PLGA-based particulate vaccine delivery systems for immunotherapy of cancer

Monteiro Garrido Castro e Silva, Ana Luisa

Chapter 5

Optimization of encapsulation of a synthetic long peptide in PLGA nanoparticles: low burst release is crucial for efficient $CD8^+$ T cell activation

Ana Luísa Silva, Rodney A. Rosalia, Arzu Sazak, Myrra G. Carstens, Ferry Ossendorp, Jaap Oostendorp, Wim Jiskoot

European Journal of Pharmaceutics and Biopharmaceutics 2013 83(3): 338-345

Abstract

Overlapping synthetic long peptides (SLP) hold great promise for immunotherapy of cancer. Poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NP) are being developed as delivery systems to improve the potency of peptide-based therapeutic cancer vaccines. Our aim was to optimize PLGA NP for SLP delivery with respect to encapsulation and release, using OVA24, a 24-residue long synthetic antigenic peptide covering a CTL epitope of ovalbumin (SIINFEKL), as a model antigen. Peptide-loaded PLGA NP were prepared by a double emulsion/solvent evaporation technique. Using standard conditions (acidic inner aqueous phase), we observed that either encapsulation was very low (1 – 30%), or burst release extremely high (> 70%) upon resuspension of NP in physiological buffers. By adjusting formulation and process parameters, we uncovered that the pH of the first emulsion was critical to efficient encapsulation and controlled release. In particular, an alkaline inner aqueous phase resulted in circa 330 nm sized NP with approximately 40% encapsulation efficiency and low (< 10%) burst release. These NP showed enhanced MHC class I restricted T cell activation *in vitro* when compared to high-burst releasing NP and soluble OVA24, proving that efficient entrapment of the antigen is crucial to induce a potent cellular immune response.

Keywords: Peptide antigen, CTL epitope, PLGA nanoparticles, cancer immunotherapy, cellular immune response

1. Introduction

In recent years there is an increased interest in the application of therapeutic vaccination for treatment of cancer [65]. Therapeutic cancer vaccines aim to induce a strong cellular response against tumor associated antigens [66]. Dendritic cells (DC) are professional antigen presenting cells (APCs) that play a major role in the initiation of such an immune response, by continuously sampling the environment for foreign antigens and establishing the communication between the innate and adaptive immune system [67, 68]. Only appropriately activated DC are capable of inducing a robust cytotoxic T cell (CTL) response, which is required for effective immunotherapy of established tumors [3, 69-71]. For this purpose, DC are the major target cells for cancer immunotherapy vaccines [3, 66].

Therapeutic vaccination with overlapping synthetic long peptides (SLP), covering the entire amino acid sequence of tumor associated protein antigens and thus containing all possible MHC class I and II epitopes, has been successfully applied in murine models and clinical therapeutic vaccination trials [66, 72-75]. Moreover, vaccination of patients suffering from human papillomavirus 16 (HPV16) induced premalignant vulvar intraepithelial neoplasia with an HPV16-based SLP vaccine resulted in complete clinical regression of the lesions in some cases [66, 74, 75].

So far, Montanide-based water-in-oil (w/o) emulsions have been applied to formulate

SLP in the majority of clinical therapeutic cancer vaccination trials [74-80]. The use of Montanide-based formulations has some important limitations, including non-biodegradability, causing significant local side effects, poorly controlled antigen release rates and limited scalability because of lack of long-term stability [81-83]. Biodegradable delivery systems based on poly(lactic-co-glycolic acid) (PLGA) offer a promising alternative strategy for peptide-based cancer vaccines. PLGA is well suited for the preparation of micro- and nanoparticles (NP) [54, 84-86], which can protect antigen from proteolytic enzymatic degradation and rapid clearance [87-90], allow co-encapsulation and simultaneous delivery of both antigen and adjuvants, and facilitate antigen uptake by DC [8, 91-93]. PLGA has a long safety record and is Food and Drug Administration (FDA) approved as an excipient, owing to its biodegradability and biocompatibility, with several slow-release formulations currently on the market [92, 94]. PLGA undergoes hydrolysis in the body to produce the original monomers, lactic acid and glycolic acid, which are natural by-products of metabolic pathways. Antigen release can be regulated e.g. by varying the lactic acid/glycolic acid ratio [95-97]. PLGA-based particulate systems can be manufactured reproducibly according to Good Manufacturing Practice conditions and have been studied extensively for the delivery of a wide variety of antigens [54, 94, 98-100]. Antigens encapsulated in PLGA microparticles have shown to induce immune responses comparable to those of antigens adjuvanted with Montanide 51 [17, 97]. PLGA is also known to have several disadvantages regarding instability of protein antigens, e.g., due to the hydrophobicity of the polymer and the local acidification of the microenvironment that occurs during degradation of the polymer at physiological pH [101-103]. However, for synthetic peptides, which do not possess a tertiary structure, this is less problematic and several peptides in PLGA microspheres have been successfully launched on the market [94, 100, 103].

The aim of this study was the pharmaceutical characterization of PLGA NP as a suitable delivery system for encapsulation of SLP for cancer immunotherapy. OVA24, a 24-residue long synthetic antigenic peptide covering a CTL epitope of ovalbumin (OVA; SIINFEKL), was studied as a model SLP, because of its proven capability to induce CTL responses *in vitro* and *in vivo* [104]. Efficient entrapment of OVA24 SLP in the polymeric matrix was obtained by exploring and fine tuning of formulation and process parameters. OVA24-loaded PLGA NP were characterized for antigen encapsulation efficiency, antigen burst release, particle size and zeta-potential, and the obtained formulations were immunologically evaluated *in vitro* for their potency to induce $CD8^+$ T cell activation.

2. Materials and methods

2.1. Materials

Synthetic long peptide 24-mer OVA24 (DEVSGLEQLESIINFEKLAAAAAK) [105],

covering the cytotoxic T lymphocyte (CTL) epitope SIINFEKL of ovalbumin (OVA) was synthesized at the interdepartmental GMP facility of the Department of Clinical Pharmacy and Toxicology of Leiden University Medical Center as described previously [73]. Poly(D,L-lactic-co-glycolic acid) [PLGA], Resomer® RG 502H was purchased from Boehringer Ingelheim (Ingelheim, Germany). 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), sodium cholate, dichloromethane (DCM), dimethyl sulfoxide (DMSO), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (ACN) and methanol (MeOH) were obtained from Biosolve BV (Valkenswaard, the Netherlands), Polyvinil alcohol (PVA) 4-88 (31 kDa) was purchased from Fluka (Steinheim, Germany). Tween 20 was purchased from Merck Schuchardt (Hohenbrunn, Germany). Sodium hydroxide was purchased from Boom (Meppel, Netherlands). Reversed phase HPLC column ReproSil-Pur C18-AQ 3 µm (150x4 mm) was purchased from Dr. Maisch HPLC GmbH (Ammerbuch-Entringen, Germany). Phosphate-buffered saline (NaCl 8.2 g/L; Na2HPO4.12 H2O 3.1 g/L; NaH2PO4.2H2O 0.3 g/L) (PBS) was purchased from B. Braun (Melsungen, Germany). Iscove's Modified Dulbecco's Medium (IMDM) was purchased from Lonza (Walkersville, USA). All other chemicals were of analytical grade and all aqueous solutions were prepared with milli Q water.

2.2. Nanoparticle preparation

2.2.1. General particle preparation process

Nanoparticles loaded with OVA24 were prepared using a double emulsion with solvent evaporation method [37]. In brief, 50 mg of PLGA dissolved in 1 ml of dichloromethane was emulsified under sonication (30 s, 20 W) with a solution containing 1.4 mg OVA24 (for solution compositions, see results). To this first emulsion (w1/o), 2 ml of an aqueous surfactant solution (for surfactant types, see results) were added immediately, and the mixture was emulsified again by sonication (30 s, 20 W), creating a double emulsion (w1/o/w2). The emulsion was then added dropwise to 25 ml of extraction medium (0.3% w/v surfactant) previously heated to 40°C under agitation, to allow quick solvent evaporation, while stirring, which was continued for 1 h. The particles were then collected by centrifugation for 15 min at 15000 g at 10°C, washed, and resuspended in deionized water, aliquoted and freeze-dried at -55oC in a Christ Alpha 1-2 freeze-drier (Osterode am Harz, Germany) for 12 hours.

As starting conditions, the method described by Slütter et al. [37] was used, with 1% v/v Tween 20 in 25 mM Hepes pH 7.4 as the aqueous phase for second emulsion, with the following modifications: DMSO used as inner phase instead of PBS pH 7.4 and 5% (w/v) PLGA was used instead of 2.5% .

