Liposome-based synthetic long peptide vaccines for cancer immunotherapy

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Chapter 6

Cationic DOTAP-based liposomes: a vaccine formulation platform for synthetic long peptides with widely different physicochemical properties

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Abstract

Synthetic long peptides (SLPs) harbouring T cell epitope sequences hold great promise for cancer immunotherapy by active vaccination. However, because of their low intrinsic immunogenicity they have to be formulated with adjuvants. Cationic DOTAP-based liposomes have been shown to be suited as a delivery system including adjuvants to improve the efficiency of the SLPs. In this study, we investigated the capacity of a DOTAP:DOPC liposomal formulation to accommodate eleven 24-residue long SLPs, all harbouring the immunodominant SIINFEKL model epitope of ovalbumin, but widely differing in hydrophobicity and isoelectric point. Following a standard optimised protocol, the eleven SLPs were successfully loaded into the cationic liposomes. The obtained particles had a size below 200 nm, a low polydispersity index (< 0.3) and a positive zeta-potential (ca. 30 mV). The SLP loading efficiency was at least 50% for all the peptides, independently of their physicochemical nature. Finally, all the different SLP-loaded liposomes efficiently delivered the long peptide to immature DCs which could process the antigenic peptide and efficiently activated SIINFEKL-specific $CD8^+$ T cells *in vitro*. The improved immunogenicity of the SLP set makes liposomes a flexible and therefore promising vehicle for the delivery of SLPs.

Keywords: cationic liposomes, synthetic long peptides, SIINFEKL antigen, therapeutic vaccine, in vitro immunogenicity

Introduction

The success of therapeutic vaccination largely depends on the capacity of the vaccine to induce a strong cellular immune response able to eradicate tumours or infections [1, 2]. In order to achieve this, specific antigens have to be efficiently delivered to and processed by properly activated DCs, which generally requires the inclusion of an adjuvant in the vaccine. Adjuvants can be generally divided into immunostimulatory compounds and delivery systems [3]. Currently several delivery systems are available for use in humans, including aluminium salts, influenza virosomes, virus-like particles (VLPs), liposomes, and oil-in-water emulsions, such as MF59, AS03 and AF03 [1,4-6]. However, the majority of these adjuvants are generally applied in prophylactic settings for induction of humoral immunity and therefore are considered less suitable for therapeutic vaccination [4].

One of the most common delivery systems that have been also traditionally used for the delivery of anti-cancer drugs, such as doxorubicin or anthracycline [7,8] are liposomes; these are small vesicles consisting of, one or several, lipid bilayers enclosing an aqueous compartment [9]. By nature, liposomes are able to accommodate a wide range of molecules-antigens. The hydrophilic heads of the phospholipids cover the inner and outer surface of the liposomes, so that hydrophilic compounds will most likely be found encapsulated in the aqueous inner compartment of liposomes, protected from enzymatic degradation by proteins and enzymes. In contrast, the hydrophobic tails of the lipid bilayer will associate or adsorb lipophilic compounds [8]. Liposomes, due to their similarity to the membrane of cells, can target the loaded drug or antigen directly into the cells [10] and by adjusting their properties – such as size and surface charge - the induced immune response can be tuned towards the desired one.

Recently we reported promising preclinical results using a cationic liposomal formulation for the delivery of SLP vaccines. Briefly, a DOTAP:DOPC formulation loaded with two model OVA SLPs or an E7-derived SLP from HPV, showed a strong efficiency to induce functional cytokines-producing antigen-specific T cells and a strong efficacy, as compared to the free SLPs, to cure mice when used as a therapeutic vaccine in two independent tumour models. In addition, the direct comparison of SLP-loaded liposomes to other particulate vaccine systems, underlined their superior immunogenicity [18].

SLP-based therapeutic vaccination has been succesfull in patients with premalignant vulvar lesions caused by oncogenic HPV type 16 by using a multiple overlapping SLP vaccine covering the entire sequences of the HPV16 E6 and E7 viral oncoproteins [11].

In addition, identification of neo-epitopes in cancer types with high mutation rates like melanoma and non-small cell lung cancer by exome sequencing of tumor cells [12,13] allows the design of personalized SLP-based cancer vaccines. For both approaches a flexible and adequate vaccine delivery system is required since the identified antigenic peptide sequences will differ in their physicochemical properties.

