A case-study investigating the physicochemical characteristics that dictate the function of a liposomal adjuvant

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A range of particulate delivery systems have been considered as vaccine adjuvants. Of these systems, liposomes offer a range of advantages, including versatility and flexibility in design format and their ability to incorporate a range of immunomodulators and antigens. Here we briefly outline research, from within our laboratories, which focused on the systematic evaluation of cationic liposomes as vaccines adjuvants. Our aim was to identify physicochemical characteristics that correlate with vaccine efficacy, with particular consideration of the interlink between depot-forming action and immune responses. A variety of parameters were investigated and over a range of studies we have confirmed that cationic liposomes, based on dimethyldioctadecylammonium bromide and trehalose 6,6'-dibehenate, formed a depot at the injection site, which stimulates recruitment of antigen presenting cells to the injection site and promotes strong humoral and cell-mediated immune responses. Physicochemical factors which promote a strong vaccine depot include the combination of a high cationic charge and electrostatic binding of the antigen to the liposome system and the use of lipids with high transition temperatures, which form rigid bilayer vesicles. Reduction in vesicle size of cationic vesicles did not promote enhanced drainage from the injection site. However, reducing the cationic nature through substitution of the cationic lipid for a neutral lipid, or by masking of the charge using PEGylation, resulted in a reduced depot formation and reduced Th1-type immune responses, while Th2-type responses were less influenced. These studies confirm that the physicochemical characteristics of particulate-based adjuvants play a key role in the modulation of immune responses.

Liposomes as Vaccine Adjuvants

Of the range of delivery systems available, liposomes were the first delivery system to be described as being able to act as immunological adjuvants.[1] Liposomes are composed of lipid molecules which, when dispersed into an aqueous phase, form bilayered vesicles (**Fig. 1**). These lipid building-blocks are composed of three sections (tail, linker and head) and due to the hydrophobic and hydrophilic natures of the tail and head respectively, the water-loving head group is directed outwards, thus forming liposomes. Liposomes can be manufactured in a wide range of morphologies from single to multi-lamellar in structure and ranging in size from ~50 nm up to several microns in size (**Fig. 1**).

Liposomes are an ideal vaccine delivery system due to their particulate nature, flexibility in formulation, ability to incorporate a range of moieties including immunogenic molecules and antigens; it is these parameters that can be used to promote a range of immune responses.[2] The geometry of liposomes is principally determined by their method of manufacture and their lipid composition. For example, size reduction of vesicles can include sonication, high-shear homogenization or high pressures whereby disruptive energy causes large vesicles to rearrange into smaller ones. An overview of a range of liposome production methods are shown in **Figure 2**. However, these traditional methods of liposome synthesis raise several difficulties e.g., mechanical stresses, difficulty in up-scaling and methods that rarely lead to size-uniform liposomes.[3] To address these issues, the area of microfluidics, and its associated development of novel lab-on-a-chip based devices, has gained increasing attention over recent decades. Besides saving time and money, the use of microfluidics methods reduces space and sample volume (**Fig. 3**).

In terms of developing liposomal adjuvants, a key consideration is their ability to carry and delivery their antigen payload to the appropriate target site. Again, a range of methods have been investigated to promote or improve antigen and liposome association e.g., the dehydration-rehydration.[4] A relatively simple and flexible option is to adopt electrostatic interactions to support antigen loading, where the liposomes are designed with the appropriate surface charge to electrostatically adsorb antigen.[5] To exploit this method, the overall charge of a peptide or protein must be known; this will be dependent on its amino acids and will change according to the pH of the solution it is suspended in. However, most protein adjuvants have an overall negative charge making cationic liposomes an appropriate design choice.