2.2.2. Optimization of formulation parameters

In order to achieve an optimum formulation, the following parameters involved in the particle preparation were varied, and their effect on peptide encapsulation efficiency was investigated.

a) Surfactant type: investigated by dissolving different surfactant types (PVA; Tween 20; sodium cholate) in the second aqueous phase (w2) during the second emulsion step.
b) Inner solvent: investigated by dissolving the peptide in different solutions (w1), i.e. DMSO; 50% ACN + 0.1% TFA; 50% ACN in 25 mM NaOH; and 50% ACN in 0.25 mM NaOH + 400 µL Hepes pH 8.0 at different concentrations).
c) Volume ratio (w1/o): investigated by varying the volume of the inner aqueous phase w1 (50 µL, 100 µL; 500 µL).

2.3. Nanoparticle characterization

2.3.1. Dynamic light scattering and zeta-potential

The Z-average size and polydispersity index (PDI) of NP were measured by dynamic light scattering, using a Zetasizer (Nano ZS, Malvern Ltd., United Kingdom). The zeta-potential was measured by laser Doppler electrophoresis, using the same device. For that purpose, NP were diluted to 2.5 mg/ml in 1 mM Hepes pH 7.4.

2.3.2. Antigen content and encapsulation efficiency

Peptide encapsulation efficiency was determined by measuring the peptide content of digested particles by reversed phase HPLC. For that purpose, 200 µL NP suspension (containing 10 mg NP) was freeze-dried overnight. The lyophilizate was then dissolved in 250 µL DMSO and the solution was agitated at 50°C for 30 min. Next, 750 µL 50% ACN with 0.1% TFA were added and the mixture was agitated at 50°C for an extra 60 min, to allow dissolution of the peptide and degradation/precipitation of PLGA, which was then eliminated by centrifugation for 10 min at 18000 g. The supernatant containing the peptide was collected and 50 µL were injected into a HPLC system equipped with a C18 column (Dr. Maisch Reprosil-Pur C18-AQ, 3 mg, 150 x 4.6 mm) and an ultraviolet detector (Waters 486). Mobile phases applied were 5% ACN in water with 0.1% TFA (solvent A), and 95% ACN in water with 0.085% TFA (solvent B). Separation was performed by applying a linear gradient from 0% to 78% solvent B over 20 min, at a flow rate of 1 ml/min, and peptide detection was performed by absorbance

at 220 nm. Peptide concentration in each sample was calculated using a calibration curve created with known concentrations of OVA24.

2.4. *In vitro* release studies

To determine burst release (BR) at time zero (t_0), freeze-dried NP were resuspended in 1x PBS, 1x IMDM cell culture medium, or 5% w/v glucose solution at a concentration of 10 mg PLGA NP/mL and the mixture was vortexed for 30 seconds at room temperature to allow complete resuspension of the particles. Then particles were centrifuged for 10 min at 18000 g, the supernatant recovered and the pellet resuspended. Both fractions were freeze-dried overnight, and extraction was performed as described previously, before being analyzed by HPLC for peptide quantification.

For longer release studies, peptide-loaded PLGA NP were prepared as described, resuspended in 1x PBS pH 7.4 at a concentration of 10 mg and maintained at 37oC in a water bath under constant tangential shaking at 100/min in a GFL 1086 shaking water bath (Burgwedel, Germany). At regular time intervals, 250 µl samples of the suspension were taken, centrifuged for 10 min at 18000 g, and the supernatant recovered. To eliminate undesirable PLGA degradation products affecting detection by HPLC, the supernatant was freeze-dried over night, and extraction was performed as described, with the final supernatant being analyzed by HPLC for peptide quantification. Peptide release profiles were generated for each NP formulation in terms of cumulative antigen release (%) over time.

2.5. *In vitro* antigen presentation

D1 cells, a long term growth factor-dependent immature splenic DC line derived from C57BL/6 (H-2Kb) mice, were cultured as described previously [106]. B3Z, cultured as described before, is a $CD8^+$ T-cell hybridoma cell line, specific for the H-2Kb–restricted ovalbumin derived CTL epitope SIINFEKL, that expresses a β-galactosidase construct under the regulation of the NF-AT element from the IL-2 promoter [107]. DC were incubated for 2.5 h with soluble OVA24 (sOVA24) or OVA24 encapsulated in PLGA NP at the indicated concentrations. Cells were washed three times with medium before the T-cell hybridoma B3Z cells were added followed by overnight incubation at 37 °C. MHC class I Ag presentation of SIINFEKL (OVA257–264) in H-2Kb was detected by activation of SIINFEKL-specific $CD8^+$ B3Z T cells. Upon T-cell receptor (TCR) ligation, lacZ protein is produced under the control of the IL-2 promoter thus allowing measurement of the IL-2 production indirectly by a colorimetric assay using chlorophenol red-β-d-galactopyranoside (CPRG) as substrate to detect lacZ activity in cell lysates. Color conversion is determined by measuring absorbance (optical density, OD) at 590 nm.

2.6. Statistical analysis

Graph Pad Prism software was used for statistical analysis. Burst release in different physiological media and the effect of inner solvent and emulsion volume on burst release between different formulations in PBS were analyzed by two-tailed unpaired Student's t-test. $CD8^+$ T cell activation of SIINFEKL-specific $CD8^+$ T cells by a two-tailed paired Student's t-test. Effect of the inner phase composition on apparent pH and the effect of Hepes concentration in the inner phase on release were analyzed using two-way ANOVA.

3. Results

3.1. Antigen encapsulation and burst release in PLGA NP

In this study, a 24-mer SLP covering a well-known CTL epitope (SIINFEKL) of ovalbumin, here designated as OVA24, was used as a model antigen to study the encapsulation of SLP in PLGA NP by a double emulsion with solvent evaporation method, as function of formulation and process parameters. As starting point, a slightly modified version of the standard double emulsion method described by Slütter et al. [37] was applied (see section 2.2.1), in which OVA24 was dissolved in DMSO and Tween 20 used as surfactant in the outer phase (**Table 1**, formulation 1). Since this method led to very low encapsulation efficiencies (ca. 1%), DMSO as inner phase was replaced by 50% ACN/0.1% TFA, which resulted in a marginal improvement (see **Table 1**, formulation 2). Attempts to dissolve OVA24 in the PLGA-containing dichloromethane phase failed (results not shown). Therefore, several process parameters were investigated in order to increase the encapsulation efficiency of OVA24 in PLGA NP. First, commonly used surfactants for making NP, sodium cholate and PVA [37, 85], were tested in replacement of Tween 20 (**Table 1**, formulations 3-7). The type of surfactant used to stabilize the second emulsion had a dramatic positive effect on the encapsulation efficiency. Using 50% ACN/0.1% TFA (with an apparent pH of 2.0) as first emulsion medium and PVA as surfactant in the second emulsion step resulted in the highest encapsulation efficiency (EE) (up to about 30%, see **Table 1**, formulations 4-7). PVA concentrations of 1% and 2% (w/v) PVA yielded comparable EE and particle size (**Table 1**, formulations 6 and 7). PVA concentrations below 1% led to a lower encapsulation efficiency and a larger particle size (**Table 1**, formulations 4 and 5). For our further studies, OVA24-loaded PLGA NP prepared with 50% ACN/0.1% TFA as inner solvent and 1% PVA as surfactant in the second emulsion (**Table 1**, formulation 6) were selected.

Table 1: Effect of first and second emulsion compositions on encapsulation efficiency of OVA24 SLP in PLGA NP.

Formulation	1st emulsion medium	2nd emulsion medium	DL (%) *	EE (%)	Z-average size (nm)	PDI	ZP (mV)
1	DMSO	1% Tween 20	0.01 ± 0.004	1 ± 0.3	319 ± 90	0.23 ± 0.07	-36 ± 7.0
2	50 µL 50% ACN 0.1% TFA	1% Tween 20	0.06 ± 0.02	4 ± 1	277 ± 5	0.20 ± 0.01	-27 ± 2.0
3	50 µL 50% ACN 0.1% TFA	1% NaCholate	0.17 ± 0.04	9 ± 2	203 ± 3	0.10 ± 0.03	-32 ± 1.1
4	50 µL 50% ACN 0.1% TFA	0.3 % PVA	0.37 ± 0.08	16 ± 1	606 ± 4	0.17 ± 0.02	-6 ± 1.5
5	50 µL 50% ACN 0.1% TFA	0.5 % PVA	0.62 ± 0.20	22 ± 7	470 ± 36	0.22 ± 0.05	-12 ± 2.1
6	50 µL 50% ACN 0.1% TFA	1 % PVA	0.86 ± 0.31	26 ± 11	372 ± 44	0.17 ± 0.01	-10 ± 1.9
7	50 µL 50% ACN 0.1% TFA	2 % PVA	0.61 ± 0.27	22 ± 3	345 ± 28	0.15 ± 0.03	-10 ± 2.4
8	100 µL 50% ACN 25 mM NaOH	1 % PVA	0.26 ± 0.22	9 ± 8	360 ± 45	0.22 ± 0.01	-12 ± 0.8
9	100 µL 50% ACN 0.25 mM NaOH	1 % PVA	0.71 ± 25	26 ± 9	379 ± 25	0.19 ± 0.02	-12 ± 0.7
10	500 µL 50% ACN 0.1% TFA	1 % PVA	0.82 ± 0.34	29 ± 12	234 ± 3	0.11 ± 0.03	-15 ± 1.3
11	500 µL 50% ACN 25 mM NaOH	1 % PVA	1.03 ± 0.15	37 ± 5	303 ± 68	0.11 ± 0.05	-11 ± 0.1
12	100 µL 50% ACN 0.25 mM NaOH + 400 µL 1 mM Hepes pH 8.0	1 % PVA	0.55 ± 0.21	20 ± 8	384 ± 7	0.18 ± 0.03	-11 ± 0.2
13	100 µL 50% ACN 0.25 mM NaOH + 400 µL 5 mM Hepes pH 8.0	1 % PVA	0.50 ± 0.20	18 ± 6	427 ± 46	0.20 ± 0.05	-10 ± 1.7
14	100 µL 50% ACN 0.25 mM NaOH + 400 µL 10 mM Hepes pH 8.0	1 % PVA	0.44 ± 0.21	16 ± 7	382 ± 69	0.18 ± 0.05	-10 ± 0.6
15	100 µL 50% ACN 0.25 mM NaOH + 400 µL 25 mM Hepes pH 8.0	1 % PVA	0.70 ± 0.09	25 ± 3	298 ± 27	0.12 ± 0.03	-10 ± 0.9
16	100 µL 50% ACN 0.25 mM NaOH + 400 µL 50 mM Hepes pH 8.0	1 % PVA	1.07 ± 0.07	38 ± 3	328 ± 50	0.22 ± 0.07	-14 ± 0.3