Considering the need for multiple peptides for the design of a therapeutic cancer vaccine, in this study we investigated the potential of this cationic DOTAP-based liposomal formulation as a platform for various synthetic long peptide (SLP) antigens. The creation of an SLP library, consisting of 24-amino acid-long peptides, all of them harbouring the ovalbumin immunodominant SIINFEKL epitope, but covering different physicochemical properties, allowed us to assess whether the earlier developed liposomes are capable of accommodating SLPs independent of their pI and hydrophobicity, without compromising their potency. In that way, a first assessment on the feasibility of a “universal” liposomal-based vaccine system for the delivery of SLP vaccines will be achieved.

Materials and Methods

Materials

The NBD-(7-nitrobenzofurazan) fluorescently labelled 24-mer SLPs (Table I) including the immunodominant cytotoxic T-lymphocyte (CTL) epitope [SIINFEKL] of ovalbumin (OVA) were produced and purified at the peptide facility of the Department of Immunohematology and Blood transfusion of Leiden University Medical Center. The lipids DOPC and DOTAP were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Acetonitrile (ACN), chloroform, and methanol were obtained from Biosolve BV (Valkenswaard, the Netherlands) and Vivaspin 2 centrifuge membrane concentrators were purchased from Sartorius Stedim Biotech GmbH (Goettingen, Germany). Iscove's modified Dulbecco's medium (IMDM; Lonza Verniers, Belgium) was supplemented with 8 % (v/v) foetal calf serum (Greiner Bioscience, Alphen a/d Rijn, the Netherlands), 50 µM 2-mercaptoehanol (Sigma-Aldrich, Zwijndrecht, Netherlands) and 2 mM glutamine (Life Technologies, Bleiswijk, the Netherlands). Deionised water with a resistivity of 18 MΩ.cm was produced by a Millipore water purification system (MQ water). Phosphate buffer was composed of 7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4, pH 7.4 (10 mM PB, pH 7.4). MQ water and 10 mM PB, pH 7.4, were filtered through a 0.22-µm Millex GP PES-filter (Millipore, Ireland) before use. Phosphate-buffered saline, (PBS: 140 mM NaCl, 8.7 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, pH 7.4), which was used for the *in vitro* MHC class I assays, was purchased from B. Braun (Meslungen, Germany).

Liposome preparation

Cationic liposomes loaded with the SLPs were prepared in two ways by using the thin film dehydration-rehydration method, followed by extrusion. In detail, 1 DOTAP: 1 DOPC (molar ratio) in chloroform was mixed in a round-bottomed flask at a concentration of 10 mg lipid per ml of final liposome dispersion. A lipid film was formed by chloroform ($CHCl_3$) evaporation in a rotary evaporator for 1 hour at 37°C. For the relatively hydrophilic SLPs (hydropathicity below -0.9; see Table 1), the film was then rehydrated with a solution of 1 mg/ml SLP in ACN/H_2O 1:1 (v/v), adjusted to a pH value of about 8.5 and after mixing in the presence of glass beads the dispersion was equilibrated for 1 h at room temperature. For the preparation of liposomes loaded with the relatively hydrophobic SLPs (hydropathicity above -0.2; see Table 1), the peptides dissolved in a $CHCl_3$: MeOH: H_2O solution (60:36:4, v/v) were added to the organic lipid solution to reach a concentration of 1 mg/ml, before the formation of the dry film. Next, the liposome dispersion (of both hydrophilic and hydrophobic SLPs) was snap-frozen in

liquid nitrogen, followed by freeze-drying in a Christ alpha 1-2 freeze-dryer (Osterode, Germany) overnight. Dehydration-rehydration liposomes were generated by gradually hydrating the freeze-dried lipid cake in 10 mM PB, pH 7.4. To prepare 2 ml liposome dispersion, two volumes of 400 µl and one volume of 1200 µl PB were successively added, with intervals of 20 minutes between each addition. The mixture was vortexed well during the rehydration steps and the resulting dispersion was kept at room temperature for at least 1 h (Figure 1).

Sizing of the obtained vesicles was performed by high-pressure extrusion at room temperature, by using a Lipex extruder (Northern Lipids Inc., Canada). To obtain monodisperse liposomes, the liposome mixture was passed four times through a 400-nm pore size membrane and four times through a 200-nm pore size membrane (polycarbonate Track Etch membranes, Nucleopore Millipore, Kent, UK).

