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

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# **Chapter 8**

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Summary,  
Discussion & Perspectives

# 1. Summary

In recent years, there has been a growing interest in therapeutic vaccination as a treatment modality against diseases such as cancer [1]. In particular, synthetic long peptides (SLPs) have been studied as well-defined antigens for immunotherapy of cancer. So far, SLPs have been formulated in Montanide ISA 51-based water-in-oil (w/o) emulsions in (pre-)clinical trials. However, the use of Montanide has some important limitations, such as suboptimal efficacy and side effects, so alternative formulations for peptide-based cancer vaccines are highly needed. The field of cancer immunotherapy, the current status of peptide-based cancer vaccines, their lack of efficacy and the need for new adjuvants are introduced in Chapter 1. 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 [2, 3]. In Chapter 2 we provide a detailed overview of the use of PLGA particulate delivery systems for the delivery of protein- and peptide-based vaccines. This chapter discusses formulation parameters influencing the adjuvanticity of these systems, such as size, charge, antigen localization, release profile, and the co-delivery of immune modulators, such as Toll-like receptor ligands (TLRLs), and/or specific targeting molecules, such as antibodies. It further outlines how these characteristics affect uptake, processing and antigen presentation by dendritic cells (DCs) and the ensuing immune response. It also provides a summary of the PLGA formulations that have been studied for the delivery of synthetic peptide-based vaccines.

The principal aim of the research described in this thesis was to investigate how PLGA-based particulate systems can act as an adjuvant for SLP-based cancer vaccines in a pre-clinical setting in order to gain 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. The main objectives of this research included:

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

The first objective was achieved in Chapter 3, where the role of particle size to induce an immune response was studied. For that purpose, we performed a comparative study between NPs versus MPs containing equivalent amounts of a model antigen, ovalbumin (OVA), and a TLRL, poly(I:C), with comparable release kinetics, and studied how their ability to be internalized by DCs affects MHC class I antigen presentation *in vitro* and the ensuing immune responses *in vivo*, in comparison to sustained release from a local subcutaneous depot. Particles were formulated to obtain NPs that could be efficiently internalized by DCs, forming intracellular depots, versus MPs with a size (>

20  $\mu\text{m}$ ) too large to be taken up, thus functioning exclusively as an extracellular depot, similarly to Montanide. We showed that efficient particle uptake is crucial to induce an immune response: NPs were efficiently taken up by DCs upon *in vitro* incubation, whereas MPs were not, resulting in increased MHC class I antigen presentation *in vitro* for NPs but not MPs. Moreover, upon subcutaneous vaccination in mice, significantly higher numbers of antigen-specific CD8<sup>+</sup> T were obtained with NPs compared to MPs or OVA emulsified in incomplete Freund's adjuvant (IFA). In addition, NP led to better antibody responses compared to MP, and induced a more balanced TH1/TH2-type antibody response than IFA. We concluded that particulate vaccines should be formulated in a nano-size range that allows efficient uptake, significant MHC class I cross-presentation and effective T and B cell responses.

Having determined the optimal size range of PLGA particles for protein-based vaccines, in Chapter 4 we describe the application of these PLGA NPs as a delivery vehicle for ex vivo loading of DCs, in order to stimulate antigen-specific CD8<sup>+</sup> T cells to be used for adoptive T cell immunotherapy. For that purpose, DCs previously incubated with PLGA NPs encapsulating model antigen OVA or the soluble protein were used to stimulate CD8<sup>+</sup> T cells, which were then transferred to mice. Ex vivo stimulation of CTLs by DCs incubated with PLGA NPs, as compared to soluble protein, resulted in a superior capacity to lyse target cells *in vivo* after adoptive transfer. Furthermore, administration of CTLs stimulated by PLGA NP-loaded DCs resulted in more efficient tumor control leading to prolonged survival of tumor bearing animals, showing that protein antigens benefit from encapsulation in PLGA-NPs, clearly enhancing MHC class I presentation and CTL activation.