* DL = drug loading, EE = encapsulation efficiency, calculated as described in section 2.3.2. PDI = polydispersity index, ZP = zeta-potential. Data are presented as average ± standard deviation of n=3 independent batches.

Formulation 6 was tested for burst release of the peptide in PBS and IMDM (cell culture medium), which are commonly used for *in vitro* and *in vivo* applications. Directly upon resuspension (t=0) in either PBS or IMDM, OVA24-loaded PLGA NP showed a very high release, ranging from 80 to 90% (**Figure 1**), indicating that initial encapsulation results were misleading. In contrast, a release study in isotonic 5% glucose solution (**Figure 1**), did not induce substantial peptide release.

With the purpose to decrease the very high burst release, new solutions were tested as inner phase (w1) in the NP preparation process, where the effect of pH was studied. Instead of using pHs below the isoelectric point (pI) of the peptide (pI = 4.3), we decided to use pHs above it, which would change the charge distribution in the peptide and thereby could affect encapsulation, as well as inner phase volume, which might affect the peptide's distribution between the inner and outer aqueous phases present during formation of the double emulsion. Therefore, instead of 50% ACN/0.1% TFA (apparent pH 2.0), OVA24 was dissolved in 50% ACN/25 mM NaOH (apparent pH 12.5).

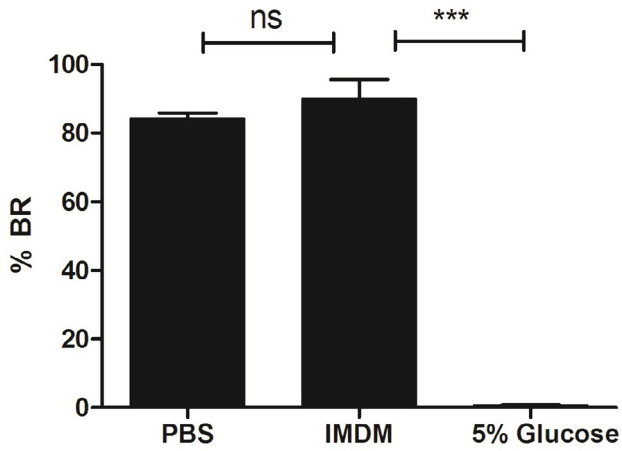

Figure 1: Burst release of OVA24 in different physiological media (PBS, IMDM cell culture medium, 5% glucose) from PLGA NP (**Table 1,** formulation 6). Data are presented as average ± standard deviation of n=3 independent batches. Data was analyzed by two-tailed unpaired Student's t-test. P values are presented as non-significant (ns) = $P > 0.05$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.0001$.

Improvement was observed in neither encapsulation efficiency nor burst release when using an inner alkaline phase volume of 100 µL (**Table 1**, formulation 9; **Figure 2a**). However, a concomitant increase of inner emulsion volume, by using an inner phase volume of 500 µL (**Table 1**, formulation 11), drastically reduced burst release from circa 90% to less than 10%, while improving encapsulation efficiency. Still, when at acidic pH, a higher inner emulsion volume (**Table 1**, formulation 10) did not affect encapsulation efficiency or burst release compared to previous values, confirming the central effect of pH in the matter. Indeed we observed that the peptide was very acidic, most likely due to remaining TFA from synthesis, drastically decreasing pH of solutions upon dissolution, with only the higher volumes containing enough base/buffer molecules to

neutralize the acid contained in the peptide (**Figure 2b**), and keep the pH above 7.

The size and zeta-potential of the prepared OVA24-loaded NP was measured in 1 mM Hepes pH 7.4. Irrespective of the preparation method, the particles were negatively charged, with zeta-potentials ranging from -10 to -15 mV (see **Table 1**), when prepared using 1% PVA as surfactant. Though the inner emulsion ratio is generally thought to influence final particle size, with a larger ratio typically yielding bigger particles [84], we did not observe significant differences in average size (**Table 1**), with final particle sizes ranging from 300 to 400 nm for higher inner phase volumes and polydispersity indices below 0.3.

Since it is well known that extreme pHs may harm peptide structure and stability, we decided to lower the working pH. Therefore, NaOH concentrations were decreased from 25 mM (apparent pH 12.5) to 0.25 mM NaOH (yielding an apparent pH of 10.5), and after dissolving the peptide in 50% ACN 0.25 mM NaOH, pH was adjusted by diluting 5 fold in Hepes buffer pH 8.0 at different Hepes concentrations (**Table 1**, formulations 12-16). We observed that a buffer concentration of at least 50 mM was necessary to maintain a basic pH in presence of peptide (**Figure 2b**), with the final pH having a direct effect on burst release (**Figure 2c**). Since the formulation with a relatively mild inner phase with an apparent pH of 8.0 (see **Table 1**, formulation 16) showed a comparable EE and burst release as the formulation with an apparent inner phase pH of 10.5 (**Table 1**, formulation 11), formulation 16 was adopted for further functional studies. With this formulation we obtained particles with an encapsulation efficiency of 38%, with an average size of 328 nm, and zeta potencial of -13.6 mV.

3.2. Release kinetics

Since the particles are meant to be delivered to DC and taken up by DC rapidly after administration, short term release properties of the peptide-loaded particles (T**able 1**, formulation 16) were assessed for 24 h. It can be observed that after 1 h OVA24 shows approximately 30% release in PBS at 37ºC under shaking 100/min (**Figure 3**). Over the next 24 h, no further release was observed. The slight decline in initial concentration of OVA24 was due to aggregation and, hence, partial precipitation during the centrifugation step. Indeed, free OVA24 dissolved in PBS at 37°C under agitation aggregated over time, as detected by DLS (results not shown). Adding Tween 80 (0.1 % w/v) to the release medium did not help. We did not further study this phenomenon, but it obviously limited the duration of the release study.