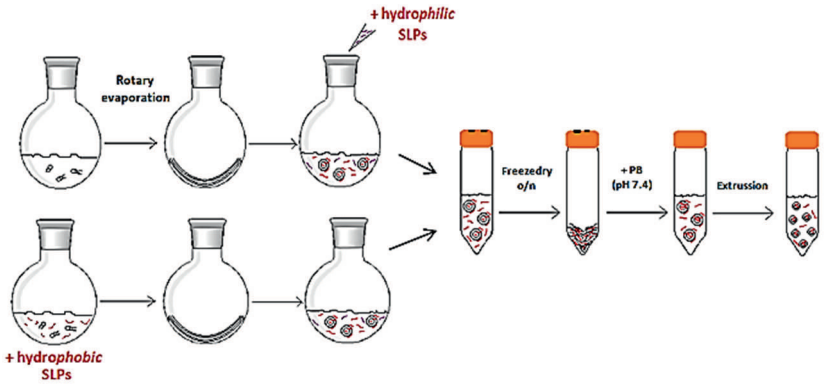

Figure 1: SLP-loaded liposomes preparation procedure. Hydrophobic SLPs were dissolved in chloroform with the lipids before the formation of the dry film, whether the solutions of hydrophilic SLPs (adjusted to a pH of 8.5) were added during the hydration of the lipid film. Overnight freeze-drying and rehydration with PB were followed by liposome sizing through filter extrusion.

Purification (removal of free SLP) and concentration of SLP-loaded liposomes were performed by using a VivaSpin 2 centrifugation concentrator (PES membrane, molecular weight cut-off (MWCO) 300 kDa): 1300 µl of the liposome dispersion were loaded in the concentrator, which was then centrifuged at 2000 rpm and 20°C for 6-7 hours, till the suspension was concentrated five-fold. The filtrate, containing the free SLP, was collected and the concentrated liposome dispersion was diluted by adding 1000 µl PB, followed again by a five-fold concentration-wash to remove most of the free peptide.

Samples of the liposome fraction and the free fractions were taken after each step for peptide content analysis (Figure 2).

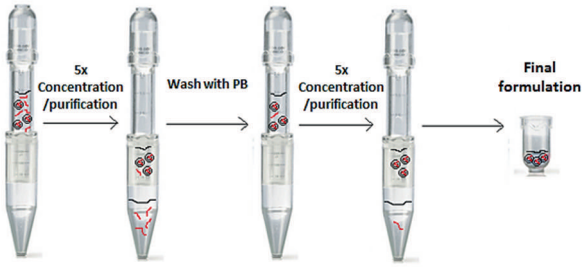

Figure 2: Purification (removal of free SLP) and concentration of SLP-loaded liposomes using VivaSpin 2 centrifugation concentrators.

Liposome characterisation

Dynamic light scattering and zeta-potential

Average diameter (Z_{ave}) and polydispersity index (PDI) of the liposomes were determined by dynamic light scattering (DLS) using a Zetasizer (NanoZS, Malvern Ltd. UK). The zeta-potential was determined by laser Doppler electrophoresis using the same device. For these measurements liposome samples were diluted 100-fold in PB.

SLP loading efficiency into the liposomes

The amount of the SLP loaded in the liposomes (t=0) was determined by measuring the fluorescence of the NBD fluorophore conjugated to the peptide (excitation wavelength 458 nm; emission wavelength 530 nm) in collected non-solubilized (i.e., intact) liposomal samples and the PB filtrates from the purification/concentration process (before wash and after wash) containing the free SLP. A calibration curve of the fluorescently labelled SLP was made and all samples were measured by using a fluorescence micro plate reader (Tecan, Salzburg, Austria).

Eight weeks (t= 8 weeks) after the first measurement of the SLP content in the liposomes, 200 µl of liposomal formulation were diluted 5x with PB buffer, followed by concentration using the VivaSpin 2 centrifugation concentrator, as previously performed. The filtrate (including free SLP) and the concentrated liposomes were collected and the loading efficiency and peptide recovery were calculated and expressed as percentage of the initial calculated loading efficiency (t=0).

