Liposomes as delivery system for allergen-specific immunotherapy

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Chapter 2
Enhanced immunogenicity of recombinant Bet v 1 through combined encapsulation in and adsorption to cationic liposomes

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Abstract

Liposomes are a commonly used vaccine adjuvant and an interesting candidate to replace aluminum hydroxide (alum) in allergy vaccines. Antigens are often either encapsulated in the aqueous core of liposomes, or adsorbed to the lipid surface. We have evaluated the effect of association method on the availability of antigen for IgE binding and the ability to induce an immune response.

Increasing amounts of Bet v 1, a recombinant version of the major allergen in birch pollen allergy, were formulated with cationic liposomes, either adsorbed or encapsulated, or a combination of both. Upon increasing the Bet v 1/lipid ratio, liposome size increased slightly, and signs of aggregation were visible starting at a protein/lipid mass ratio of 0.15. With increasing Bet v 1 concentration, the association efficiency decreased. Encapsulated allergen was approximately 8-fold less effective at binding IgE than Bet v 1 in buffer, as determined by ImmunoCAP inhibition. Bet v 1 adsorbed to or encapsulated in cationic liposomes was able to induce an antigen-specific IgG1 response, but liposomes with both encapsulated and adsorbed Bet v 1 resulted in a stronger IgG1 and response as well as a stronger cytokine production upon stimulation.

In conclusion, encapsulation of Bet v 1 resulted in the most hypo-allergenic formulation. The combination of adsorption and encapsulation resulted in the most efficient antigen association to cationic liposomes, as well as the strongest immune response.

Introduction

Subcutaneous allergen immunotherapy (SCIT) has been used to treat allergies for more than 100 years [1]. During immunotherapy, the ongoing immune response is redirected from a T helper (Th)2 response towards a response that suppresses Th2-driven allergy symptoms by induction of Th1 or regulatory Th cells (Treg). The treatment usually consists of weekly subcutaneous injections of allergen extracts in the build-up phase and monthly injections in the maintenance phase and requires 3-5 years to achieve sustained therapeutic effect [2]. The long duration of the therapy and frequent (local) side effects are associated with low therapy adherence [3].

In mice, the desired protective immune response has been reported to be characterized by IL-10 and IgG2a, a mixed Treg/Th1 response [4, 5]. Apart from being an adjuvant, adsorption to aluminium hydroxide (alum) also decreases the access of IgE to allergen, which results in less side-effects [6]. An alternative adjuvant should ideally also contribute to achieving a similar or preferably higher degree of hypo-allergenicity, in order to prevent allergic adverse events. Many innovative ideas are explored to improve the efficacy and safety of SCIT, among which recombinant hypoallergenic allergens and new adjuvants [7]. Nanoparticles such as liposomes are an example of a new adjuvant that could replace alum [8].

Liposomes consist of at least one lipid bilayer and an aqueous core and are a versatile delivery system and adjuvant for vaccines [9-11]. The versatility is related to the large variety of synthetic and natural (phospho)lipids that are available and can be incorporated in the lipid bilayer [10, 12, 13]. Cationic liposomes are considered to be taken up more efficiently by antigen-presenting cells (APCs) than neutral or anionic counterparts, which is ascribed to the ability to interact with anionic cell surfaces [14, 15]. As uptake in APCs is a crucial first step to induce an immune response, cationic, rather than anionic, liposomes are often used in combination with an antigen for vaccination against a wide variety of diseases [11, 16, 17].

For vaccination purposes, antigens (= allergens in case of allergy vaccines) are commonly associated with liposomes in either of the following two ways: via adsorption to the surface of the liposome or by encapsulation in the aqueous core of the vesicle [18]. Association to the liposome surface is an easy method, in which antigen and pre-formed liposomes are mixed. The efficiency of adsorption, however, depends on the physicochemical properties of the antigen and the liposomes and often relies on electrostatic interactions [19, 20]. Upon *in vivo* administration, however competition with endogenous compounds, such as salts and proteins, may lead to rapid antigen desorption from the liposome

[21, 22]. Encapsulation in the aqueous core ensures that antigen and liposomes stay associated for a longer time *in vivo*. However, the manufacturing is often a laborious and inefficient process, in which both precious antigen and liposomes can be lost [16].

In order to assess the effect of association method on the induced immune response, we formulated Bet v 1, the main allergen in birch pollen allergy [23], with cationic liposomes via adsorption onto the surface of the lipid bilayer, encapsulation in the aqueous core, or a combination with Bet v 1 both encapsulated and adsorbed. We assessed the effect of allergen concentration in the initial formulation on particle size, zeta potential and final allergen association. Subsequently, we immunized mice with 10 µg bet v 1 in the different liposomal Bet v 1 formulations and compared the immune response to that of Bet v 1 adsorbed to alum (Bet v 1-alum). We observed that liposomal Bet v 1 formulations induced stronger antibody responses than Bet v 1-alum. The cationic liposome formulation with Bet v 1 both adsorbed on the surface and encapsulated in the core induced the strongest humoral immune response.

Materials & Methods

Chemicals and reagents

Cholesterol (CHOL), 1,2-distearoyl-*sn*-glycero-3-phosphocoline (DSPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Lipids. Sucrose, HEPES and sodium azide were obtained from Sigma Aldrich, and aluminum hydroxide (Imject ® Alum) from Thermo Scientific. Recombinant Bet v 1 (isoform Bet v 1.0101) was purchased from the Department of Molecular Biology of the University of Salzburg (Salzburg, Austria).