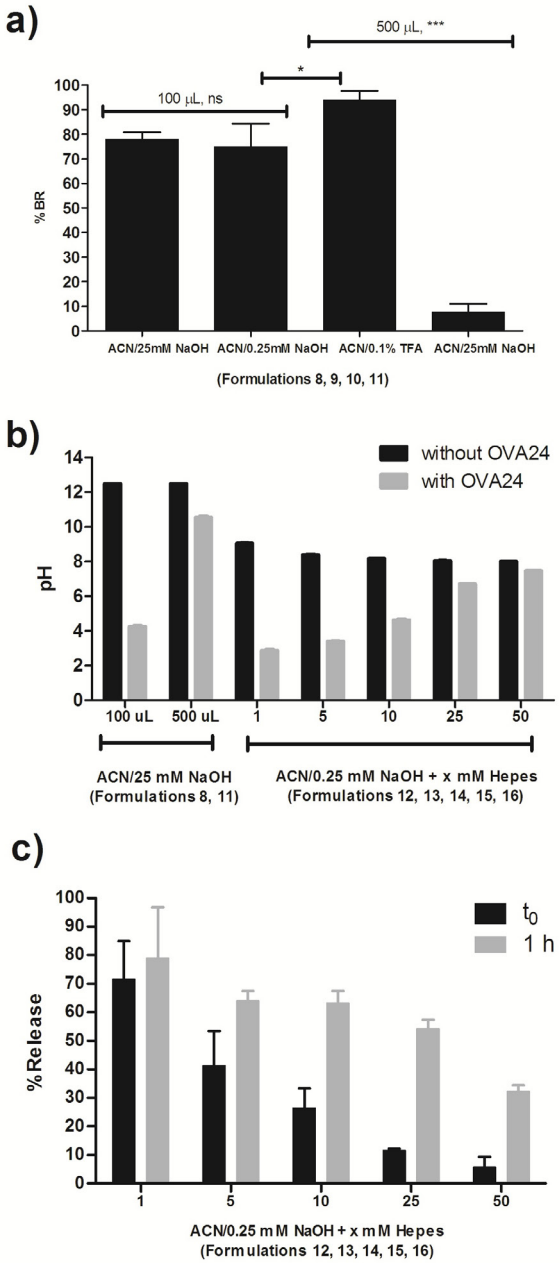

Figure 2: A) Effect of inner solvent and emulsion volume on burst release of OVA24 in PLGA NP, assessed at to upon resuspension in PBS (Formulations 8-11). **B)** Effect of the inner phase composition on apparent pH before and after the addition of OVA24 (P<0.0001) (formulations 8, 11- 16). **C)** Effect of Hepes concentration in the inner phase on release, assessed at t0 (burst release) and 1 h after resuspension (P<0.0001) (formulations 12-16). Data are presented as average ± standard deviation of n=3 independent batches. Formulation numbers are according to Table 1. P values are presented as non-significant (ns) = P > 0.05; * = P < 0.05; ** = P < 0.01; *** = P < 0.0001.

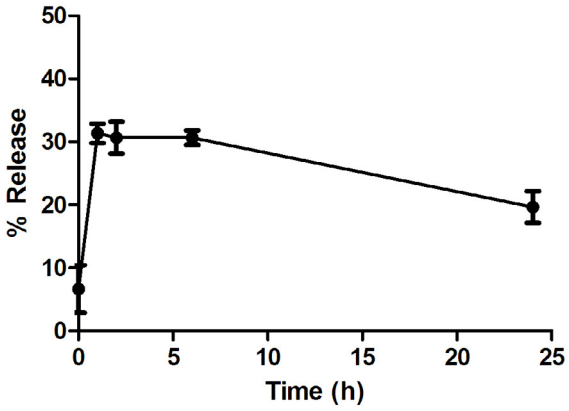

Figure 3: Release kinetics of OVA24 from PLGA NP (Table 1, formulation 16) in PBS at 37oC under shaking 100/min. Data are presented as average ± standard deviation of n=3 independent batches.

3.3. *In vitro* antigen presentation of SLP antigen encapsulated in NP

The effect of encapsulation of OVA24 on the efficiency of DC uptake and processing into MCH class I for antigen cross-presentation resulting into activation of $CD8^+$ T cells was tested *in vitro* (**Figure 4**). For that purpose, the different formulations were incubated with DC for 2.5 h, washed to remove excess unbound antigen, followed by co-culture in the presence of OVA-specific B3Z $CD8^+$ T cells. OVA24-containing PLGA NP with low (<10%) burst release (**Table 1**, formulation 16) were compared with those exhibiting high (>75%) burst release (**Table 1**, formulation 9), against soluble OVA24, empty particles, and a mixture of soluble OVA24 and empty particles. Encapsulation of OVA24 in PLGA-NP resulted in significantly enhanced activation of B3Z $CD8^+$ T cells compared to soluble OVA24. Although both tested NP formulations enhanced MHC class I antigen cross-presentation, we observed that delivery of OVA24 via encapsulation in PLGA NP with low (<10%) burst release resulted in improved T cell responses in comparison to OVA24-containing PLGA NP with high (ca. 75%) burst release. The addition of empty NP to soluble OVA24 did not show an effect on T cell activation in comparison to soluble OVA24 alone, indicating the strict necessity of peptide encapsulation in NP for enhanced processing in MHC class I antigen presentation pathways. Taken together, these data demonstrate that effective entrapment of antigen in PLGA with low burst release is crucial for efficient antigen cross-presentation by DC.

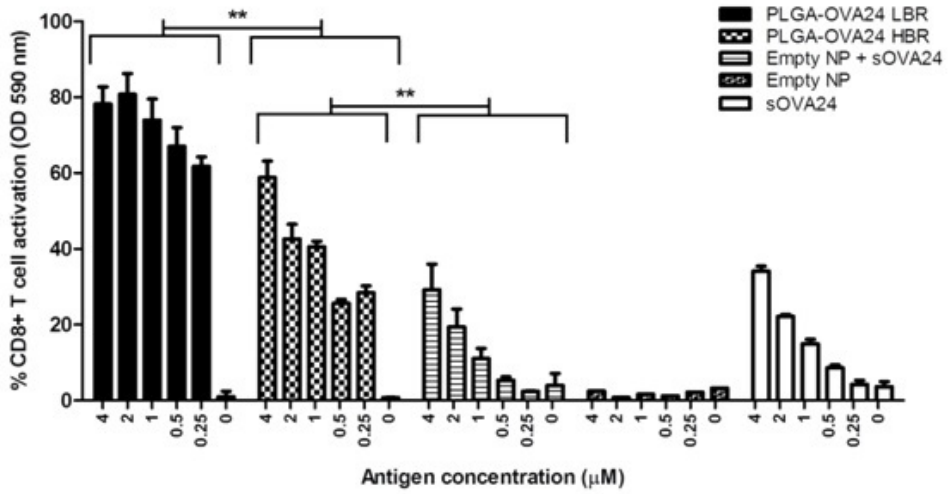

Figure 4: Normalized CD8⁺ T cell activation of SIINFEKL-specific CD8⁺ T cells (B3Z) after co-culturing with DC incubated for 2.5 hours with titrated amounts of different OVA24 SLP formulations: low-burst releasing (LBR) PLGA NP loaded with OVA24 (Table 1, formulation 16), high-burst releasing (HBR) PLGA NP loaded with OVA24 (Table 1, formulation 9), soluble OVA24 mixed with empty NP (sOVA24 + Empty NP), empty NP, and soluble OVA24 (sOVA24). Data are presented as average ± SD of triplicate measurements. Representative results from one out of 3 experiments are shown. Graphs depict normalized T cell activation data based on the maximal OD 590 nm value/T cell activation measured upon incubation of DC with 1 μM of the minimal H2-Kb epitope OVA8/SIINFEKL-peptide (OD 590 nm value of 0.918 = 100%) as positive control. P values are presented as * = $P < 0.05$; ** = $P < 0.01$.

4. Discussion

The aim of this study was to develop a method to efficiently encapsulate OVA24 in PLGA, and perform a full characterization of the obtained formulation. This approach may result in antigen-bearing biodegradable particles that can be actively taken up by DC, generating specific T cell immunity and improving the efficacy of synthetic peptide-based cancer vaccines.

Whereas hydrophilic and hydrophobic peptides have been successfully encapsulated in PLGA NP and/or microparticles in the past [85, 97, 100, 108], efficient entrapment of the moderately hydrophobic OVA24 proved to be challenging. OVA24 does not readily dissolve in aqueous solutions and is insoluble in solvents like DCM or chloroform. However, it can be dissolved in a suitable matrix such as 50% v/v ACN in water, as well as in the commonly used solvent DMSO, prior to dilution in aqueous solutions. Standard encapsulation procedures generally used for encapsulation of hydrophilic or hydrophobic antigens led to low encapsulation efficiencies and high burst release once NP were resuspended in isotonic solutions at physiological pH. Therefore,

several formulation parameters, particularly inner and outer emulsion compositions, were studied in order to increase encapsulation. The positive effect of using PVA in the outer aqueous phase on EE may be due to fact that it is not a classical surfactant with a distinct hydrophobic tail and hydrophilic headgroup, but a polymer that coats the surface, stabilizing the emulsion. In contrast, both Tween 20 and cholate are able to form micelles at concentrations above their critical micelle concentrations (0.07 and 0.6% w/v for Tween 20 and cholate, respectively), that may capture and solubilize the peptide during the emulsification process and thereby favor its extraction in the external water phase. A measurable difference between NP formulated with Tween 20 or sodium cholate (**Table 1**, formulations 1-3) and those formulated with PVA (**Table 1**, formulations 5-16) was that the negative zeta-potential of the former formulations was significantly higher. While the relatively high zeta-potential values obtained with cholate can be explained by the negative charge of the surfactant, we can only speculate that the nonionic surfactant Tween 20, due to its lower molecular size, does not shield the negative charge of the PLGA as much as PVA coating does.