In vitro MHC class I antigen (SLP) presentation

The immunogenicity of the SLP-loaded formulations was tested in an *in vitro* read-out, regarding their efficiency to activate immature DCs and present SIINFEKL to $CD8^+$ antigen specific T cells, leading to their activation. Immature D1 cells were incubated in 96-well flat-bottomed plates at 37°C in supplemented IMDM with SLP-loaded liposome formulations or plain SLPs in PBS at different concentrations. After 2.5 hours the plates were washed three times with supplemented IMDM culture medium, in order to remove excess antigen. Subsequently, T cell hybridoma B3Z cells ($50x10^5$/well) were added, followed by overnight incubation at 37°C [14]. Chlorophenol red-β-galactopyranoside (CPRG) was used as lacZ substrate in cell lysates and the colour conversion was measured by detecting absorbance at 590 nm.

Results

Using the online ExPASy ProtParam tool that allows for the calculation of various physical and chemical parameters, a series of fifteen SLPs was designed. The eleven 24-amino acid long peptides, presented in table I, cover a wide range of theoretical pIs (from 4.02 to 9.40) and the grand average of hydropathicity (GRAVY) values (from -1.208 to +0.713), representative to SLPs properties used in previous clinical studies [11]. From the theoretical pI one can judge the net charge of the peptides throughout the whole process of the liposomal preparation, while the hydropathicity factor indicates whether they are considered to be more hydrophilic or hydrophobic.

Table I: The SLP library with calculated SLPs properties based on the online ExPASy tool

	Peptide sequence NBD-G-XXXXXXXXXX-SIINFEKLAAAK	**Theoretical pI ***	**Hydropathicity (GRAVY) ****
Hydro*phili*c SLPs			
SLP 1	DEDKDKDDEEA	4.16	-1.208
SLP 2	DEEEKEGKEKA	4.82	-1.096
SLP 3	RKDDKDDKDLA	6.23	-0.963
SLP 4	RKHDHEHEHHA	7.16	-1.171
SLP 5	EDKKKSEKESA	8.34	-1.017
SLP 6	DEKRKKERELA	9.40	-1.004
Hydro*phobic* SLPs			
SLP 7	DELYDLYDELA	4.02	-0.079
SLP 8	DEGLLRHLDEA	4.90	-0.163
SLP 9	DAKHDHLLHAA	7.02	-0.104
SLP 10	LDKKLLEKELA	8.38	-0.008
SLP 11	RIDIRLIIEIA	8.59	0.713

** The isoelectric point -pI was calculated for the unlabelled peptide (11-mer varying sequence+ SIINFEKLAAAK) of which the N-terminus was not blocked, so the real theoretical pI values are expected to be slightly lower for the labelled peptide*

*** The sum of hydropathicity values of all amino acids, divided by the number of residues in the sequence. Hydrophilic proteins/peptides have values < - 1*

Four rather hydrophobic SLPs, with a pI range from 4.18 to 9.55 (supplementary table I) were excluded from the study, because of solubility problems. In an attempt to test the potential of a DOTAP-based formulation, liposomes were prepared following the dehydration-rehydration lipid film method. Hydrophilic SLPs, with a GRAVY hydropathicity value around or below -1, were loaded during the dry film rehydration, whereas hydrophobic SLPs were added to the lipid mixture before the formation of the film, since their lipophilic nature led to a by eye extensive aggregation upon mixture with aqueous solvents or physiological buffer (PB).

With regard to the encapsulation of the hydrophilic SLPs into the liposomes, the peptide's pH was adjusted at a value of about 8.5, thus above the isoelectric point (>pI) of most of the peptides where they are expected to be negatively charged. Based on our previous observations (Chapters 3-4, this thesis), this appeared to be of vital importance in order to achieve a high loading efficiency.

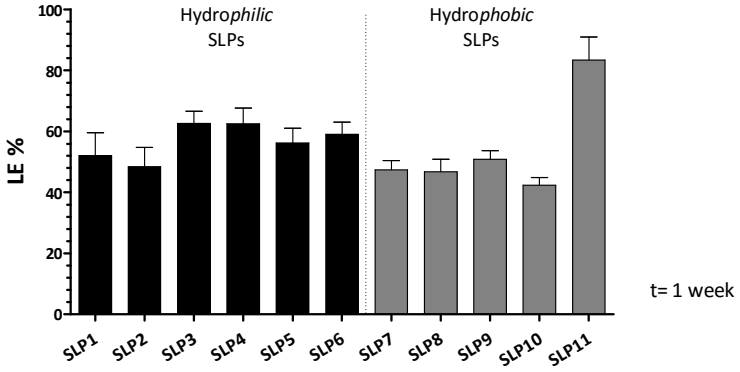

Figure 3: Loading efficiencies of hydrophilic and hydrophobic SLPs into the cationic liposomal formulation, expressed as a percentage based on the initial amount loaded (100 % LE = 1300 μg).

