing the dispersed antigen when injected subcutaneously. These emulsions are known to elicit long-lasting IgG responses as well as stimulate the activation of CTL and Th cells [53, 76-80], its adjuvant activity being attributed to depot formation and the ability to trigger inflammation at the site of injection [81], while emulsion droplets can promote antigen uptake by DCs [82, 83].

However, the use of Montanide w/o emulsions is far from ideal, not only since they have poorly defined immunogenic properties, but also because their non-biodegradability causes significant local side effects, such as tenderness, swelling, granuloma, local pain, erythema and discomfort, reason why they have not been approved for routine human immunotherapy but are only being used in investigational clinical trials [17, 54, 81, 84]. Furthermore, their use as an adjuvant has some other important limitations: they show poor control of the peptide release rate and lack specific DC-activating capacity. Moreover, the pharmaceutical development and scalability of w/o emulsions is hampered by limited long-term stability and the complexity of the manufacturing process. In addition, formulation of peptides is hampered by the need for organic solvents (like dimethylsulfoxide) to dissolve them prior to parental administration, whereas distribution of peptides in the emulsion is dependent on peptide polarity, making the release of a multipeptide vaccine difficult to predict and control. Thus, there is an urgent medical need for the development of better adjuvants with improved safety and efficacy profiles that can be formulated into vaccine products that are stable during preparation, storage and administration. This can be achieved via different formulation strategies which allow co-delivery of antigens and adjuvants, such as encapsulation in biodegradable particles [79, 85], use of antibody-mediated targeting [86, 87] and/or SLP-TLRL conjugates [78, 88].

## 1.4. Adjuvants

Adjuvants can be categorized into two groups: immunostimulatory molecules and delivery systems. Immunostimulatory molecules play a major role in directing immunity towards either a bacterial or viral/tumoral defense pathway [1]. Though vaccine delivery systems are often sufficient to induce a long-lasting protective immunity, poorly immunogenic antigens, such as synthetic peptides, are often unable to induce strong responses when incorporated alone, requiring the inclusion of immune-potentiating molecules [89]

### 1.4.1. Immunostimulatory molecules

DCs are critically involved in the generation and maintenance of an effective CTL response. Therefore, vaccines need to mimic the most successful natural triggers of

DC activation. Immunostimulatory molecules play a major role in directing immunity towards either a bacterial or viral/tumoral defense pathway [1]. Therefore, selecting 'the right' immunostimulatory molecule(s) is crucial in the design of a successful subunit vaccine.

TLRs are the most commonly used target as TLR stimulation is critically involved in the uptake and processing of antigens by DCs, and are able to trigger cross-presentation. TLR activation induces the secretion of proinflammatory cytokines and type I interferon, and leads to upregulation of CD40, CD80 and CD86 costimulatory molecules on the surface of APCs, as well as release of Th1 cytokines such as IL-1, IL-2, IL-6, and TNF, leading to T cell activation [90]. There are at least 10 types of TLRs in humans, which can either be surface bound or expressed intracellularly, recognizing different extracellular or intracellular pathogenic components (Table 1) [1, 8].

Recent research is aimed at activating DCs by targeting TLRs [6, 53, 91-94]. TLRs can be located either on the cell surface or inside intracellular compartments, ultimately indicating their roles at detecting extracellular or intracellular pathogens [8]. TLR1, 2 and 6 are mainly surface TLRs that detect surface bacterial lipoproteins and lipopeptides, which are usually recognized by TLR2 dimerized with TLR1 when triacylated, and TLR2 dimerized with TLR6 when diacylated [8]. A well-known TLR1/2 agonist is Pam3CSK4, a synthetic tripalmitoylated lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins, whereas Pam2CSK4, being a diacylated lipopeptide, induces signaling through TLR2/6. Lipopolysaccharide (LPS), an endotoxin from Gram-negative bacteria, is a potent activator of TLR4 with the subsequent induction of NF-κB and the production of pro-inflammatory cytokines. TLR5 recognizes flagellin, the protein monomer that constitutes the filament of bacterial flagellae, found on nearly all motile bacteria. TLR3, and 7 to 9 are expressed intracellularly, within the endosomal compartments, and are able to detect bacterial and viral nucleic acids. TLR3 recognizes viral double-stranded RNA (dsRNA), a molecular pattern associated with viral infection, for which polyinosinic-polycytidylic acid (poly(I:C)) is a synthetic analog. TLR7 and TLR8 recognize single-stranded RNA (ssRNA) and small synthetic molecules like imidazoquinolines and nucleoside analogs, such as the imidazoquinoline drug compounds resiquimod (R848) and imiquimod. Finally, TLR9 recognizes unmethylated CpG (cytosine–phosphate–guanine) oligonucleotide (ODN) motifs in DNA molecules, common elements in viruses and bacteria, but not in humans.