The observed high burst release in the formulations with acidic inner phase led us to the notion that the majority of the OVA24 molecules might not be encapsulated in the NP's polymeric matrix, but instead were adsorbed to their surface, masking real encapsulation efficiencies. This would explain the rapid release of peptide from the NP in presence of salt by disruption of electrostatic interactions between the peptide and the PLGA surface, due to the presence of counter ions that may shield the charges, and/or alter the peptide's solubility. Indeed, the high burst release is highly consistent with observed release profiles for a tumor necrosis factor alpha blocking peptide adsorbed to PLGA NP [99], occurring once exposed to salt-based isotonic solutions at physiological pH. Increasing particle hydrophobicity by using different types of PLGA with higher molecular weights and lactic acid/glycolic acid ratio (PLGA 75:25), as well as using DMSO as solvent, creating an oil-in-water emulsion, instead of water-in-oil-in-water to reduce porosity [109, 110], did not result in a decrease of the burst release (data not shown), concurring with the adsorption hypothesis, in opposition to diffusion due to high porosity. Moreover, resuspension in either water or isotonic 5% (w/v) glucose solution did not lead to substantial burst release (**Figure 1**), which provides further evidence that the instant release was not due to high particle porosity and peptide diffusion due to osmosis.

A clear correlation between burst release and pH of the inner phase was observed (**Figure 2**), confirming that the pH of the inner phase is of primary importance for efficient peptide encapsulation. The effect of the higher inner phase volume on the burst release is mainly pH related, by providing a higher number of base/buffer molecules able to neutralize the acidic peptide and maintain an alkaline pH, whereas a higher acidic inner volume showed no effect (**Table 1**, formulation 10).

In order to better understand the physicochemical characteristics of the peptide and the effect of pH of the inner (w1) phase on the observed encapsulation efficiency and burst release, the sequence of the peptide was analyzed using ProtParam, the online protein identification and analysis software that is available through the ExPASy World Wide Web server [111] Using this tool, the theoretical isoelectric point of the OVA24 peptide

was determined as 4.3, and the grand average of hydropathicity (GRAVY) as 0.087 [112]. According to this index, amino acids are separated into hydrophilic (negative GRVAY value) and hydrophobic (positive GRAVY value). The amino acids with GRAVY values closer to zero correspond to the least hydrophobic and hydrophilic ones, which are respectively alanine (1.8) and glycine (-0.4). The slightly positive value of the OVA24 peptide confirms its slight hydrophobic nature.

Hydrophobic protein domains have shown to adsorb to polymer surfaces by electrostatic and/or hydrophobic interactions, and particle surface properties can influence adsorption [113]. Though for the OVA24/PLGA system the mechanism of encapsulation versus surface localization is still not clear, we hypothesize that efficient encapsulation at a higher pH of the inner phase may result from a more favorable partitioning of the peptide between internal/external aqueous phases and interfaces. A closer look at the peptide sequence and position of its charged residues (D-E-VSGLE-QLE-SIIN-FE-K^+LAAAAAK$^+$) allows us to divide it into two sections, with distinct characteristics. At low pHs, below the peptide's pI, the DEVSGLEQLESIINFE part should be mostly neutral, with the exception of the N-terminal amine group, and therefore rather hydrophobic. On the other hand, the K^+LAAAAAK$^+$ sequence might act as a charged headgroup, thereby rendering the molecule surface active and prone to migrate to the surface. Free peptide in the external phase may also adsorb to the particles through electrostatic interactions between positively charged residues and negatively charged PLGA NP surfaces. Once exposed to saline solutions at higher pHs, the 'hydrophobic tail' will become negatively charged, hence more hydrophilic, by which the molecule may loose its surface activity, as well as repel from negatively charged PLGA, whereas the electrostatic interactions between the positively charged residues and PLGA may also be displaced by the presence of counter ions, which could explain the release in PBS pH 7.4, but not in water (i.e., during washing of the particles during preparation) or isotonic glucose solutions with acidic pHs.

Previous studies with the short synthetic peptide SIINFEKL in PLGA microparticles showed nearly total release of the peptide within 24 hours in PBS [54]. Studies with longer peptides (13 to 43 amino acids) or recombinant human growth hormone in microparticles showed 20-70% release over the first 24 h [114-117], whereas insulin also shows 30-40% release from PLGA NP within 24 h [118]. With our method we were able to encapsulate OVA24 with nearly 40% EE in NP showing minimal burst release and a total peptide release of circa 30% over 24 h. The better retention of SLP will allow delivery of encapsulated peptide to DC, which is a great improvement compared to release over the same period of time of high releasing formulations. This is especially important if we consider subsequent development of this particulate system for delivery of SLP, which may include co-encapsulation of adjuvants, such as Toll-like receptor ligands (TLRL), or even surface modification with targeting moieties that require covalent binding to the particles at controlled pH such as described by Cruz et al. [85] without losing most of the antigen during the manufacturing process or shortly after administration.

The effect of OVA24 encapsulation on antigen cross-presentation to activate $CD8^+$ T cells was tested *in vitro* with conclusive results. OVA24-containing PLGA NP with low

burst release showed significantly higher capacity of $CD8^+$ T cell activation comparing to those with high burst release (**Figure 4**). Likewise, soluble peptide mixed with empty particles did not show any improvement when compared to soluble peptide, further proving the need of effective entrapment of antigen in PLGA to increase antigen cross presentation by DC. Furthermore, we showed that not only encapsulation of OVA24 in PLGA NP is required, but also the release characteristics are of vital importance, with low burst release being fundamental to induce a potent cellular immune response. This is probably due to enhanced uptake and/or processing by DC of particulate antigen. When incubated with low-burst releasing particles, DC may efficiently uptake the antigen cargo still encapsulated in PLGA particles, whereas with high-burst releasing particles, DC will take up less encapsulated peptide, thereby lowering the overall efficiency by which the peptide is internalized and routed into MHC class I antigen presentation pathways. The improved MHC class I antigen presentation observed with low-burst releasing particles is indicative of sustained release of the peptide inside the DC, similar to other antigen delivery systems to DC [119].

5. Conclusion

In this study we described a method for efficient encapsulation of a model SLP in PLGA NP, resulting in a particle delivery system able to enhance $CD8^+$ T cell activation *in vitro*. This encapsulation method, employing an apparent inner phase pH above the pI of the encapsulated SLP, may be a promising approach for encapsulation of peptides with amphiphilic and/or hydrophilic properties, and may be considered as a firm basis for the development of NP formulations for SLP-based immunotherapy of cancer. Preliminary studies showed that the method is applicable to other SLP as well (unpublished results). Additionally, this study shows the importance of thorough characterization of peptide encapsulation process in PLGA NP to achieve a successful formulation. In conclusion, this study has shown that encapsulation and release characteristics are strongly dependent on the pH of the first emulsion, whereas a direct comparison between NP with similar physicochemical characteristics in terms of charge, size and antigen loading, but different release profiles, uncovered the importance of low burst release to induce a potent cellular immune response.

Acknowledgements

This study was supported by grants from Immune System Activation (ISA) Pharmaceuticals and the Leiden University Medical Center. We thank Jeroen van Smeden for his help with the statistical analysis.

Conflict of Interest

The authors declare that they have no conflict of interest.

6. References

1. Ada, G., Overview of vaccines and vaccination. Mol Biotechnol, 2005. 29(3): p. 255-72.
2. Arens, R., T. van Hall, S.H. van der Burg, F. Ossendorp, and C.J. Melief, Prospects of combinatorial synthetic peptide vaccine-based immunotherapy against cancer. Seminars in immunology, 2013. 25(2): p. 182-90.
3. Melief, C.J., Cancer immunotherapy by dendritic cells. Immunity, 2008. 29(3): p. 372-83.
4. Waeckerle-Men, Y., E.U. Allmen, B. Gander, E. Scandella, E. Schlosser, G. Schmidtke, . . . M. Groettrup, Encapsulation of proteins and peptides into biodegradable poly(D,L-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. Vaccine, 2006. 24(11): p. 1847-57.
5. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, . . . C.J. Melief, Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. The New England journal of medicine, 2009. 361(19): p. 1838-47.
6. Atanackovic, D., N.K. Altorki, E. Stockert, B. Williamson, A.A. Jungbluth, E. Ritter, . . . S. Gnjatic, Vaccine-induced CD4^{+} T cell responses to MAGE-3 protein in lung cancer patients. J Immunol, 2004. 172(5): p. 3289-96.
7. Heit, A., F. Schmitz, T. Haas, D.H. Busch, and H. Wagner, Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. European journal of immunology, 2007. 37(8): p. 2063-74.
8. Schlosser, E., M. Mueller, S. Fischer, S. Basta, D.H. Busch, B. Gander, and M. Groettrup, TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. Vaccine, 2008. 26(13): p. 1626-37.
9. Rosalia, R.A., A.L. Silva, M. Camps, A. Allam, W. Jiskoot, S.H. van der Burg, . . . J. Oostendorp, Efficient ex vivo induction of T cells with potent anti-tumor activity by protein antigen encapsulated in nanoparticles. Cancer Immunol Immunother, 2013. 62(7): p. 1161-73.
10. Braun, M., C. Jandus, P. Maurer, A. Hammann-Haenni, K. Schwarz, M.F. Bachmann, . . . P. Romero, Virus-like particles induce robust human T-helper cell responses. European journal of immunology, 2012. 42(2): p. 330-40.
11. Rosalia, R.A., L.J. Cruz, S. van Duikeren, A.T. Tromp, A.L. Silva, W. Jiskoot, . . . F. Ossendorp, CD40-targeted dendritic cell delivery of PLGA-nanoparticle vaccines induce potent anti-tumor responses. Biomaterials, 2014.
12. Shen, H., A.L. Ackerman, V. Cody, A. Giodini, E.R. Hinson, P. Cresswell, . . . D.J. Hanlon, Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. Immunology, 2006. 117(1): p. 78-88.
13. Tran, K.K., X. Zhan, and H. Shen, Polymer blend particles with defined compositions for targeting antigen to both class I and II antigen presentation pathways. Advanced healthcare materials, 2014. 3(5): p. 690-702.
14. Tacken, P.J., I.J. de Vries, R. Torensma, and C.G. Figdor, Dendritic-cell