Adopting a cationic change is not just beneficial for enhanced antigen loading; cationic systems can also enhance antigen delivery and immunostimulatory potency. For example, the cationic lipid dimethyldioctadecylammonium (DDA) was discovered as an adjuvant by Gall in the mid-1960s.[6] DDA is a
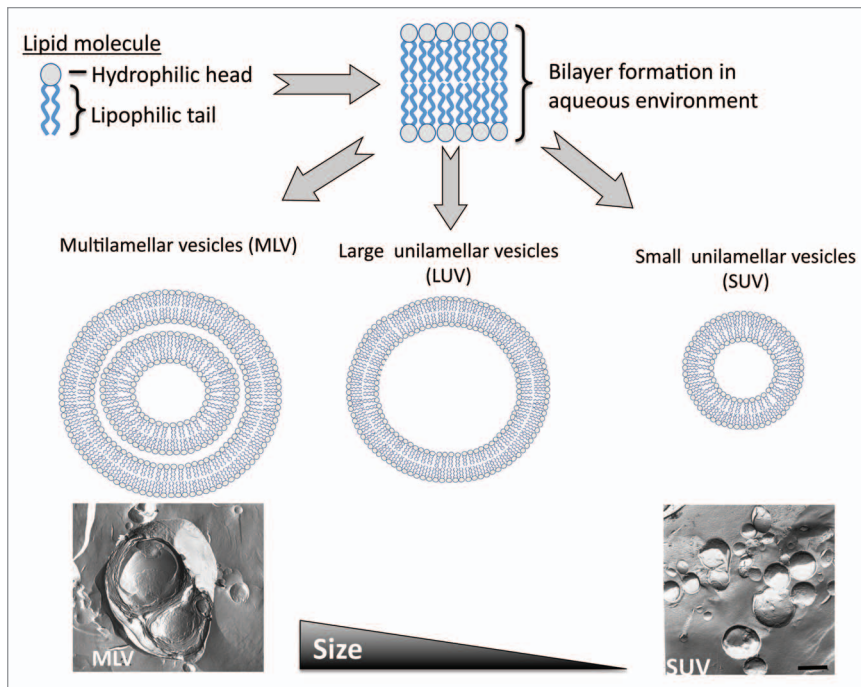
Figure 1. Schematic outline of the formation of liposomes. Lipid molecules, when dispersed in an aqueous phase form bilayers vesicles which can be prepared in a range of sizes from larger multilamellar vesicles, through to small unilamellar vesicles.

for a T-cell receptor recognizing a major histocompatibility complex (MHC) class II-restricted OVA-epitope (OVA323–339). Both proliferation and IFNγ production was increased upon interaction with either murine BMDCs or purified B-cells, stimulated with OVA adsorbed to DDA.[17,18]

To further enhance the potency of DDA liposomes, a second component, D-(+)-trehalose 6,6'-dibehenate (TDB) can be added.[13] TDB is a synthetic analog of trehalose-6,6-dimycolate, a immunostimulatory component of *Mycobacterium tuberculosis*. TDB has two saturated fatty-acid chains of 22 carbons (behenyl), each replacing the branched mycobacterial mycolic acids of > 70 carbons (**Fig. 4**). These two behenyl chains are linked by ester bonds to carbon number 6 of each of the two glucopyranose rings making up the trehalose head group (**Fig. 4**). TDB has been shown to retain much of the bioactivity of the native form, while showing less toxicity as a result of the shorter fatty acid chains.[19,20] In combination with DDA, it is an efficient adjuvant for TB subunit vaccines,[13] inducing a strong IFNγ response, considered to be the key cytokine for induction of a Th1 immune response and essential for effective anti-mycobacterial immunity.[21,22]