Preparation of liposome formulations

The liposomes consisted of DSPC, DOTAP and cholesterol in a 2:1:1 molar ratio. These lipids were dissolved in chloroform and mixed in the desired ratio. Subsequently, the organic solvent was evaporated in a rotary evaporator at 37 ° C and 180 mbar, leaving a lipid film. This film was hydrated at 37 ° C in the presence of glass beads with 1 mL of 10 mM HEPES (pH 7.4), 280 mM sucrose buffer (H/S buffer). After hydration, the suspension was snap-frozen and lyophilized resulting in a fluffy cake. The lipid cake was rehydrated at 37 ° C with filtered Milli-Q water to a final volume of 2 mL and homogenized by using a LIPEX extruder (Evonik, Canada) over a stacked 400-nm and 200-nm Nucleopore Track-Etch membrane (Whatman, the Netherlands).

For adsorbed Bet v 1, liposomes were prepared as described above. To adsorb Bet v 1, liposomes were mixed with varying amounts of Bet v 1 and incubated at

ambient temperature for at least 15 minutes prior to sample analysis or injection.

Encapsulated Bet v 1 liposomes were prepared as described above, but varying amounts of Bet v 1 were dissolved in the H/S buffer in the hydration step. After preparation, any free and bound Bet v 1 was removed with centrifuge membrane concentrators. Three repeated wash-steps were performed, in which the liposomes were concentrated approximately 5-fold, reconstituted with H/S to the original volume, and unbound fractions were collected.

For a combination of adsorbed and encapsulated Bet v 1, liposomes were prepared as for encapsulated Bet v 1, with varying amounts of Bet v 1 in the H/s buffer in the hydration step. Here, however, no purification step was performed to remove free and bound Bet v 1. The differences between these formulations are schematically depicted in Figure 1.

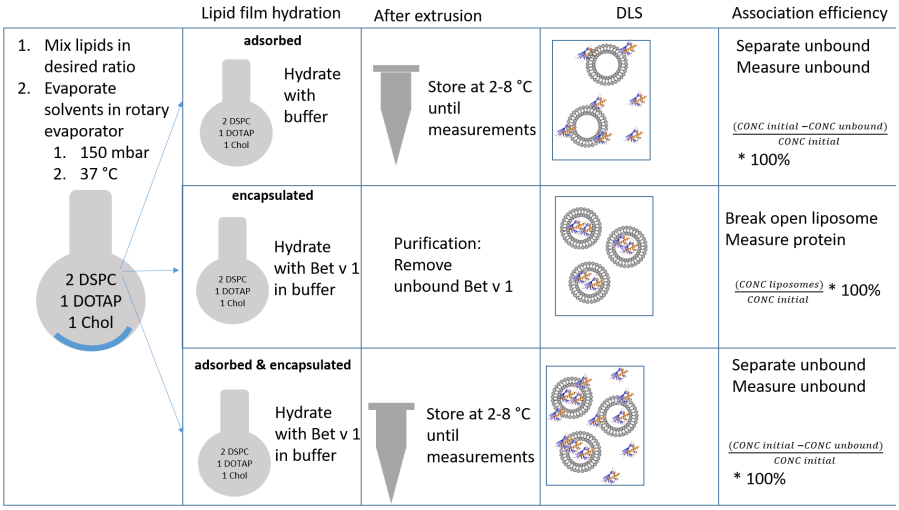

Figure 1. Overview of the different formulations. The differences in the preparation process and schematically what is measured in the characterization. For adsorbed Bet v 1, no antigen is added in the liposome preparation. Only liposomes with encapsulated Bet v 1 were purified directly after they were formed in the extrusion process. For adsorbed and adsorbed and encapsulated formulation, there is also a fraction of unbound protein present.

Liposome characterization

Hydrodynamic diameter (Z-average diameter) and polydispersity index were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The zeta potential was measured using laser Doppler electrophoresis (lDe) on the same machine with a Zeta Dip Cell (Malvern Instruments Ltd.). Each sample was diluted 100-fold in 10 mM HEPES buffer (pH 7.4, 0.2-µm filtered) before measurement.

Determination of association efficiency

To determine the association efficiency of the different formulations, unbound protein was separated from liposomes with centrifuge membrane concentrators (Vivaspin2, 300.000 MWCO, Sartorius) by spinning down at 500 x g at 5 °C until concentrated approximately 5-fold. The flow through, which contained unbound Bet v 1 was collected, while the concentrate was diluted to the original volume with H/S buffer.

For liposomes with Bet v 1 adsorbed, the unbound fraction was removed as described above. The unbound fraction was collected and used to determine the percentage of unbound protein. Adsorption efficiency was calculated as:

$$Adsorption\ efficiency = \frac{(CONC\ initial - CONC\ unbound)}{CONC\ initial} * 100\%$$

For encapsulated Bet v 1, the liposomes were purified directly after the extrusion step. The unbound protein was separated as described above. This process was repeated 3 times, to ensure that > 99% of unbound protein was removed. Subsequently, the liposomes were dissolved in methanol and the protein content was determined as described below. Encapsulation efficiency is calculated as:

$$Encapsulation\ efficiency = \frac{CONC\ in\ liposomes}{CONC\ initial} * 100\%$$

For the combination of encapsulated and adsorbed Bet v 1, the association efficiency was determined as for adsorbed Bet v 1. The unbound fraction was removed as described above and the protein content of this fraction was determined. Subsequently, the association efficiency was calculated:

$$Association\ efficiency = \frac{(CONC\ initial - CONC\ unbound)}{CONC\ initial} * 100\%$$