immunotherapy: from ex vivo loading to *in vivo* targeting. Nature reviews. Immunology, 2007. 7(10): p. 790-802.
15. Hailemichael, Y., Z. Dai, N. Jaffarzad, Y. Ye, M.A. Medina, X.F. Huang, . . . W.W. Overwijk, Persistent antigen at vaccination sites induces tumor-specific CD8(+) T cell sequestration, dysfunction and deletion. Nat Med., 2013. 19(4): p. 465-72. doi: 10.1038/nm.3105. Epub 2013 Mar 3.
16. Parker, R., S. Deville, L. Dupuis, F. Bertrand, and J. Aucouturier, Adjuvant formulation for veterinary vaccines: Montanide™ Gel safety profile. Procedia in Vaccinology, 2009. 1(1): p. 140-147.
17. Mueller, M., E. Schlosser, B. Gander, and M. Groettrup, Tumor eradication by immunotherapy with biodegradable PLGA microspheres--an alternative to incomplete Freund's adjuvant. Int J Cancer, 2011. 129(2): p. 407-16.
18. Mundargi, R.C., V.R. Babu, V. Rangaswamy, P. Patel, and T.M. Aminabhavi, Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. Journal of controlled release : official journal of the Controlled Release Society, 2008. 125(3): p. 193-209.
19. Newman, K.D., P. Elamanchili, G.S. Kwon, and J. Samuel, Uptake of poly(D,L-lactic-co-glycolic acid) microspheres by antigen-presenting cells *in vivo*. Journal of biomedical materials research, 2002. 60(3): p. 480-6.
20. Putney, S.D. and P.A. Burke, Improving protein therapeutics with sustained-release formulations. Nat Biotechnol, 1998. 16(2): p. 153-7.
21. Kasturi, S.P., I. Skountzou, R.A. Albrecht, D. Koutsonanos, T. Hua, H.I. Nakaya, . . . B. Pulendran, Programming the magnitude and persistence of antibody responses with innate immunity. Nature, 2011. 470(7335): p. 543-7.
22. Badiee, A., N. Davies, K. McDonald, K. Radford, H. Michiue, D. Hart, and M. Kato, Enhanced delivery of immunoliposomes to human dendritic cells by targeting the multilectin receptor DEC-205. Vaccine, 2007. 25(25): p. 4757-66.
23. Zhang, X.Q., C.E. Dahle, N.K. Baman, N. Rich, G.J. Weiner, and A.K. Salem, Potent antigen-specific immune responses stimulated by codelivery of CpG ODN and antigens in degradable microparticles. Journal of immunotherapy, 2007. 30(5): p. 469-78.
24. Pulendran, B. and R. Ahmed, Immunological mechanisms of vaccination. Nature immunology, 2011. 12(6): p. 509-17.
25. Demento, S.L., N. Bonafe, W. Cui, S.M. Kaech, M.J. Caplan, E. Fikrig, . . . T.M. Fahmy, TLR9-targeted biodegradable nanoparticles as immunization vectors protect against West Nile encephalitis. J Immunol, 2010. 185(5): p. 2989-97.
26. Link, A., F. Zabel, Y. Schnetzler, A. Titz, F. Brombacher, and M.F. Bachmann, Innate immunity mediates follicular transport of particulate but not soluble protein antigen. J Immunol, 2012. 188(8): p. 3724-33.
27. Johansen, P., T. Storni, L. Rettig, Z. Qiu, A. Der-Sarkissian, K.A. Smith, . . . T.M. Kundig, Antigen kinetics determines immune reactivity. Proc Natl Acad Sci U S A, 2008. 105(13): p. 5189-94.
28. Simon, L.C. and C.M. Sabliov, The effect of nanoparticle properties, detection method, delivery route and animal model on poly(lactic-co-glycolic) acid nanoparticles biodistribution in mice and rats. Drug metabolism reviews, 2013.
29. Oyewumi, M.O., A. Kumar, and Z. Cui, Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses.

Expert Rev Vaccines, 2010. 9(9): p. 1095-107.
30. Memish, Z.A., G. Dbaibo, M. Montellano, V.P. Verghese, H. Jain, A.P. Dubey, . . . J.M. Miller, Immunogenicity of a single dose of tetravalent meningococcal serogroups A, C, W-135, and Y conjugate vaccine administered to 2- to 10-year-olds is noninferior to a licensed-ACWY polysaccharide vaccine with an acceptable safety profile. The Pediatric infectious disease journal, 2011. 30(4): p. e56-62.
31. Joshi, V.B., S.M. Geary, and A.K. Salem, Biodegradable particles as vaccine delivery systems: size matters. AAPS J, 2013. 15(1): p. 85-94.
32. Tran, K.K. and H. Shen, The role of phagosomal pH on the size-dependent efficiency of cross-presentation by dendritic cells. Biomaterials, 2009. 30(7): p. 1356-62.
33. Winzler, C., P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, . . . P. Ricciardi-Castagnoli, Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. The Journal of experimental medicine, 1997. 185(2): p. 317-28.
34. Schuurhuis, D.H., A. Ioan-Facsinay, B. Nagelkerken, J.J. van Schip, C. Sedlik, C.J. Melief, . . . F. Ossendorp, Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific $CD8^+$ CTL responses *in vivo*. J Immunol, 2002. 168(5): p. 2240-6.
35. Sanderson, S. and N. Shastri, LacZ inducible, antigen/MHC-specific T cell hybrids. International immunology, 1994. 6(3): p. 369-76.
36. Rosalia, R.A., A.L. Silva, M. Camps, A. Allam, W. Jiskoot, S.H. van der Burg, . . . J. Oostendorp, Efficient ex vivo induction of T cells with potent anti-tumor activity by protein antigen encapsulated in nanoparticles. Cancer immunology, immunotherapy : CII, 2013.
37. Slutter, B., S. Bal, C. Keijzer, R. Mallants, N. Hagenaars, I. Que, . . . W. Jiskoot, Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen. Vaccine, 2010. 28(38): p. 6282-91.
38. Sah, H., A new strategy to determine the actual protein content of poly(lactide-co-glycolide) microspheres. Journal of pharmaceutical sciences, 1997. 86(11): p. 1315-8.
39. Silva, A.L., R.A. Rosalia, A. Sazak, M.G. Carstens, F. Ossendorp, J. Oostendorp, and W. Jiskoot, Optimization of encapsulation of a synthetic long peptide in PLGA nanoparticles: low-burst release is crucial for efficient $CD8(^+)$ T cell activation. Eur J Pharm Biopharm, 2013. 83(3): p. 338-45.
40. Kreutz, M., P.J. Tacken, and C.G. Figdor, Targeting dendritic cells--why bother? Blood, 2013. 121(15): p. 2836-44.
41. Schliehe, C., C. Redaelli, S. Engelhardt, M. Fehlings, M. Mueller, N. van Rooijen, . . . M. Groettrup, CD8- dendritic cells and macrophages cross-present poly(D,L-lactate-co-glycolate) acid microsphere-encapsulated antigen *in vivo*. J Immunol, 2011. 187(5): p. 2112-21.
42. Fifis, T., A. Gamvrellis, B. Crimeen-Irwin, G.A. Pietersz, J. Li, P.L. Mottram, . . . M. Plebanski, Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol, 2004. 173(5): p.