DOTAP/DOPC liposomes were successfully loaded with both hydrophilic and hydrophobic SLPs. Encapsulation of the SLPs yield an excellent loading efficiency above 40 % for all of the peptides, while the SLP11, the most hydrophobic SLP based on its GRAVY value, was the most readily loaded-adsorbed into the liposomes (about 80% LE) (Figure 3).

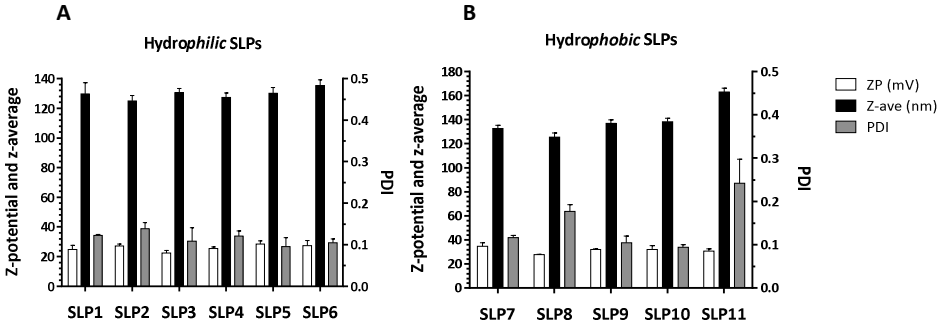

Figure 4: Physicochemical properties of the liposomal formulations loaded with (A) hydrophilic and (B) hydrophobic SLPs. Data are average ± SD of 3 independent batches. *ZP* = zeta-potential measured in mV, *Z-ave* = Z-average particle diameter in nanometres, *PDI* = polydispersity index.

Particles characterization showed that all produced liposomes had a size below 200 nm – around 130 nm – (Figure 4), a relatively low PDI (ca. < 0.3) and a positive zeta potential (ca. 30 mV). Up to at least 8 weeks of storage at 4°C, all the three parameters did not change significantly (results not shown).

The efficacy of the liposomal SLP-formulations to be engulfed by DCs and process the loaded peptide for MHC class I antigen presentation was tested in an *in vitro* immunoassay by activation of SIINFEKL-specific $CD8^+$ T cell hybridoma B3Z. Immature DCs were incubated with free SLPs and SLP-loaded liposomes, and after the removal of