As we showed that protein antigen delivery through encapsulation in PLGA NPs is an efficient way to stimulate potent anti-tumor T cells, in Chapter 5 we studied the feasibility of the application of encapsulation in PLGA NPs to SLPs, achieving the second objective. Using OVA24, a 24-residue long synthetic antigenic peptide covering a CTL epitope of OVA (SIINFEKL), as a model antigen, our aim was to define the formulation parameters required to successfully encapsulate an SLP in PLGA NPs, and to optimize PLGA NPs for SLP delivery with respect to encapsulation and release kinetics, to improve the efficacy of SLP cross-presentation by DCs. When using the standard "double emulsion with solvent evaporation" encapsulation techniques, we observed that either encapsulation was very low (< 30%), or burst release extremely high (> 70%). By adjusting formulation and process parameters, we uncovered that the pH of the first emulsion was critical to efficient encapsulation and sustained release. By using an alkaline inner aqueous phase rather than an acidic one, we were able not only to optimize the encapsulation of the SLP but also to reduce its burst release, finally obtaining stable NPs of approximately 330 nm, with an encapsulation efficiency of circa 40% and a burst release lower than 10%. Encapsulation of OVA24 in PLGA NP resulted in enhanced MHC class I restricted T cell activation *in vitro* when compared to high-burst releasing NP and soluble OVA24, revealing the importance of low burst release to induce a potent cellular immune response. This encapsulation method may be a promising approach for encapsulation of peptides with amphiphilic and/or hydrophilic properties, and has been successfully applied to other SLPs as well. This study underscores the importance of optimizing the encapsulation process for the

development of an effective and stable delivery system, and may be considered as a basis for the development of NP formulations for SLP-based immunotherapy of cancer.

Subsequently, the third objective was addressed in Chapter 6, where we studied the co-encapsulation of SLP OVA24 and TLR2L Pam3CSK4 in PLGA NPs, which were used to characterize the intracellular mechanisms via which DC process PLGA-SLP NPs and to determine the study the immunological effects the combination of SLPs with an adjuvant. We showed that TLR 2 stimulation enhanced MHC class I presentation of SLP by DCs *in vitro*. DCs loaded with PLGA-SLP(TLR2L) NPs internalized them into endolysosomal compartments and not the cytosol as occurs with soluble SLP. Moreover, encapsulated SLP could be detected for long periods inside DCs endolysosomal compartments, resulting in prolonged MHC class I presentation for up to 96 h. PLGA-SLP NPs and especially PLGA-SLP/TLR2L NPs induced sustained CD8<sup>+</sup> T cell proliferation *in vivo* after adoptive transfer of PLGA-SLP(TLR2L) NP-loaded DCs. These findings demonstrate that CD8<sup>+</sup> T cell response is enhanced when the antigen is cross-presented in MHC class I molecules in a sustained manner, and that co-encapsulation of a TLRL further boosts these effects, and thus supports the use of PLGA NPs co-encapsulating SLPs and TLRLs as anti-cancer vaccines.

Finally, in Chapter 7 we investigated the potential of PLGA NPs and cationic liposomes as delivery systems for SLP-based vaccines for the induction of cell-mediated immunity. For that purpose, we studied the co-delivery of two SLPs containing the CTL (OVA24) and the Th (OVA17) epitopes of OVA together with TLRLs Pam3CSK4 and/or poly(I:C) in a direct comparison to the clinically used adjuvants Montanide (a water-in-oil emulsion) and a squalene-based oil-in-water emulsion analog to MF59. The obtained formulations were assessed *in vitro* and *in vivo* for their potency to induce CD8<sup>+</sup> and CD4<sup>+</sup> T cell immune responses. The liposomal and PLGA NP formulations were able to enhance antigen uptake by DCs and subsequent activation of T cells *in vitro*. Subcutaneous vaccination of mice showed that the efficiency of the SLP-loaded liposomes and PLGA NPs to induce functional antigen-specific T cells *in vivo*, was at least as good (PLGA NPs) or better (cationic liposomes) than that of the emulsion-based formulations, while liposomes induced T cells with the highest killing capacity of transferred target cells in mice, outperforming PLGA NPs. Considering the questionable safety profile of the currently clinically used adjuvant Montanide, these findings indicate that both particulate systems are promising biodegradable delivery vehicles for clinical application of SLP-based cancer immunotherapy.