3148-54.
43. Gutierro, I., R.M. Hernandez, M. Igartua, A.R. Gascon, and J.L. Pedraz, Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres. Vaccine, 2002. 21(1-2): p. 67-77.
44. Demento, S.L., W. Cui, J.M. Criscione, E. Stern, J. Tulipan, S.M. Kaech, and T.M. Fahmy, Role of sustained antigen release from nanoparticle vaccines in shaping the T cell memory phenotype. Biomaterials, 2012. 33(19): p. 4957-64.
45. Xiang, S.D., A. Scholzen, G. Minigo, C. David, V. Apostolopoulos, P.L. Mottram, and M. Plebanski, Pathogen recognition and development of particulate vaccines: does size matter? Methods, 2006. 40(1): p. 1-9.
46. Sharp, F.A., D. Ruane, B. Claass, E. Creagh, J. Harris, P. Malyala, . . . E.C. Lavelle, Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. Proc Natl Acad Sci U S A, 2009. 106(3): p. 870-5.
47. Mata, E., M. Igartua, R.M. Hernandez, J.E. Rosas, M.E. Patarroyo, and J.L. Pedraz, Comparison of the adjuvanticity of two different delivery systems on the induction of humoral and cellular responses to synthetic peptides. Drug delivery, 2010. 17(7): p. 490-9.
48. Zeestraten, E.C., F.M. Speetjens, M.J. Welters, S. Saadatmand, L.F. Stynenbosch, R. Jongen, . . . C.J. Melief, Addition of interferon-alpha to the p53-SLP(R) vaccine results in increased production of interferon-gamma in vaccinated colorectal cancer patients: a phase I/II clinical trial. International journal of cancer. Journal international du cancer, 2013. 132(7): p. 1581-91.
49. Yeo, Y. and K. Park, Control of encapsulation efficiency and initial burst in polymeric microparticle systems. Arch Pharm Res, 2004. 27(1): p. 1-12.
50. Leelarasamee, N., S.A. Howard, C.J. Malanga, L.A. Luzzi, T.F. Hogan, S.J. Kandzari, and J.K. Ma, Kinetics of drug release from polylactic acid-hydrocortisone microcapsules. Journal of microencapsulation, 1986. 3(3): p. 171-9.
51. Mansour, H.M., M. Sohn, A. Al-Ghananeem, and P.P. Deluca, Materials for pharmaceutical dosage forms: molecular pharmaceutics and controlled release drug delivery aspects. Int J Mol Sci, 2010. 11(9): p. 3298-322.
52. Cruz, L.J., R.A. Rosalia, J.W. Kleinovink, F. Rueda, C.W. Lowik, and F. Ossendorp, Targeting nanoparticles to CD40, DEC-205 or CD11c molecules on dendritic cells for efficient CD8 T cell response: A comparative study. Journal of controlled release : official journal of the Controlled Release Society, 2014. 192C: p. 209-218.
53. Cruz, L.J., P.J. Tacken, R. Fokkink, B. Joosten, M.C. Stuart, F. Albericio, . . . C.G. Figdor, Targeted PLGA nano- but not microparticles specifically deliver antigen to human dendritic cells via DC-SIGN *in vitro*. Journal of controlled release : official journal of the Controlled Release Society, 2010. 144(2): p. 118-26.
54. Fischer, S., E. Schlosser, M. Mueller, N. Csaba, H.P. Merkle, M. Groettrup, and B. Gander, Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response. J Drug Target, 2009. 17(8): p. 652-61.
55. Krishnamachari, Y. and A.K. Salem, Innovative strategies for co-delivering antigens and CpG oligonucleotides. Advanced drug delivery reviews, 2009.

61(3): p. 205-17.
56. Finkelman, F.D., J. Holmes, I.M. Katona, J.F. Urban, Jr., M.P. Beckmann, L.S. Park, . . . W.E. Paul, Lymphokine control of *in vivo* immunoglobulin isotype selection. Annual review of immunology, 1990. 8: p. 303-33.
57. Romagnani, S., Th1/Th2 cells. Inflamm Bowel Dis, 1999. 5(4): p. 285-94.
58. Eckl-Dorna, J. and F.D. Batista, BCR-mediated uptake of antigen linked to TLR9 ligand stimulates B-cell proliferation and antigen-specific plasma cell formation. Blood, 2009. 113(17): p. 3969-77.
59. van de Weert, M., W.E. Hennink, and W. Jiskoot, Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharm Res, 2000. 17(10): p. 1159-67.
60. Samadi, N., A. Abbadessa, A. Di Stefano, C.F. van Nostrum, T. Vermonden, S. Rahimian, . . . W.E. Hennink, The effect of lauryl capping group on protein release and degradation of poly(D,L-lactic-co-glycolic acid) particles. Journal of controlled release : official journal of the Controlled Release Society, 2013. 172(2): p. 436-43.
61. Schirmbeck, R., W. Bohm, and J. Reimann, Injection of detergent-denatured ovalbumin primes murine class I-restricted cytotoxic T cells *in vivo*. European journal of immunology, 1994. 24(9): p. 2068-72.
62. Huang, C.F., T.C. Wu, Y.H. Chu, K.S. Hwang, C.C. Wang, and H.J. Peng, Effect of neonatal sublingual vaccination with native or denatured ovalbumin and adjuvant CpG or cholera toxin on systemic and mucosal immunity in mice. Scand J Immunol, 2008. 68(5): p. 502-10.
63. Peng, H.J., Z.N. Chang, L.C. Tsai, S.N. Su, H.D. Shen, and C.H. Chang, Heat denaturation of egg-white proteins abrogates the induction of oral tolerance of specific Th2 immune responses in mice. Scand J Immunol, 1998. 48(5): p. 491-6.
64. Rosalia, R.A., E.D. Quakkelaar, A. Redeker, S. Khan, M. Camps, J.W. Drijfhout, . . . C.J. Melief, Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. Eur J Immunol, 2013. 43(10): p. 2554-65.
65. Perrie, Y., A.R. Mohammed, D.J. Kirby, S.E. McNeil, and V.W. Bramwell, Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens. International Journal of Pharmaceutics, 2008. 364(2): p. 272-80.
66. Welters, M.J., G.G. Kenter, S.J. Piersma, A.P. Vloon, M.J. Lowik, D.M. Berends-van der Meer, . . . S.H. van der Burg, Induction of tumor-specific $CD4^+$ and $CD8^+$ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. 14(1): p. 178-87.
67. Steinman, R. and K. Inaba, Immunogenicity: role of dendritic cells. Bioessays, 1989. 10(5): p. 145-52.
68. Steinman, R.M., The dendritic cell system and its role in immunogenicity. Annu Rev Immunol, 1991. 9: p. 271-96.
69. Inaba, K., J.W. Young, and R.M. Steinman, Direct activation of $CD8^+$ cytotoxic T lymphocytes by dendritic cells. The Journal of experimental medicine, 1987. 166(1): p. 182-94.
70. Trumpfheller, C., M.P. Longhi, M. Caskey, J. Idoyaga, L. Bozzacco, T. Keler, . .

. R.M. Steinman, Dendritic cell-targeted protein vaccines: a novel approach to induce T-cell immunity. Journal of internal medicine, 2012. 271(2): p. 183-92.
71. Vulink, A., K.J. Radford, C. Melief, and D.N. Hart, Dendritic cells in cancer immunotherapy. Advances in cancer research, 2008. 99: p. 363-407.
72. Zwaveling, S., S.C. Ferreira Mota, J. Nouta, M. Johnson, G.B. Lipford, R. Offringa, . . . C.J. Melief, Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. J Immunol, 2002. 169(1): p. 350-8.
73. Ossendorp, F., E. Mengede, M. Camps, R. Filius, and C.J. Melief, Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. J Exp Med, 1998. 187(5): p. 693-702.
74. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, . . . C.J. Melief, Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. 14(1): p. 169-77.
75. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, . . . C.J. Melief, Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. The New England journal of medicine, 2009. 361(19): p. 1838-47.
76. Melief, C.J., Synthetic vaccine for the treatment of lesions caused by high risk human papilloma virus. Cancer J, 2011. 17(5): p. 300-1.
77. Melief, C.J., Treatment of established lesions caused by high-risk human papilloma virus using a synthetic vaccine. Journal of immunotherapy, 2012. 35(3): p. 215-6.
78. Chiang, C.L., L.E. Kandalaft, and G. Coukos, Adjuvants for enhancing the immunogenicity of whole tumor cell vaccines. International reviews of immunology, 2011. 30(2-3): p. 150-82.
79. van Driel, W.J., M.E. Ressing, G.G. Kenter, R.M. Brandt, E.J. Krul, A.B. van Rossum, . . . J.B. Trimbos, Vaccination with HPV16 peptides of patients with advanced cervical carcinoma: clinical evaluation of a phase I-II trial. European journal of cancer, 1999. 35(6): p. 946-52.
80. Slingluff, C.L., Jr., G. Yamshchikov, P. Neese, H. Galavotti, S. Eastham, V.H. Engelhard, . . . W.G. Ross, Phase I trial of a melanoma vaccine with gp100(280-288) peptide and tetanus helper peptide in adjuvant: immunologic and clinical outcomes. Clinical cancer research : an official journal of the American Association for Cancer Research, 2001. 7(10): p. 3012-24.
81. Aucouturier, J., S. Ascarateil, and L. Dupuis, The use of oil adjuvants in therapeutic vaccines. Vaccine, 2006. 24 Suppl 2: p. S2-44-5.
82. Aucouturier, J., L. Dupuis, S. Deville, S. Ascarateil, and V. Ganne, Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. Expert Rev Vaccines, 2002. 1(1): p. 111-8.
83. Leenaars, P.P., M.A. Koedam, P.W. Wester, V. Baumans, E. Claassen, and C.F. Hendriksen, Assessment of side effects induced by injection of different adjuvant/antigen combinations in rabbits and mice. Laboratory animals, 1998.