Several synthetic TLRLs have been shown to enhance antigen-specific CTL responses in mice when mixed or conjugated with antigen (see Table 1 for examples) [6, 8]. The co-delivery of TLR agonists and antigens through co-encapulation in delivery systems can be an efficient strategy to increase the strength and modulate the quality of the immune response against antigens. Furthermore, as most pathogens present multiple TLR agonists to APCs, simultaneous stimulation of multiple TLRs can result in a synergistic upregulation of inflammatory cytokine production. Hence, the combination of multiple TLRLs may be a promising strategy to induce strong immune responses [6].

In this thesis, either TLR2L Pam3CSK4 or TLR3L Poly(I:C), or a combination of both, have been co-encapsulated with antigen into delivery systems in order to enhance the

**Table 1: Human toll-like receptors and their natural and synthetic ligands.**

| TLR | Location | PAMP recognized | Pathogens | Synthetic agonists | Immune response |
|---|---|---|---|---|---|
| TLR 1+2 | cell membrane | triacylated lipoproteins | bacteria | Pam3Cys | Induce production of inflammatory cytokines |
| TLR 2+6 | cell membrane | diacylated lipoproteins<br>peptidoglycans<br>LTA<br>fungal β –glucans | mycoplasma bacteria<br>Gram-positive bactéria<br>Fungi | Pam2Cys<br>MALP-2 | Induce production of inflammatory cytokines |
| TLR 3 | endosome | double stranded RNA | viruses | Poly(I:C)<br>Poly(A:U) | Synthesis of type 1 interferons |
| TLR 4 | cell membrane | LPS<br>heat shock proteins<br>hyaluronic acid<br>heparan sulfate<br>fibrinogen | Gram-negative bacteria | MPLA<br>LPS analogs | Synthesis of type 1 interferons |
| TLR 5 | cell membrane | flagellin | bacteria | - | Induces production of TNF- α |
| TLR 7 & 8 | endosome | single stranded RNA | viruses | Resiquimod (R848)<br>Imiquimod (R837)<br>Gardiquimod<br>Loxoribine (Guanosine analogs) | Anti-viral response |
| TLR 9 | endosome | unmethylated CpG motifs | Bacteria<br>Viruses<br>protozoa | CPG ODN | Dependent on type of CpG:<br>Type A/D induces IFN- α<br>Type B/K induces IL-12 and TNF- α production |
| TLR 10 | endosome | profillin-like proteins | unknown | unknown | unknown |

CpG ODN: Cytosine-guanine rich oligonucleotide; LPS: Lipopolysaccharide; LTA: Lipoteichoic acid; MALP-2: Macrophage-activating lipoprotein-2; MPLA: Monophosphoryl lipid A; Pam2Cys: Dipalmitoyl-S-glyceryl cysteine; Pam3Cys: Tripalmitoyl-S-glyceryl cysteine; poly(I:C): Polyribo(inosinic-cytidylic) acid; TLR: Toll-like receptor

potency of anti-cancer vaccines.

## 1.4.2. Particulate delivery systems

Most peptide-based cancer vaccine strategies have used Montanide as an adjuvant to increase specific T cell immunity. Over the recent years, because of its drawbacks, alternatives from various types of biomaterials have been used to formulate particulate vaccine carriers, such as liposomes (vesicles formed from phospholipid bilayers) and micro- (MPs) or nanoparticles (NPs) made from polymers like PLGA, poly-lactic acid (PLA) or (derivatives of) chitosan, a polysaccharide obtained by deacetylation of chitin [74, 81, 95]. These particulate systems are biocompatible and biodegradable and can be manufactured reproducibly according to Good Manufacturing Practice (GMP). Furthermore, studies have shown that DC targeted vaccination strategies benefit from encapsulation of antigens in biodegradable particulate systems, as they (i) are able to protect the antigen from premature enzymatic degradation and clearance by the kidneys, increasing their residence time; (ii) serve as depot for sustained antigen release; (iii) are able to co-encapsulate multiple components, delivering antigen and adjuvant together to DCs; and (iv) can be efficiently targeted to specific tissues or immune receptors through targeting moieties coupled to the particle surface [1, 87]. Moreover, particulate antigens enhance antigen-specific humoral and cellular responses more efficiently than soluble ones, as particulate formulations mimic the size and structure of a pathogen facilitating uptake by DCs, and prolong antigen cross-presentation owing to sustained intracellular release of the antigen following uptake [2, 32, 96-99]. These features make biodegradable particles attractive candidates as vaccine delivery systems for peptide-based cancer immunotherapy.