Protein concentration determination

Protein concentration was determined with a Micro BCA assay kit (Boster Biological Technology, Pleasanton, CA, USA) according to the manufacturer's protocol. This kit was chosen as it is compatible with methanol. Flow through fractions after separation with Vivaspin2 columns were measured without sample preparation. For encapsulated fraction, a modified Bligh-Dyer extraction was performed as described previously [24]. In short, 100 µL of liposome suspension were mixed with 250 µL of methanol and 125 µL of chloroform. The mixture was vigorously vortexed for 10 seconds before 125 µL of chloroform and 250 µL of 0.1 M HCl were added to the mixture. The mixture was vigorously vortexed for 20 seconds and centrifuged during 5 minutes at 2100 rpm and room temperature. After centrifugation, the upper phase (methanol-water), containing the Bet v 1, was collected and its protein content was determined.

ImmunoCAP inhibition assay

The IgE binding potency of various liposome formulations was determined by ImmunoCAP IgE inhibition assay [25]. To that end, liposomes were serially diluted (dilution factor 10) in 10 mM HEPES, 280 mM sucrose, pH 7.4. A pool of 36 sera from birch pollen allergic patients (from a reference serum bank at AMC [26, 27]) was diluted to 12 kU/mL of specific IgE against Bet v 1 and added 1:1 (v/v) to all serial dilutions, followed by incubation at room temperature for one hour. Uncomplexed IgE in the samples was measured on a Phadia-250 machine (ThermoFisher Scientific, Uppsala, Sweden) loaded with rBet v 1 ImmunoCAPs (catalogue code t215, ThermoFisher Scientific). The percentage inhibition was calculated on a scale from 100% inhibition (no serum) to an 0% inhibition (PBS + serum). The concentration at which 50% inhibition occurred (IC50) was determined by non-linear regression fit with variable slope (4 parameters: no restriction for top, bottom was set to 0, Hill coefficient was set to "shared for all data sets", and IC50 must be greater than 0).

In vivo immunogenicity

Mice were immunized subcutaneously at day 0, 7 and 14 with 10 µg Bet v 1, as determined by BCA, in various formulations containing liposomes or alum (1 mg per injection). Serum for antibody detection was collected at days -1, 6, 13 and 20. At day 27, 28 and 29 the animals received an intranasal challenge under 3% (v/v) isoflurane anesthesia with 100 µg/mL birch pollen extract (BPE, HAL Allergy, Netherlands) in PBS to induce lung inflammation. On day 31, the mice were sacrificed, and blood was collected to analyze Bet v 1 specific levels of IgG1 and IgG2a in serum. Moreover, lung draining lymph nodes were collected to determine the production of IL-4, IL-5, IL-13, IL-10 and IFN-γ cytokines after stimulation with Bet v 1.

Determination of Bet v 1 specific antibodies

Serum was analyzed by ELISA for the level of Bet v 1-specific IgG1 and IgG2a (IgG1: Opteia, BD, San Diego, CA, USA, IgG2a: eBioscience) as previously described [4]. In short, Maxisorp plates were coated with recombinant Bet v 1 overnight and subsequently washed. After blocking with 10% fetal calf serum, serum samples were diluted 100-fold, prior to addition to the microtiter plate and incubated for 2 hours at room temperature. Subsequently, detecting biotin-conjugated antibodies specific for IgG1 or IgG2a, respectively, were added, followed by an streptavidin-HRP and TMB substrate detection step. Coloring reaction was stopped by addition of H_2SO_4, according to the manufacturer's instructions.

Ex vivo re-stimulation of lung draining lymph node cells

Lung draining lymph node cell suspensions were plated in a 96-well round bottom plate at a density of 2 x 10^5 cells per well in RPMI supplemented with gentamicin,

fetal calf serum and β-mercaptoethanol. The cells were re-stimulated for 4 days with 10 µg/mL recombinant Bet v 1. Expression levels of cytokines IL-4, IL-5, IL-10, IL-13 and IFN-γ were determined in the supernatant by ELISA (eBioscience).

Statistics

Data was processed and analyzed in GraphPad v8 (Prism) for Windows. Statistical analysis was performed with the same program and the method of analysis is indicated in the figure legends.

Results

Increasing amounts of Bet v 1 adsorbed to cationic liposomes ultimately results in aggregation

The association method of Bet v 1 with liposomes may affect the colloidal stability of the liposomes. Therefore, we evaluated how Bet v 1 loading of liposomes via adsorption or encapsulation affected the size and charge of liposomes. Empty liposomes consisting of DSPC, DOTAP and cholesterol had a hydrodynamic diameter of circa 200 nm and a zeta potential of > 35 mV. Mixing of increasing