## 2. Discussion & Perspectives

At the Leiden University Medical Center (LUMC), several (pre-)clinical studies have been conducted, showing that SLP-based vaccines efficiently induce the immune system against cancer [4-6]. In a clinical setting, a vaccine consisting of 13 overlapping SLPs covering the entire sequence of the E6 and E7 oncogenic proteins of high-risk human papillomavirus 16 (HPV16) emulsified in Montanide ISA 51 was administered to women suffering from HPV16-induced (pre-)malignant vulvar intraepithelial neoplasia, resulting in robust immunogenicity in end-stage cervical cancer patients, and complete regression of pre-malignant lesions in 9 of 20 women [4-6]. However, Montanide, a clinical grade version of IFA, has some important limitations: it shows poor control of the antigen release rate, lacks specific DC-activating capacity, and the mineral oil component, which is non-biodegradable, has been associated with significant local side effects [4, 7, 8]. Moreover, though SLP-vaccines showed potent therapeutic efficacy against pre-malignant lesions, they failed to achieve durable clinical responses in late-stage cancer patients, underlining the necessity for better SLP-vaccine formulations that could enhance their therapeutic efficacy. This was the starting point for designing the PhD project described in this thesis, aimed at optimizing the efficacy of SLP vaccines via the encapsulation in poly-(lactic-co-glycolic acid) (PLGA) particles, while reducing the side effects associated with the current (pre-)clinical administration of SLP vaccines emulsified in water-in-oil preparations.

This thesis describes the research toward the optimization of PLGA particulate systems for the delivery of synthetic long peptide (SLP)-based vaccines for immunotherapy of cancer as an alternative to Montanide. For this purpose we explored the use of PLGA particles as delivery systems for SLPs from the pharmaceutical formulation to the immunological evaluation.

Two main approaches have been used to target DCs in cancer immunotherapy: administration of ex vivo TAA-loaded DCs and *in vivo* delivery of TAAs. In Chapter 4 we showed the superior ability of particulate delivery systems compared to soluble antigen to activate DCs *in vitro*, resulting in better CD8<sup>+</sup> T cell priming and increased tumor protection *in vivo*. However, though ex vivo-loaded DC-based cancer vaccines have been successfully used in therapeutic settings, each vaccine needs to be specifically prepared for each individual, requiring the harvest and *in vitro* culture of their own DCs, which is time-consuming and requires extensive logistics, thus making these vaccines extremely expensive [9]. By using *in vivo* delivery of tumor associated antigens (TAAs) and adjuvant together to DCs, cancer vaccines could be widely applied to cancer patients. Therefore, our aim was to study formulations to be applied *in vivo*.

Several aspects concerning particulate vaccine formulations have been studied in this thesis, in particular the characterization of the formulations and their immunological effects *in vitro* and *in vivo*. Depending on their physicochemical characteristics, delivery systems can modulate the immune response, mainly due to direct influence in the following mechanisms: facilitated uptake by DCs, regulation of the internalization and presentation pathways, and interaction with specific receptors that mediate the immune

response towards humoral or cellular bias.

One of the most important factors playing a crucial role in vaccine efficacy is size. While smaller particles tend to be more easily taken up by DCs, larger particles can form stable extracellular depots from where the antigen can be slowly released [1]. In Chapter 3, we studied the role of particle size to induce an immune response by comparing NPs that could be internalized by DCs with MPs that could not. Although previous studies had investigated the role of size in particle uptake and ensuing immune response, the extreme differences in antigen release kinetics observed between the formulations would most likely have affected the results [10]. By comparing NPs and MPs with similar sustained release profiles, we were able to get better insight on the actual differences between antigen release from intracellular versus extracellular depots at a similar rate. Indeed, NPs were effectively taken up by DCs, inducing more potent immune responses than MPs, and more importantly than IFA, pointing to the importance of efficient particle uptake by DCs.