### 1.4.2.1. Liposomes

Liposomes are artificially-prepared spherical vesicles, with particle sizes ranging between 30 nm to several microns, composed of a lipid bilayer. This bilayer usually consists of synthetic and/or naturally occurring, biodegradable phospholipids and cholesterol, which can be manipulated in order to influence the liposome's physicochemical characteristics, such as size, surface charge and rigidity [32, 98-102]. Liposomes have been extensively studied because of their ability to carry both hydrophilic and hydrophobic compounds, as hydrophilic molecules can be encapsulated in the inner aqueous phase, whereas hydrophobic compounds can be dissolved into the lipidic membrane, and amphiphilic ones can partition between both phases [103, 104]. This ability has rendered interest in liposomal vaccines, as they are suitable to carry a variety of molecules with different characteristics, such as antigens and immunomodulatory compounds; moreover, targeting moieties can be embedded in or covalently attached to the liposomal bilayer to facilitate targeted delivery to DCs [104].

The adjuvant effect of liposomes is highly dependent on their physicochemical properties,

such as vesicle size, surface charge, composition of phospolipid head groups, length and saturation of the lipid tail, and rigidity of the bilayer, which can be customized by adjusting their composition and method of preparation. The surface charge is of particular importance, as cationic liposomes have shown superior adjuvant capacity compared to neutral or negatively charged ones, probably because of electrostatic interactions with the negatively charged surface of DCs [99, 105]. Furthermore, cationic liposomes facilitate adsorption of negatively charged antigens to their surface, such as synthetic peptides, proteins and nucleic acids.

Numerous other liposomal vaccines have been in clinical trials, with shown efficacy, including cancer vaccine candidates, most prominent being Stimuvax® (or L-BLP25, Oncothyreon) against non-small cell lung cancer (NSCLC); and MAGE-A3 (GlaxoSmithKline) against metastatic MAGE-A3-positive melanoma and NSCLC [105-107].

#### 1.4.2.2. PLGA nanoparticles

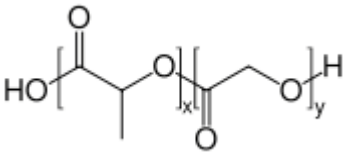
Polymeric particulate delivery systems can consist of bio-degradable or non-biodegradable materials, and can derive from either natural or synthetic sources. Natural polymers include starch, gelatin, alginate, or chitosan, and offer the benefits of being biocompatible, hydrophilic and inexpensive, but their production often results in batch-to-batch inconsistency and incidence of unwanted impurities or contaminants [13]. In contrast, synthetic polymers can be manufactured with high purity and reproducibility and can be tailored to achieve the desired molecular weight, co-polymer composition and rate of degradation [108].

Synthetic particles made from synthetic polymers have been widely explored as peptide antigen delivery vehicles as they offer many advantages over other systems. They are biodegradable and biocompatible and can act as antigen depots, slowly releasing peptides for a pre-determined period of time that can range from a few days to several months, depending on the customized particle degradation rate, potentially eliminating the need for booster doses [6].

PLGA (**Figure 1**) is a copolymer which is Food and Drug Administration (FDA) approved for therapeutic devices and drug delivery systems, owing to its biodegradability and biocompatibility, with several slow-release formulations currently on the market [109-111]. These properties have made PLGA a common choice in the production of a variety of biomedical devices, such as sutures, implants and prosthetic devices, as well as delivery systems, such as micro- and nanoparticles [112, 113].

PLGA undergoes hydrolysis in the body to produce the original monomers, lactic acid and glycolic acid, which, under normal physiological conditions, are by-products of various metabolic pathways in the body. Since the body effectively deals with the two monomers, there is minimal systemic toxicity associated with using PLGA for medical applications.