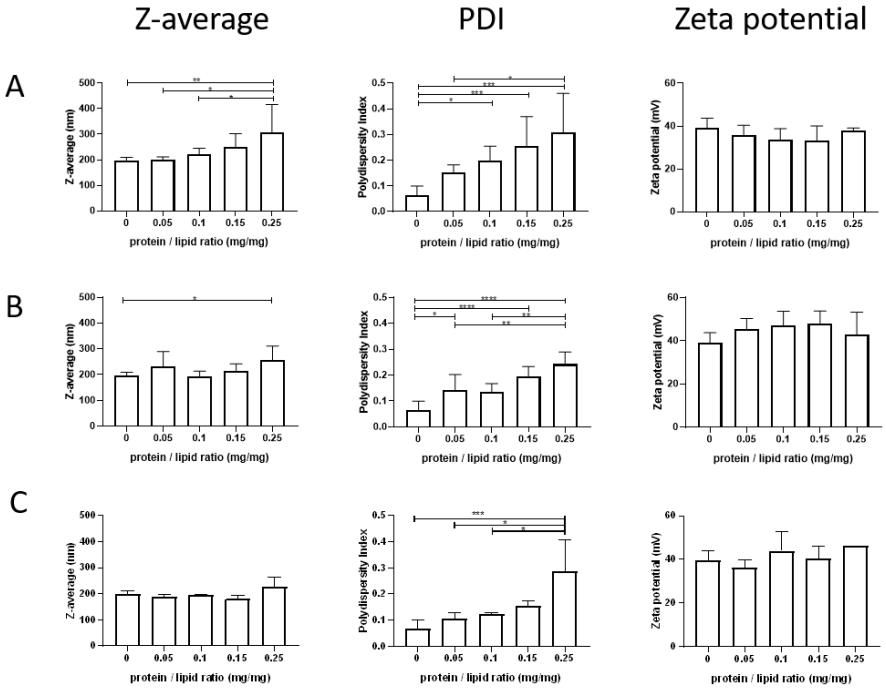

*Figure 2. Liposome properties: Z-average diameter (left column), polydispersity index (middle column) and zeta potential (right column) of liposomes with increasing amounts of Bet v 1 adsorbed (A) or encapsulated (B) or both adsorbed and encapsulated (C). Mean values with standard deviations are plotted (n=2-6). Means were compared with a 1-way ANOVA and Tukey's multiple comparison post-test. * = p < 0.05, ** p = < 0.01, *** = p < 0.001, **** = p < 0.0001.*

amounts of Bet v 1 (resulting in final Bet v 1 concentrations of 65 – 325 µg/mL) with empty liposomes (final concentration 1.25 mg lipid/mL) resulted in a slightly increased size and polydispersity index, while the zeta potential was not significantly changed (Figure 2A). While the size and zeta potential did not vary with increasing amounts of Bet v 1 encapsulated, the polydispersity index increased in a similar manner as for adsorption (Figure 2B). For a combination of adsorbed and encapsulated Bet v 1, the only changes observed were in the polydispersity index (Figure 2C). Although the effect on size seems low, from a protein/lipid ratio between 0.10 and 0.15 a second peak is visible in the size-intensity plot after adsorption, encapsulation and the combination of both (Supplementary Figure 1, 2 and 3 respectively), suggesting that some aggregation had occurred.

Increasing amounts of Bet v 1 results in decreased association efficiency, but increased amount of associated antigen

Next, we set out to investigate how increasing Bet v 1/lipid ratios affects the allergen association efficiency with liposomes for all formulations. Regardless of association method, more Bet v 1 was associated to liposomes with increasing amounts of initial Bet v 1 (Figure 2). The association efficiency, however, was decreased as more Bet v 1 was added for adsorption (Figure 2A) and encapsulation (Figure 2B), while the association efficiency was constant for the combination of encapsulation and association (Figure 2C). To have the highest possible allergen-association, without any visible aggregation, subsequent formulations for immunoCAP and the animal study were prepared with a protein/lipid ratio of 0.1 (w/w).

Bet v 1 encapsulation in cationic liposomes decreases IgE binding

In allergic patients, binding of IgE to Bet v 1 can cause potentially severe side effects. An ImmunoCAP assay was performed to evaluate whether the different association methods had effect on the IgE binding potency of Bet v 1. Adsorbed Bet v 1 should be accessible for IgE binding, while encapsulated Bet v 1 theoretically should not, unless liposomes break open or allergen can leak out. As shown in Figure 3, Bet v 1 adsorbed to liposomes bound IgE in a similar, dose-dependent manner as free Bet v 1. Bet v 1 which was encapsulated in liposomes showed an 8-fold reduction in IgE binding. This suggests that not all Bet v 1 has been removed from the outside of the liposomes. Based on figure 3, the liposomes with both encapsulated and adsorbed Bet v 1, should have approximately 50% of the Bet v 1 encapsulated, and 50% available for binding IgE. The IgE inhibition of liposomes with adsorbed and encapsulated Bet v 1 showed an inhibition curve in between that of adsorbed and encapsulated Bet v 1 (Figure 4).