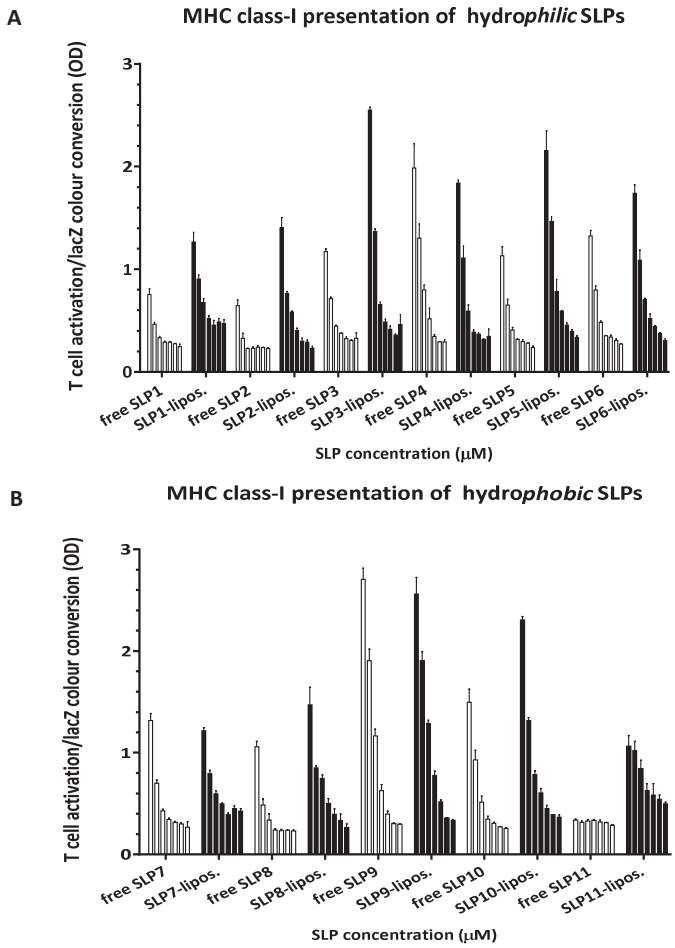

Figure 5: Activation of SIINFEKL-specific B3Z $CD8^+$ T cells after overnight culture with DCs pulsed for 2.5 h with titrated amounts of free SLPs and SLP-loaded formulations (8.0-0.125 μM). Graphs depict T cell activation based on the optical density (OD) at 590 nm measured after colour conversion of cell lysates after addition of CPRG (lacZ substrate) in samples of hydrophilic (A) and hydrophobic (B) SLPs.

the excessed antigen, B3Z cells were added to the culture for overnight incubation.

Figure 5 shows that *in vitro* activation of SIINFEKL-specific $CD8^+$ T cells by liposomes loaded with hydrophilic or hydrophobic SLPs, is similar or improved than the free peptides, showing that the antigenicity of the SLPs after their encapsulation into the liposomes has been retained; and for the lipophilic SLP 11 antigen presentation is strongly enhanced.

Finally, eight weeks after storage of the SLP-loaded liposome at 4°C, their (storage) stability was checked, by diluting the formulations 5 times in PB and then using the Vivaspin columns to separate the released free peptide from the particles. Although, for both hydrophilic and hydrophobic SLP-loaded particles, the size, PDI and z-potential remained comparable, their peptide content was reduced. More specifically after the final quantification, the SLP content in the liposomal formulations varied between 45 and 55% (Figure 6). Therefore, in all liposomes about half of the initial peptide content was still preserved after two months of storage irrespective on the peptide's nature.

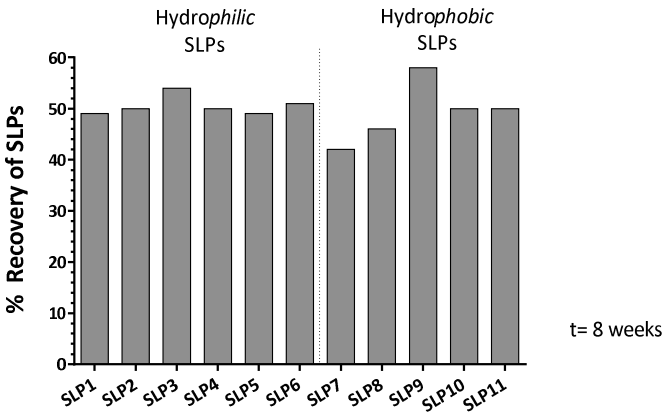

Figure 6: Recovery efficiency of hydrophilic and hydrophobic SLPs in the liposomes measured eight weeks after preparation. SLP recovery is expressed relative to the peptide loading efficiency presented in figure 3.

Discussion

In this study we investigated the potential of our DOTAP-cationic liposomal formulation for the loading of synthetic long peptide antigens and we tested their immunogenicity *in vitro*. Although liposomes have been widely used for the delivery of drugs, therapeutic agents and antigens, the development of a universal formulation for the delivery of both hydrophilic and hydrophobic compounds has not been reported yet. Using a library of 11 SLPs in total, six peptides expected to be hydrophilic and five peptides expected to be hydrophobic according to the obtained GRAVY values, the feasibility of a liposomal-based "platform" was tested.

The backbone of our vaccine delivery system is a DOTAP-based liposomal formulation. It is known that positively-charged liposomes can enhance DC maturation and subsequent T cell priming, and as a result they improve the strength of the vaccine-induced immune response, compared to negative or neutral liposomes [15]. Nevertheless, a second neutral lipid, such as DOPC in this case, with a helper role is required to preserve the stability of the formulation [16].

With regard to the loading of the hydrophilic SLPs, the standard optimized protocol for the preparation and encapsulation into liposomes procedures was followed that requires the adjustment of the SLP's solution to a pH around 8.5. Previously reported data on the DOPC: DOTAP liposomes preparation loaded with the so-called OVA24 peptide showed that a decrease of the SLP's pH below its isoelectric point led to a significant reduction of the loading efficiency [17], suggesting that the peptide entrapment in the cationic liposomes is significantly dependent on electrostatic interactions. In addition to that, the importance of the DOTAP-cationic formulation can be pointed out, since negative or neutral liposomes would negatively influence the entrapment of the hydrophilic SLPs, as we have previously observed with other SLPs.