Several forms of PLGA with different properties can be obtained, either depending on the ratio of lactide to glycolide used for the polymerization, or the type of end group (ester-terminated (capped) or carboxylic acid terminated (uncapped)), offering the possibility to tailor the polymer degradation time and regulate drug release [114-117]. Degradation rate of PLGA is related to the lactide/glycolide ratio: the higher the content of glycolic acid, the more hydrophilic it becomes and, consequently, the faster its hydrolysis rate. Also, unlike the homopolymers of lactic and glycolic acid (polylactide and polyglycolide, respectively) which show poor solubilities, PLGA can be dissolved in a wide range of common organic solvents.




PLGA
(x: lactic; y: glycolic)

**Figure 1:** Chemical structure of poly-(lactic-co-glycolic) acid, with x standing for the number of lactide monomers and y for the number of glycolide monomers.

Consequently, biodegradable particles composed of PLGA have many desirable features for antigen delivery. These particle systems are biocompatible and can be manufactured reproducibly according to Good Manufacturing Practice in a scalable, affordable and reproducible way. Importantly, PLGA is very well suited for making particles of different size, resulting in either MPs or NPs, with controllable release properties [108]. For these reasons, PLGA particles have been studied extensively for the delivery of a wide variety of macromolecules, from DNA to proteins and peptides [53, 81, 112, 118]. Moreover, antigen loaded PLGA particles have been shown to induce cellular immune responses comparable to those elicited with Montanide 51 or IFA [119-121].

PLGA particles can be prepared by a variety of different methods, most commonly used for protein and peptide antigens being the double emulsion with solvent evaporation method [108]. In brief, the polymer is dissolved in an organic volatile solvent (e.g. dichloromethane, ethyl acetate) to which the antigen is added in aqueous solution (the inner water phase, w1) followed by emulsification by homogenization or sonication; this first water in oil (w1/o) emulsion is added to an external aqueous phase (w2) containing a surfactant (e.g., polyvinyl alcohol, polysorbate 20), followed by a second emulsification step, forming the double emulsion (w1/o/w2); the second emulsion is then transferred to an aqueous phase under magnetic stirring at room or reduced temperature to allow for solvent evaporation, leading to precipitation of the polymer and solidification of the particles, which are finally recovered by centrifugation and then freeze-dried [122].

The use of PLGA particles for vaccine delivery offers several advantages from both pharmaceutical and immunological perspectives as compared to soluble antigens or w/o emulsions. Several immunotherapeutic approaches have successfully used both

PLGA MPs and NPs to target DCs [10, 81, 97]. Particles allow for the concomitant delivery of antigen and adjuvant [76, 79], and vaccination with PLGA NPs containing TAA and TLRL has been demonstrated to be as effective as vaccination with IFA [66, 81, 123]. Particles also facilitate endosomal escape, which is a known mechanism leading to antigen cross-presentation by DCs [64, 124]. Furthermore, they allow the coupling of targeting molecules to specific receptors such as CD40 or DEC-205, which can efficiently direct particles to DCs, increasing specific uptake and cellular immune responses [42, 87, 114].

Several factors affect the efficiency of PLGA particles as vaccine delivery vehicles. Particle size is a critical factor, as it can influence bio-distribution and uptake efficiency by APCs. Particles can be defined as NPs or MPs according to their size ranges: NPs for particles within the nano range (10 nm to 1 mm); and MPs for particles in the micron range (1 mm to 1000 mm). It is generally accepted that NPs are advantageous over MPs for drug targeting, as NPs can more easily permeate biological barriers and efficiently reach target tissues [125-127]. However, the ideal size for vaccine delivery is still not consensual [116, 128, 129]. Particle size is known to influence the efficiency and mechanism of uptake, depot formation, and release kinetics [129-132]. The way a cell takes up an antigen-loaded particle can determine how it processes the antigen: MPs are mainly phagocytosed, whereas NPs are generally taken up by endocytosis or pinocytosis. Sustained release of antigen and adjuvants is essential to properly stimulate DCs, whereas a low burst release eliminates potential loss of antigen before the particle is taken up by DCs, increasing antigen presentation and $CD8^+$ T cell activation [31, 32].