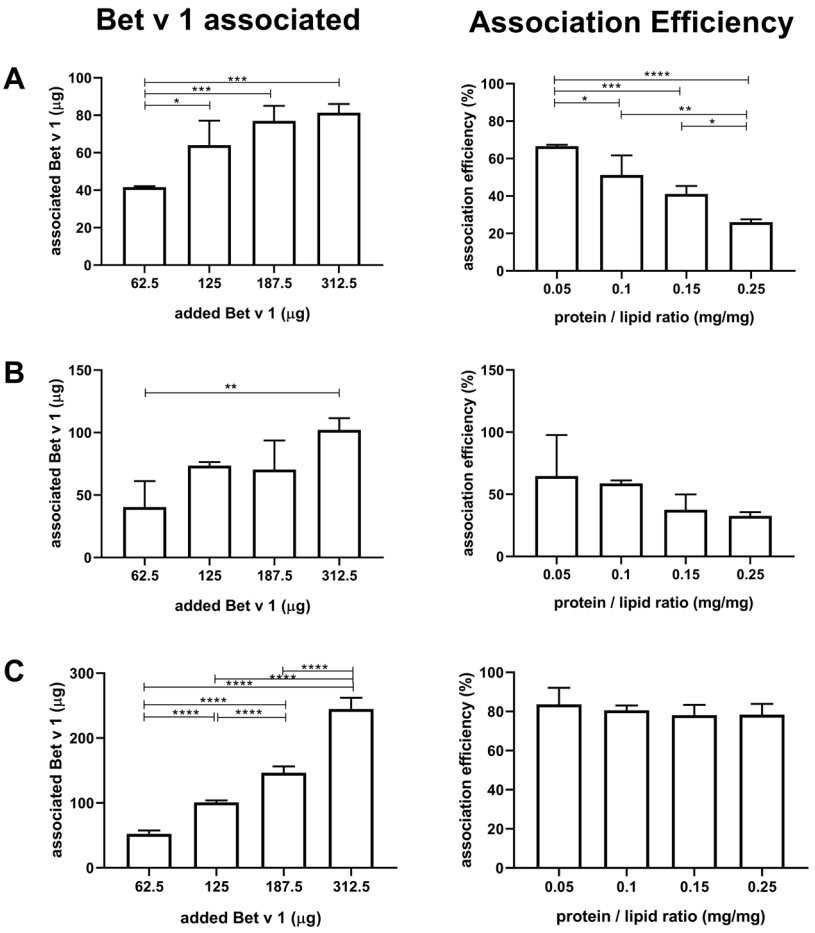

*Figure 3. Total amount of antigen associated to liposomes (left panel) and association efficiency (right panel) after increasing amounts of Bet v 1 were adsorbed to (A), encapsulated in (B), or adsorbed to and encapsulated in (C) liposomes. Mean values with standard deviations are plotted (n=2-6). Means were compared with a 1-way ANOVA and Tukey's multiple comparison post-test. * = p < 0.05, ** p = < 0.01, *** = p < 0.001, **** = p < 0.0001.*

Cationic liposomes with Bet v 1 on surface and in core induced strongest IgG1 response in mice

To assess the impact of the method of allergen association to liposomes on immunogenicity, mice were immunized with 10 µg of Bet v 1 in the different liposome formulations. The liposomal Bet v 1 formulations were compared to a formulation of Bet v 1 adsorbed to alum. Bet v 1-specific IgG1, IgG2a and IgE were measured in the mouse sera at the end of the experiment. At the (100-fold) dilution used for IgG detection, liposomes without allergen and alum-adsorbed Bet v 1 did not induce detectable antigen-specific antibodies (Figure 5A). In contrast, all liposomal Bet v 1 formulations induced antigen-specific IgG1.

The liposomal formulation in which Bet v 1 was both encapsulated and adsorbed induced the strongest IgG1 response (4 out of 5 responders). None of the tested formulations induced detectable amounts of Bet v 1-specific IgG2a or IgE.

	Bet v 1	Adsorbed	Encapsulated	Adsorbed + encapsulated
IC50	0.001700	0.002247	0.01225	0.006104

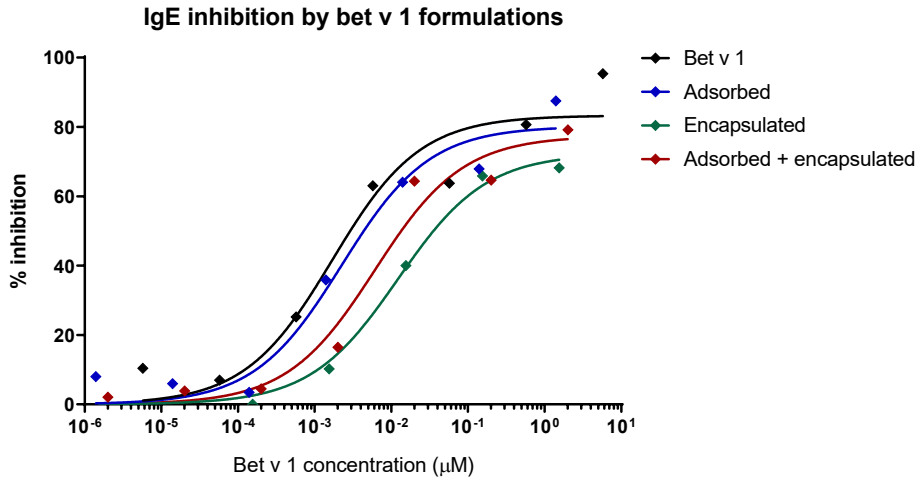

Figure 4. The ability of free Bet v 1 (black), or Bet v 1 adsorbed to (blue), encapsulated in (green), both adsorbed to and encapsulated in (red) liposomes to bind patient-derived Bet v 1 specific IgE.