While MPs and IFA allow the formation of a depot that can trigger inflammation and the local recruitment of immune cells to the site of injection, to which the antigen and TLR ligand can be delivered extracellularly in soluble form, the findings in Chapter 6 show that the mechanisms of uptake and processing soluble or particulate antigen by DCs do differ. DCs internalized NPs into the endolysosomal compartments and not the cytosol, as occurs with soluble SLPs. Encapsulated SLPs could be detected for long periods inside DCs' endolysosomal compartments, resulting in prolonged MHC class I presentation in comparison to soluble SLPs. Indeed, these observations concur with the results described in Chapter 4, where *ex vivo* PLGA NP-loaded DCs more effectively primed CD8<sup>+</sup> T cells to confer tumor protection after adoptive transfer *in vivo*. Moreover, they are also in line with the observations in Chapter 5, where we compared NP with similar physicochemical characteristics in terms of charge, size and antigen loading, but different release profiles, having determined that a low-burst release, with the majority of antigen being delivered to DCs still encapsulated in PLGA NPs, was crucial to enhance MHC class I presentation.

The importance of release kinetics and especially the burst release points to the importance of thorough characterization and optimization of SLPs encapsulation in PLGA NPs in order to obtain a successful vaccine formulation. In Chapter 5, we studied the encapsulation of OVA24 SLP in PLGA NP as function of formulation and process parameters. Whereas hydrophilic and hydrophobic peptides have been successfully encapsulated in PLGA NP and/or microparticles in the past [11-13], efficient entrapment of the moderately hydrophobic OVA24 proved to be challenging. The observed high burst release indicated that most of the OVA24 molecules might not be encapsulated in the NP's polymeric matrix, but instead were adsorbed to their surface, being quickly released when resuspended in physiological buffers. Efficient entrapment of OVA24 SLP in the polymeric matrix was obtained by exploring and fine-tuning of formulation and process parameters, in particular the composition of the inner aqueous phase, as encapsulation and release characteristics were strongly dependent on the pH of the first emulsion. Our novel PLGA NP formulation method allowed us to achieve up to 40% encapsulation efficiency of OVA24, exhibiting minimal burst release. Moreover,

this novel method was also successfully applied to encapsulate OVA17, encoding the T helper epitope of OVA, and gp100, encoding an immunodominant CTL epitope present in melanoma. These encouraging results illustrate the applicability of our encapsulation process to different SLPs, and the possibility to be applied in the future to encapsulate the 13 overlapping SLPs encoding the HPV16 E6 and E7 oncoproteins in PLGA NPs to be tested in pre-clinical models and eventually in the clinic.

The formulation method developed in Chapter 5 was also applied to co-encapsulate OVA24 SLP with TLR2L agonist Pam3CSK4 in the PLGA NPs studied in Chapter 6. The prolonged CD8<sup>+</sup> T cell activation observed indicates that the internalized NPs slowly release the encapsulated SLPs inside the endolysosomal compartments. The released antigen is then gradually processed in the cytosol, after escape from the endosome, where it is continuously transferred to the proteasome and degraded into smaller peptides to be loaded on MHC class I molecules, resulting in sustained MHC class I Ag presentation. The enhanced MHC class I presentation observed is likely related to the protection from the rapid degradation that occurs with soluble SLPs in the cytosol, which were barely detectable after 24 h, whereas encapsulated SLPs were detected up to 72 h after antigen loading. Co-encapsulation of Pam3CSK4 significantly enhanced the capacity for DCs to prolong MHC class I Ag presentation and CD8<sup>+</sup> T cell activation *in vivo*. TLR stimulation is critically involved in the uptake and processing of antigens by DCs, and can trigger cross-presentation [14, 15]. 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 leading to T cell activation [16]. Moreover it increases the half-life of MHC class I-peptide complexes on the cell surface [17], which together with the prolonged presence of encapsulated antigen inside endolysosomal compartments can account for the sustained CD8<sup>+</sup> T cell activation we described using PLGA-SLP/TLR2L NPs.