32(4): p. 387-406.
84. Astete, C.E. and C.M. Sabliov, Synthesis and characterization of PLGA nanoparticles. J Biomater Sci Polym Ed, 2006. 17(3): p. 247-89.
85. Cruz, L.J., P.J. Tacken, R. Fokkink, B. Joosten, M.C. Stuart, F. Albericio, . . . C.G. Figdor, Targeted PLGA nano- but not microparticles specifically deliver antigen to human dendritic cells via DC-SIGN *in vitro*. J Control Release, 2010. 144(2): p. 118-26.
86. Mundargi, R.C., V.R. Babu, V. Rangaswamy, P. Patel, and T.M. Aminabhavi, Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. J Control Release, 2008. 125(3): p. 193-209.
87. Vlieghe, P., V. Lisowski, J. Martinez, and M. Khrestchatisky, Synthetic therapeutic peptides: science and market. Drug discovery today, 2010. 15(1-2): p. 40-56.
88. Woodley, J.F., Enzymatic barriers for GI peptide and protein delivery. Critical reviews in therapeutic drug carrier systems, 1994. 11(2-3): p. 61-95.
89. Schall, N., N. Page, C. Macri, O. Chaloin, J.P. Briand, and S. Muller, Peptide-based approaches to treat lupus and other autoimmune diseases. Journal of autoimmunity, 2012.
90. Witt, K.A., T.J. Gillespie, J.D. Huber, R.D. Egleton, and T.P. Davis, Peptide drug modifications to enhance bioavailability and blood-brain barrier permeability. Peptides, 2001. 22(12): p. 2329-43.
91. De Temmerman, M.L., J. Rejman, J. Demeester, D.J. Irvine, B. Gander, and S.C. De Smedt, Particulate vaccines: on the quest for optimal delivery and immune response. Drug discovery today, 2011. 16(13-14): p. 569-82.
92. Fahmy, T.M., S.L. Demento, M.J. Caplan, I. Mellman, and W.M. Saltzman, Design opportunities for actively targeted nanoparticle vaccines. Nanomedicine, 2008. 3(3): p. 343-55.
93. Elamanchili, P., C.M. Lutsiak, S. Hamdy, M. Diwan, and J. Samuel, "Pathogen-mimicking" nanoparticles for vaccine delivery to dendritic cells. J Immunother, 2007. 30(4): p. 378-95.
94. Jain, S., D.T. O'Hagan, and M. Singh, The long-term potential of biodegradable poly(lactide-co-glycolide) microparticles as the next-generation vaccine adjuvant. Expert review of vaccines, 2011. 10(12): p. 1731-42.
95. van der Lubben, I.M., J.C. Verhoef, G. Borchard, and H.E. Junginger, Chitosan and its derivatives in mucosal drug and vaccine delivery. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 2001. 14(3): p. 201-7.
96. Jiang, W., R.K. Gupta, M.C. Deshpande, and S.P. Schwendeman, Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. Adv Drug Deliv Rev, 2005. 57(3): p. 391-410.
97. Mata, E., M. Igartua, R.M. Hernandez, J.E. Rosas, M.E. Patarroyo, and J.L. Pedraz, Comparison of the adjuvanticity of two different delivery systems on the induction of humoral and cellular responses to synthetic peptides. Drug delivery, 2010. 17(7): p. 490-9.
98. Slutter, B., L. Plapied, V. Fievez, M.A. Sande, A. des Rieux, Y.J. Schneider, . . . V. Preat, Mechanistic study of the adjuvant effect of biodegradable nanoparticles

in mucosal vaccination. J Control Release, 2009. 138(2): p. 113-21.
99. Yang, A., L. Yang, W. Liu, Z. Li, H. Xu, and X. Yang, Tumor necrosis factor alpha blocking peptide loaded PEG-PLGA nanoparticles: preparation and *in vitro* evaluation. Int J Pharm, 2007. 331(1): p. 123-32.
100. Wischke, C. and S.P. Schwendeman, Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. Int J Pharm, 2008. 364(2): p. 298-327.
101. Liu, Y. and S.P. Schwendeman, Mapping Microclimate pH Distribution inside Protein-Encapsulated PLGA Microspheres Using Confocal Laser Scanning Microscopy. Molecular pharmaceutics, 2012. 9(5): p. 1342-50.
102. van de Weert, M., W.E. Hennink, and W. Jiskoot, Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharm Res, 2000. 17(10): p. 1159-67.
103. Jiskoot, W., T.W. Randolph, D.B. Volkin, C.R. Middaugh, C. Schoneich, G. Winter, . . . J.F. Carpenter, Protein instability and immunogenicity: roadblocks to clinical application of injectable protein delivery systems for sustained release. Journal of pharmaceutical sciences, 2012. 101(3): p. 946-54.
104. Bijker, M.S., S.J. van den Eeden, K.L. Franken, C.J. Melief, R. Offringa, and S.H. van der Burg, $CD8^+$ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. J Immunol, 2007. 179(8): p. 5033-40.
105. Khan, S., M.S. Bijker, J.J. Weterings, H.J. Tanke, G.J. Adema, T. van Hall, . . . F. Ossendorp, Distinct uptake mechanisms but similar intracellular processing of two different toll-like receptor ligand-peptide conjugates in dendritic cells. J Biol Chem, 2007. 282(29): p. 21145-59.
106. Winzler, C., P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, . . . P. Ricciardi-Castagnoli, Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. J Exp Med, 1997. 185(2): p. 317-28.
107. Sanderson, S. and N. Shastri, LacZ inducible, antigen/MHC-specific T cell hybrids. Int Immunol, 1994. 6(3): p. 369-76.
108. Wieber, A., T. Selzer, and J. Kreuter, Characterisation and stability studies of a hydrophilic decapeptide in different adjuvant drug delivery systems: a comparative study of PLGA nanoparticles versus chitosan-dextran sulphate microparticles versus DOTAP-liposomes. International Journal of Pharmaceutics, 2011. 421(1): p. 151-9.
109. Luan, X. and R. Bodmeier, Influence of the poly(lactide-co-glycolide) type on the leuprolide release from in situ forming microparticle systems. Journal of controlled release : official journal of the Controlled Release Society, 2006. 110(2): p. 266-72.
110. Bao, W., J. Zhou, J. Luo, and D. Wu, PLGA microspheres with high drug loading and high encapsulation efficiency prepared by a novel solvent evaporation technique. Journal of microencapsulation, 2006. 23(5): p. 471-9.
111. Gasteiger, E., A. Gattiker, C. Hoogland, I. Ivanyi, R.D. Appel, and A. Bairoch, ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res, 2003. 31(13): p. 3784-8.
112. Kyte, J. and R.F. Doolittle, A simple method for displaying the hydropathic character of a protein. Journal of molecular biology, 1982. 157(1): p. 105-32.
113. Jung, T., W. Kamm, A. Breitenbach, G. Klebe, and T. Kissel, Loading of tetanus toxoid to biodegradable nanoparticles from branched poly(sulfobutyl-

polyvinyl alcohol)-g-(lactide-co-glycolide) nanoparticles by protein adsorption: a mechanistic study. Pharmaceutical research, 2002. 19(8): p. 1105-13.
114. Sales-Junior, P.A., F. Guzman, M.I. Vargas, S. Sossai, V.A. Patarroyo, C.Z. Gonzalez, and J.H. Patarroyo, Use of biodegradable PLGA microspheres as a slow release delivery system for the Boophilus microplus synthetic vaccine SBm7462. Veterinary immunology and immunopathology, 2005. 107(3-4): p. 281-90.
115. Rafi, M., S.M. Singh, V. Kanchan, C.K. Anish, and A.K. Panda, Controlled release of bioactive recombinant human growth hormone from PLGA microparticles. Journal of microencapsulation, 2010. 27(6): p. 552-60.
116. Burton, K.W., M. Shameem, B.C. Thanoo, and P.P. DeLuca, Extended release peptide delivery systems through the use of PLGA microsphere combinations. Journal of biomaterials science. Polymer edition, 2000. 11(7): p. 715-29.
117. Buyuktimkin, B., Q. Wang, P. Kiptoo, J.M. Stewart, C. Berkland, and T.J. Siahaan, Vaccine-like controlled-release delivery of an immunomodulating peptide to treat experimental autoimmune encephalomyelitis. Molecular pharmaceutics, 2012. 9(4): p. 979-85.
118. Yang, J., H. Sun, and C. Song, Preparation, characterization and *in vivo* evaluation of pH-sensitive oral insulin-loaded poly(lactic-co-glycolicacid) nanoparticles. Diabetes, obesity & metabolism, 2012. 14(4): p. 358-64.
119. van Montfoort, N., M.G. Camps, S. Khan, D.V. Filippov, J.J. Weterings, J.M. Griffith, . . . F. Ossendorp, Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. Proc Natl Acad Sci U S A, 2009. 106(16): p. 6730-5.