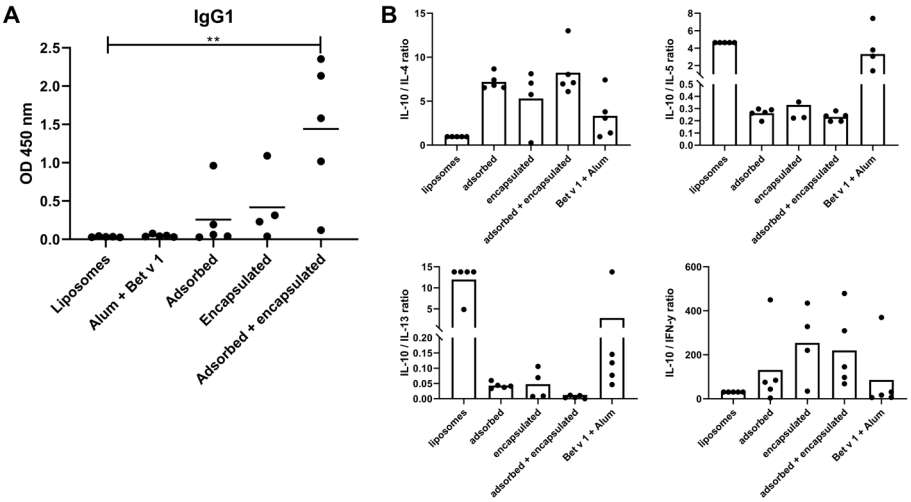

*Figure 5. A. Bet v 1-specific IgG1 in serum after immunization of mice with Bet v 1 associated to liposomes in different ways. Group mean and standard deviation are plotted (n = 4-5 mice/ group). B. Production of IL-4, IL-5, IL-10, IL-13 and IFN-γ by lung draining lymph node cells from immunized mice after exposure to Bet v 1. Group mean and standard deviation are plotted (n = 4-5 mice/group). Groups were compared with a Kruskal-Wallis test followed by Dunn's correction for multiple comparisons. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.*

After sacrifice, lung draining lymph nodes were isolated and the production of cytokines by lymph node cells was measured after exposure to Bet v 1. The cytokine production trend followed that of the antibody responses. No cytokines were detected for the groups immunized with liposomes alone, or alum-adsorbed Bet v 1. The highest IL-5, IL-10 and IL-13 levels were observed after immunization with a combination of adsorbed and encapsulated Bet v 1, while encapsulated Bet v 1 resulted in higher cytokine levels than adsorbed Bet v 1 (Supplementary Figure 4), except for IFN-γ, which had low levels in all groups. In cytokine ratios (IL-10 / IL-4, IL-5, IL-13 and IFN-γ) all liposome formulations induce the same pattern compared to liposomes without antigen or alum-adsorbed Bet v 1 (Figure 5B). The IL-10/IL-4 ratio is increased compared to empty liposomes or alum-adsorbed Bet v 1, while the IL-10/IL-5 and IL-10/IL-13 ratio is decreased. The IL-10/IFN-γ ratio is very high in all groups, as very little IFN-γ was detected.

Discussion

In this study we set out to explore the effect of allergen association method on liposome properties and immunogenicity of the allergen (Bet v 1). Allergen adsorption to liposomes is most efficient with opposite charges, which happens in the case of cationic liposomes with negatively charged Bet v 1 (isoelectric point = 5.4 [28]). The adsorption of anionic model antigens (α-lactalbumin, ovalbumin and bovine serum albumin) to cationic liposomes has been reported to result in up to almost 100% adsorption efficiencies. There is a maximum antigen adsorption capacity of liposomes and a threshold above which aggregation starts occurring, which depends on properties of both protein and formulation. The aggregation which was detected for Bet v 1/lipid ratios larger than 0.1-0.15 (w/w) is in line with previous studies [19, 20]. The association efficiency increased with decreasing antigen / lipid ratio, which is in line with other studies [29].

Successful removal of unbound Bet v 1 in encapsulated Bet v 1 was confirmed by the results of the ImmunoCAP assay. While adsorbed Bet v 1 resulted in practically the same IgE binding as Bet v 1 in buffer, encapsulated Bet v 1 resulted in an 8-fold lower IgE binding. This suggests that approximately 12% of all Bet v 1 was present on the surface of the liposomes or had leaked out under the assay conditions. The presence of adsorbed Bet v 1 on the liposome surface might be an inevitable consequence of the formulation process because of incomplete removal of adsorbed Bet v 1 despite multiple washing steps. In the formulation where the washing steps were omitted (adsorbed + encapsulated, figure 4), the IC50 of IgE binding was in between the IC50 of liposomes with adsorbed and encapsulated Bet v 1, respectively. This is in line with the Bet v 1 encapsulation efficiency of approximately 50% that was found for a 0.1 antigen / lipid ratio. With regard to safety, encapsulated antigen would be the preferred option over

adsorption, or a combination, as approximately 8-fold more Bet v 1 was required to achieve a similar IgE binding.

Formulations with adsorbed allergen also had a fraction of unbound, free allergen. Based on the ImmunoCAP assay, even the formulation which was washed repeatedly to remove any bound and free Bet v 1, was able to bind Bet v 1-specific IgE, albeit to a lesser extent than adsorbed Bet v 1. This unbound fraction is not likely to interfere in DLS measurements, as proteins are much smaller than liposomes and therefore will hardly contribute to the light scattering signal [30]. The unbound fraction is definitely found in the ImmunoCAP assay, where also surface-adsorbed Bet v 1 seems to be detected as free Bet v 1. This unbound fraction may be problematic when applying these formulations for immunotherapy, where free allergen is associated with side effects [5, 31]. The unbound Bet v 1 is however not expected to contribute significantly to the induced immune response, as free Bet v 1 has previously shown to not induce an immune response in naïve mice [data not shown, Leboux et al., Chapter 6].
The immune responses that were reported in this manuscript were unexpected, both for the cationic liposomes as for aluminium hydroxide. Cationic liposomes reportedly induce a strong Th1 skewed immune response, of which antigen-specific IgG2a and IFN-y are hallmarks [10, 11, 32]. Surprisingly, no IgG2a induction in mice by any of the liposome formulations was observed. Moreover, barely any IFN-y was detected after stimulation of lung draining lymph node cells *ex vivo*. This poor IFN-y production is not in line with previous observations [33, 34]. However, it is important to note that others used spleen-derived cells while in this study lung draining lymph node cells were used, because a strong response in these cells was expected after the intranasal boost that was administered at the end of the experiment. These cells produced large quantities of IL-5, IL-10, IL-13, and to a lesser extent IL-4, which are signature cytokines for a Th2-skewed response [35].