However, there are also disadvantages of the use of PLGA, as encapsulation in PLGA particles may result in loss of antigen integrity during preparation and storage, but also after administration, especially regarding protein antigens, where denaturation or degradation can occur due to local acidification of the inner core due to the hydrolysis of the polymer [133]. Though peptides do not require a tertiary structure and may be less susceptible to acidic degradation, their encapsulation in PLGA particles can lead to accelerated deamidation or formation of peptide adducts due to acylation of lysine residues with lactic and glycolic units [134]. These issues are more predominant for MPs than NPs, as their inner core takes longer to degrade the polymer and release the antigen, allowing higher accumulation of acidic molecules [133]. Furthermore, PLGA particles must be freeze-dried to ensure long-term stability during storage and must be re-hydrated prior to administration. Still, formulation of SLPs in PLGA NPs may be advantageous, provided that the formulation is carefully designed and optimized. Nevertheless, despite encouraging results obtained in clinical trials, there are still no PLGA-based particulate vaccines on the market [1, 135].

Incorporating multi-SLP antigens together with TLRL in PLGA particles may result in a ‘pathogen-mimicking’ particle that displays slow antigen release and may be actively phagocytosed by DC, representing a promising approach for generating specific T cell immunity. In the present thesis, PLGA particles were studied to gain better insight into the critical factors influencing delivery and immunogenicity in order to formulate a potent particulate delivery system to improve the safety and efficacy of SLP-based cancer vaccines.

# 2. Aim and outline of this thesis

The aim of this thesis is to gain a better insight into the role of the formulation of particulate delivery systems in the effectiveness of cancer vaccines through the preparation, physicochemical characterization, and immunological evaluation of a biodegradable, PLGA-based particulate delivery system, incorporating protein or SLP antigens as well as TLRL. The main objectives of this research include:

- Determination of the best size range of particles for vaccine delivery
- Development of SLP loaded PLGA NP formulations
- Development of formulations based on PLGA NPs co-encapsulating SLPs and TLRLs

The overall goal is gaining fundamental insight into how to improve the immunogenicity, clinical efficacy and safety of SLP-based vaccines for cancer immunotherapy, to be used as a safer and more effective alternative to Montanide ISA 51.

In **Chapter 2** the literature regarding PLGA-based particulate systems for protein- and peptide-based vaccine delivery is reviewed, with a strong focus on particle properties affecting immunogenicity.

In **Chapter 3 and 4** of this thesis we use ovalbumin (OVA), a 45-kDa protein purified from chicken egg, as a model protein antigen to conduct initial studies regarding the formulation of PLGA particles as vaccine delivery vehicles. The following chapters (Chapters 5-7) focus on the development of formulations for SLP delivery, using SLPs derived from OVA covering CTL and/or Th epitopes as our model antigens.

The optimal size range of PLGA-based particulate vaccines in order to achieve the most efficient cellular immune responses is studied in **Chapter 3**, where MPs and NPs encapsulating OVA and a TLR3L are compared head-to-head for their capacity to activate B and T cell responses. Having determined that NPs are more efficiently delivered to DCs than MPs, resulting in more effective immune responses *in vivo*, in Chapter 4 we describe the application of PLGA-OVA NPs as a delivery vehicle for *ex vivo* loading of DCs, in order to enhance DC-mediated stimulation of antigen-specific T cells to be used for adoptive T cell immunotherapy.

In **Chapter 5** the development of a new method for SLP encapsulation in PLGA NPs is studied, focusing on formulation conditions required to successfully encapsulate an SLP in PLGA NPs, and optimization of the formulation to improve the efficacy of cross-presentation by DCs of PLGA-SLP NPs in comparison to soluble SLP *in vitro*. Subsequently, in **Chapter 6** we study the co-encapsulation of SLP and TLR2L in PLGA NPs, which are used to study the intracellular mechanisms via which DC process PLGA-SLP NPs and to determine the *in vivo* vaccine potency of PLGA-SLP-TLRL NPs in comparison to soluble SLP and TLRL.

In **Chapter 7** we study the co-encapsulation of two SLPs covering the CTL and Th epitopes of model antigen OVA and two TLRLs in PLGA NPs and liposomes, which were used to ascertain the *in vivo* vaccine efficacy in a direct comparison to Montanide and MF59 analogs, with properties that are very similar to adjuvants currently used in the clinic.

Finally, in **Chapter 8** we discuss the achievements described in this thesis and the perspectives for further research on nanoparticulate delivery systems for SLP-based cancer vaccines.

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