In contrast, for the loading of the expected hydrophobic SLPs a different preparation procedure had to be followed, especially due to the lipophilic character of the peptides that did not allow their mixture with the ACN: H_2O solution. As a result, the SLPs had to be added with the lipid mixture before the dry film formation, allowing them to be readily entrapped in the lipid bilayer. In contrast, hydrophilic peptides will more likely be encapsulated in the liposomes' aqueous core and/or associated with the outer part of the lipid membrane.

Interestingly, although it would be expected that the different preparation method for the loading of the (hydrophilic and hydrophobic) SLPs would influence the

physicochemical properties of the particles, we did not observe any significant difference between the hydrophilic and hydrophobic-loaded liposomes, regarding their zeta-potential, size and polydispersity index. Furthermore, the loading efficiency did not vary significantly among the different SLPs, being around 50% for all of them, apart from the extreme hydrophobic SLP11 that yielded a LE around 80%. The reason for this improved loading efficiency that was not further investigated is not really clear; however, it could be speculated that is due to the strong hydrophobic forces between particles and the SLP11. Moreover, SLP11 showed an improved *in vitro* capacity to be processed and presented by the pulsed DCs to antigen-specific $CD8^+$ T cells compared to free SLP: SLP11 might be poorly soluble in saline buffer and therefore appears to be more antigenic when adsorbed into the liposomes. In this case, liposomes may be an advantage for extremely hydrophobic antigenic peptide sequences. The rest of the SLPs showed a comparable or better efficiency between the free and the liposomal-loaded SLP, result indicating that the functionality of the SLPs is maintained after their association with the liposomes and that their antigenicity is independent of their hydrophilic or hydrophobic nature.

Although further studies and *in vivo* data are needed to validate the immunogenicity of multiple peptide liposomes, the data support the feasibility of this platform for the delivery of SLPs with varying physicochemical properties. This is important especially for the design of a vaccine where multiple overlapping peptides are needed. As it has been previously reported, the clinical product used for the treatment of vulvar intraepithelial neoplasia consisted of 13 HPV E6/E7-derived SLPs, all of them covering a wide range of pIs (3.76 – 11.54) and hydropathicity (-1.587- +0.466) (supplementary table II), comparable to the ranges of the eleven model peptides used in our study. Provided the superior immunogenicity of liposomes compared to the Montanide ISA-51, vehicle which is used in the latter study, we propose the use of cationic liposomes loaded with SLPs as a flexible platform for therapeutic vaccination-based immunotherapy.

Conclusions

In this study, we described the efficient loading of eleven model SLPs in cationic liposomes composed of DOTAP and DOPC. The SLP-loaded liposomes were physicochemically characterised and considering the in *vitro* immunogenicity assay results we showed that the same liposomal formulation can be used for the accommodation of both hydrophilic and hydrophobic peptides, resulting in a particulate delivery system that can be used as a vaccine delivery platform for multiple antigenic peptides.