The weak response in mice that received alum-adsorbed Bet v 1 was also unexpected. In several reports, alum-adsorbed Bet v 1 has been described to induce a strong, allergy-like pathology in mice [4, 36-38]. For this purpose however, the formulation is typically administered intraperitoneally (i.p.). It has been reported that subcutaneous administration of alum-adsorbed Bet v 1 is less efficient at inducing antigen-specific antibodies than i.p. administration [38, 39]. Perhaps another dosing regimen, or increased allergen dose per injection would have increased the immune response. Altogether, the formulations used in this manuscript did not induce an immune response which is associated with relief of allergy symptoms in mice [4], but give insight into the effect of antigen association method on the induced immune response.

Similarly to the results presented in this manuscript, no significant differences were found between either adsorption or encapsulation of influenza antigens with cationic liposomes in a previous study [40], but a combination of both methods was not explored. Tetanus toxoid either mixed or encapsulated (but not purified) in liposomes in both cases induced similar IgG1, IgG2a and IgG2b levels. The antibody response lasted longer in mice injected with liposomes that contained encapsulated antigen. The combination of adsorption and encapsulation, as tested in our study, may induce the strongest response because there is more and potentially longer antigen exposure. First, surface antigen is (partially) desorbed from the liposomes and can quickly migrate away from the injection site [19, 21]. Subsequently, cationic liposomes containing antigen remain at the injection site and are removed by APCs that process and present antigen fragments as well. This may be beneficial, because a humoral response requires intact antigen (quickly desorbed antigen), which is enhanced by helper T-cell stimulation with antigen derived peptides (from processed encapsulated antigen) [41]. The same mechanism is assumed for colloidal aluminum salt-based adjuvants, which slowly release antigen from the injection site [42].

In conclusion, we have shown that preparing cationic liposomes with increasing amounts of Bet v 1 results in more association to liposomes, but a lower association efficiency. Bet v 1 causes aggregation between an antigen/lipid ratio of 0.10 and 0.15 (w/w) regardless of association method. Encapsulation of Bet v 1 in liposomes seems to result in a hypo-allergenic product, as the ability of Bet v 1 to bind IgE was reduced 8-fold. Unpurified liposomes in which Bet v 1 was both adsorbed and encapsulated (and partly unbound) were more allergenic than the ones with only encapsulated Bet v 1, but induced a stronger IgG1 response as well as a stronger cellular response to *ex vivo* stimulation, than otherwise associated Bet v 1. This demonstrates that the association method not only affects the association efficiency, but also the subsequent (hypo)allergenicity and immunogenicity. The most hypo-allergenic formulation is the one with encapsulated Bet v 1, while liposomes with both encapsulated and adsorbed Bet v 1 showed the highest immunogenicity.