For successful implementation of PLGA NPs as delivery systems for SLP-based vaccines in a clinical setting, PLGA NPs should be applicable to co-encapsulate multiple SLPs with different physicochemical properties, such as the 13 overlapping long peptides encoding the HPV16 E6 and E7 oncoproteins. Furthermore, as most pathogens present multiple TLR agonists to APCs, the combination of multiple TLRs can result in a synergistic effect in order to induce strong immune responses [15]. In Chapter 7 we studied the potential of liposomes and PLGA NPs, co-encapsulating two SLPs containing the CTL (OVA24) and the Th (OVA17) epitopes of OVA together with TLRs Pam3CSK4 and/or poly(I:C), as alternatives to clinically used Montanide- and squalene-based emulsions in a direct comparison. Both liposomal and PLGA NP formulations were able to enhance antigen uptake by DCs and subsequent activation of T cells *in vitro*, and induce functional antigen-specific T cells *in vivo*, with at least equivalent or even better efficacy than the emulsion-based formulations after subcutaneous vaccination in mice. Liposomes outperformed PLGA NPs, showing the highest killing capacity after transfer of target cells in mice. The differences observed between liposomes and PLGA NPs may be attributed to the combination of two main factors, particle size and surface charge. The size of particulate adjuvants is crucial for their adjuvant activity and the immunogenicity, as small particles (10-150 nm) can easily penetrate the extra-cellular matrix and be transported via the lymphatic

system into the lymph nodes where they come in contact with resident DCs [18], while particles smaller than 200 nm, such as the liposomes used in this study, but not the PLGA NPs, will likely be taken up by DCs more efficiently than bigger particles [19]. Furthermore, as immune cells are negatively charged, cationic particles are more efficiently uptaken due to electrostatic interactions [20], while polycations can also aid in phagosomal/endosomal escape after being internalized by APCs [2], potentially influencing the antigen presentation pathway and type of immune response. Therefore cationic liposomes are in advantage in comparison with PLGA NPs, which are both bigger and negatively charged. Making smaller PLGA NPs similar to liposomes and/or coating them with cationic surfactants may be a way to increase their adjuvanticity. Still, both liposomes and PLGA NPs were used successfully to co-encapsulate up to four components, showing their ability to deliver (multiple) SLPs and TLRs together to DC, and outperforming both clinically used Montanide and squalene-based emulsions.

For successful implementation of PLGA NPs as a delivery vehicle for SLP-based vaccines to human use, the pharmaceutical formulation should be straightforward, reproducible, stable, and meet GMP quality requirements and regulations. Important factors influencing vaccine efficacy include size; release kinetics; surface characteristics; concomitant delivery of antigen and immunostimulants, allowing DCs to associate danger signals with the antigen, while co-encapsulation of multiple TLRs may result in a synergistic effect; coating or coupling of DC-specific targeting moieties, increasing DC uptake and enhancing antigen presentation to T cells. Future developments in vaccine delivery will likely involve the combination of (multiple) TLRs with delivery vehicles modified with DC-specific targeting ligands/antibodies to significantly enhance the delivery of SLP-vaccines to DCs.

Taking into consideration particle size, antigen release kinetics, adjuvanticity and *in vivo* uptake, the findings described in this thesis indicate that robust cellular immune responses can be obtained by using small NPs rather than MPs, with low-burst sustained release, positively charged and co-encapsulating (multiple) antigens and TLRs (Chapters 3, 5, 6 and 7).

In conclusion, the results described in this thesis present evidence that PLGA NPs may be successfully used as a delivery system for SLP-vaccines as a suitable replacement for emulsions-based formulations for cancer immunotherapy.

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