References

1. Leroux-Roels, G., *Unmet needs in modern vaccinology: adjuvants to improve the immune response.* Vaccine, 2010. 28 Suppl 3: p. C25-36.
2. 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.
3. Hamdy, S., et al., *Targeting dendritic cells with nano-particulate PLGA cancer vaccine formulations.* Adv Drug Deliv Rev, 2011. 63(10-11): p. 943-55.
4. Foged, C., J. Hansen, and E.M. Agger, *License to kill: Formulation requirements for optimal priming of CD8(+) CTL responses with particulate vaccine delivery systems.* Eur J Pharm Sci, 2012. 45(4): p. 482-91.
5. Mbow, M.L., et al., *New adjuvants for human vaccines.* Curr Opin Immunol, 2010. 22(3): p. 411-6.
6. Rappuoli, R., *Bridging the knowledge gaps in vaccine design.* Nat Biotechnol, 2007. 25(12): p. 1361-6.
7. Cai, S., et al., *Lymphatic drug delivery using engineered liposomes and solid lipid nanoparticles.* Adv Drug Deliv Rev, 2011. 63(10-11): p. 901-8.
8. Al-Jamal, W.T. and K. Kostarelos, *Liposomes: from a clinically established drug delivery system to a nanoparticle platform for theranostic nanomedicine.* Acc Chem Res, 2011. 44(10): p. 1094-104.
9. Ben-Yehuda, A., et al., *Immunogenicity and safety of a novel liposomal influenza subunit vaccine (INFLUSOME-VAC) in young adults.* J Med Virol, 2003. 69(4): p. 560-7.
10. Slutter, B., et al., *Adjuvant effect of cationic liposomes and CpG depends on administration route.* J Control Release, 2011. 154(2): p. 123-30.
11. Kenter, G.G., et al., *Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia.* N Engl J Med, 2009. 361(19): p. 1838-47.
12. Linnemann, C., et al., *High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma.* Nat Med, 2015. 21(1): p. 81-5.
13. Kreiter, S., et al., *Mutant MHC class II epitopes drive therapeutic immune responses to cancer.* Nature, 2015. 520(7549): p. 692-6.
14. Sanderson, S. and N. Shastri, *LacZ inducible, antigen/MHC-specific T cell hybrids.* Int Immunol, 1994. 6(3): p. 369-76.
15. Ma, Y., et al., *The role of surface charge density in cationic liposome-promoted dendritic cell maturation and vaccine-induced immune responses.* Nanoscale, 2011. 3(5): p. 2307-14.
16. Campbell, R.B., S.V. Balasubramanian, and R.M. Straubinger, *Phospholipid-cationic lipid interactions: influences on membrane and vesicle properties.* Biochim Biophys Acta, 2001. 1512(1): p. 27-39.
17. Varypataki, E.M., et al., *Cationic liposomes loaded with a synthetic long Peptide and poly(i:C): a defined adjuvanted vaccine for induction of antigen-specific T cell cytotoxicity.* Aaps J, 2015. 17(1): p. 216-26.
18. Varypataki, E.M., et al., *Synthetic long peptide based vaccine formulations for induction of cell mediated immunity: a comparative study of cationic liposomes and PLGA nanoparticles*. J Control Release, 2016. 226: p. 98-106.

Supplementary data

Supplementary table I: The properties of the SLPs that were excluded from the study *

	Peptide sequence NBD-G-XXXXXXXXXXX-SIINFEKLAAAK	Theoretical pI	Hydropathicity (GRAVY)
	Hydro*phobic* SLPs		
SLP 12	GSAAESASGSA	6.14	0.126
SLP 13	RDKSLKELLSA	9.53	-0.113
SLP 14	ELIDIIDIEIA	4.18	0.796
SLP 15	DLKLADLLALA	6.12	0.771

*the freeze-dried product of the above SLPs was not soluble in ACN:H_20, or $CHCl_3$: MeOH: H_2O or pure DMSO (+ TFA) even when low concentrations were tried

Supplementary table II: The properties of the 9 HPV16-E6 and 4 HPV16-E7 used in the clinic [11]

	Peptide sequence	Theoretical pI *	Hydropathicity (GRAVY) *
	Hydro*philic* SLPs		
E71-35	MHGDTPTLHEYMLDLQPETTDLYCYEQLNDSSEEE	3.76	-1.100
E722-56	LYCYEQLNDSSEEEDEIDGPAGQAEPDRAHYNIVT	3.76	-1.091
E61-32	MHQKRTAMFQDPQERPRKLPQLCTELQTTIHD	8.00	-1.250
E6109-140	RCINCQKPLCPEEKQRHLDKKQRFHNIRGRWT	10.08	-1.587
E6127-158	DKKQRFHNIRGRWTGRCMSCCRSSRTRRETQL	11.54	-1.569
	Hydro*phobic* SLPs		
E619-50	LPQLCTELQTTIHDIILECVYCKQQLLRREVY	5.53	0.056
E691-120	YGTTLEQQYNKPLCDLLIRCINCQKPLCPEEK	6.23	-0.591
E764-98	TLRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP	6.41	0.466
E685-109	HYCYSLYGTTLEQQYNKPLCDLLIR	6.73	-0.400
E743-77	GQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIR	6.89	-0.277
E641-65	KQQLLRREVYDFAFRDLCIVYRDGN	8.17	-0.536
E655-80	RDLCIVYRDGNPYAVCDKCLKFYSKI	8.59	-0.165
E671-95	DKCLKFYSKISEYRHYCYSLYGTTL	8.71	-0.536

*SLPs properties were calculated as previously according to the online ExPASy tool