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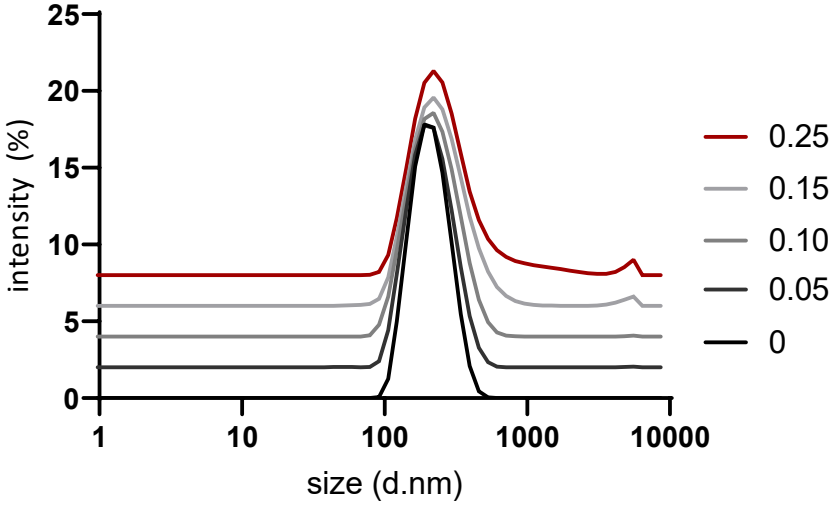
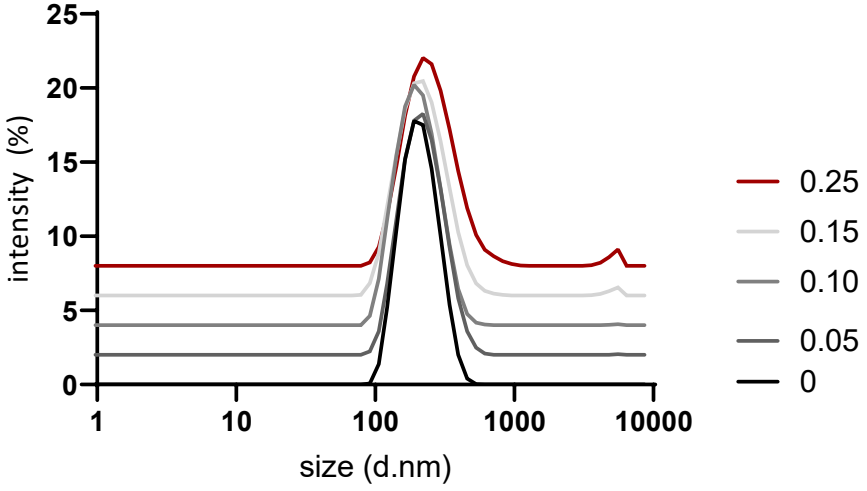
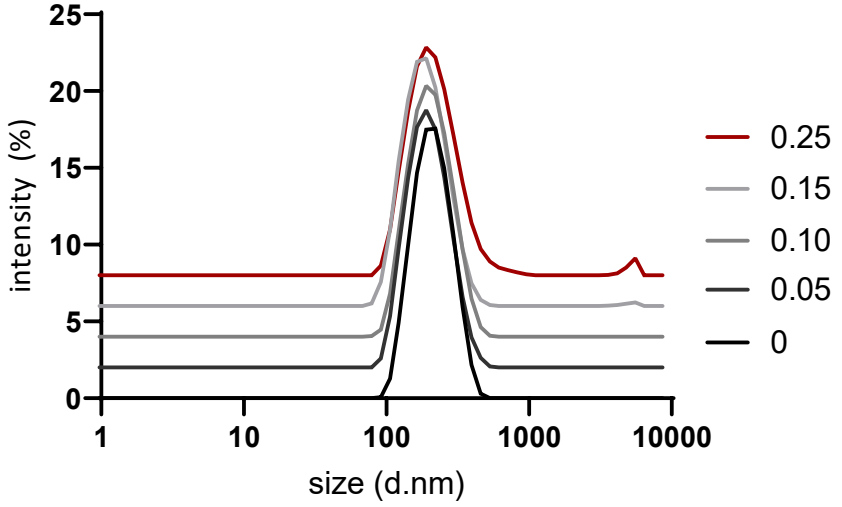
Supplementary Figures



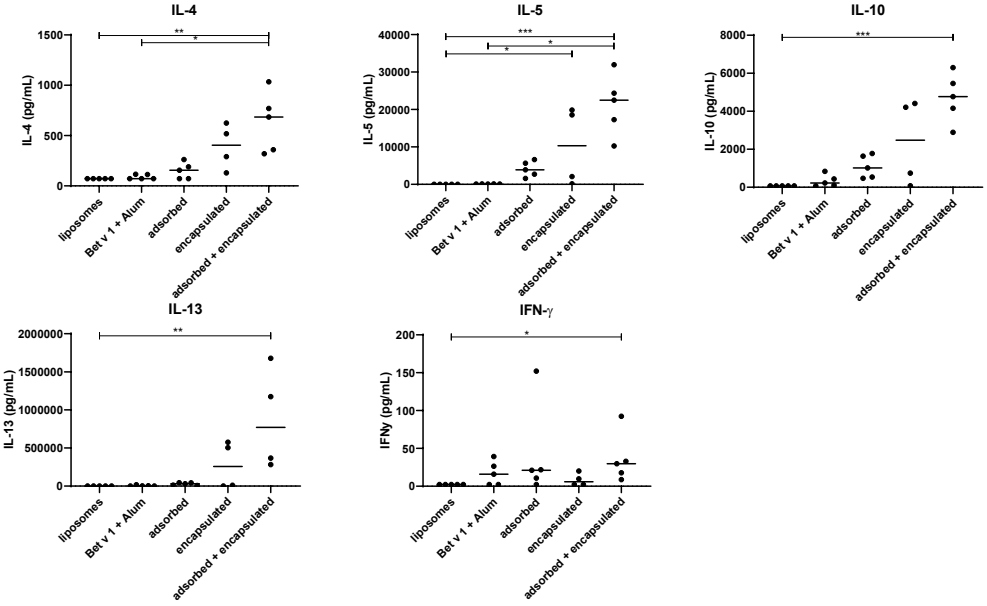
Supplementary Figure 1. Effect of protein/lipid ratio on particle size distribution of pre-formed cationic liposomes mixed with Bet v 1. The graphs shown are the average of 4-5 separate experiments, in which 3 repeated measurements were made. The graphs were artificially nudged by 2 y-axis values for sake of clarity.



Supplementary Figure 2. Effect of protein/lipid ratio on particle size distribution of cationic liposomes with encapsulated Bet v 1. The graphs shown are the average of 4-6 separate experiments, in which 3 repeated measures were made. The graphs were artificially nudged by 2 y-axis values for sake of clarity.



Supplementary Figure 3. Effect of protein/lipid ratio on particle size distribution of cationic liposomes with both adsorbed and encapsulated Bet v 1. The graphs shown are the average of 2 separate experiments, in which 3 repeated measures were mad. The graphs were artificially nudged by 2 y-axis values for sake of clarity.



*Supplementary Figure 4. Production of IL-4, IL-5, IL-10, IL-13 and IFN-y by lung draining lymph node cells from immunized mice after exposure to Bet v 1. Group mean and standard deviation are plotted (n = 4-5 mice/group). Groups were compared with a Kruskal-Wallis test followed by Dunn's correction for multiple comparisons. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.*