Interestingly TDB does not only enhance the immunogenicity of the DDA liposomal systems.[7] Having shown that DDA:TDB could be effectively prepared in a sterile and stabile format via lyophilization and gamma-sterilization,[23,24] the contributing role of TDB to the DDA membranes both in an aqueous[25] and freeze-dried format[26] was shown to result from the higher attractive forces between the trehalose head group of TDB and water compared with the quaternary ammonium head group of DDA and water.[25]
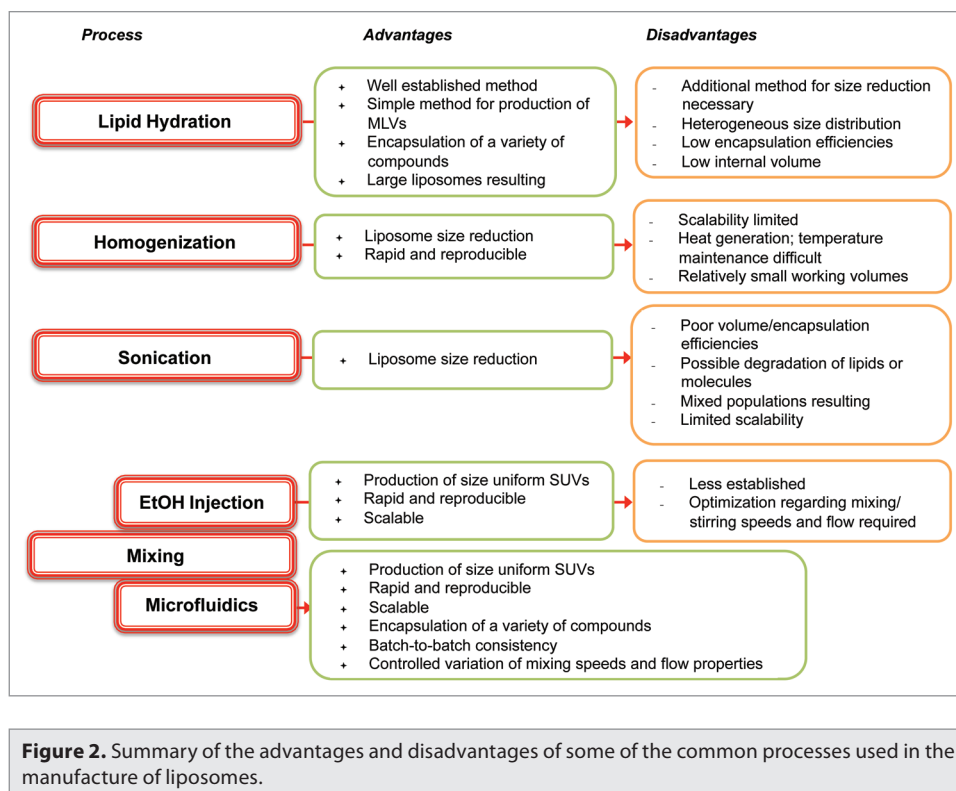
Antigen Delivery and Immune-Stimulation

Mycobacteria are well known to exert a number of immunostimulatory effects and are a good source of adjuvants e.g., Freund's complete adjuvant (an oil emulsion and heat killed mycobacteria). Purified components of mycobacteria such as TDB have also been shown to be effective. Holten-Andersen et al.[13] first investigated the combination of DDA:TDB, and studied the ability of seven different immunostimulators, including four mycobacteria-derived immunostimulators, to increase the protective efficacy of DDA, using ESAT-6 as a possible TB antigen. An effective IFNγ response was obtained with the combination of DDA with monophosphoryl lipid A (MPL) and/or TDB. This combination also induced protection in mice similar to that obtained after BCG vaccination. The protective efficacy of DDA:TDB using Ag85B-ESAT-6 was also shown[7] and the adjuvant activity of DDA:TDB was compared with aluminum hydroxide. These studies showed

synthetic amphiphile, which contains a quaternary ammonium group with two 18-carbon-long alkyl chains forming the hydrophobic moiety and two methyl groups, which together with the ammonium group form the polar head group (**Fig. 4**). The positively charged head group carries a monovalent counterion, typically bromide or chloride. Due to its amphiphilic character, DDA can form liposomal structures when dispersed in aqueous media at temperatures above its gel-to-liquid phase transition temperature (~47°C).[7] DDA is known to induce cell-mediated immunity and delayed-type hypersensitivity[8] and, along with its cationic nature and surfactant properties, has been shown to be an effective adjuvant in numerous applications, including mucosal immunization,[9] gene delivery[10,11] and subunit vaccine delivery.[12-15]

The mechanism of action behind the adjuvant effect of DDA has been attributed to its positive surface charge and its ability to associate antigens.[16] This was confirmed and further elaborated by using ovalbumin (OVA) as a model antigen.[17] Stimulation of immature bone marrow-derived dendritic cells (BMDCs) with fluorescently labeled OVA showed that adsorption of OVA onto DDA enhanced the cellular acquisition of the antigen. Further, inhibition of active cellular processes by OVA stimulation at 4°C or by the addition of cytochalasin D reduced the cellular uptake, suggesting that active actin-dependent endocytosis is the predominant uptake mechanism.[17] DDA-mediated OVA uptake was also associated with a functional enhancement of the antigen presenting cells (APC). This was shown by measuring the increase in gamma interferon (IFNγ) production and cellular proliferation of purified autologous DO11.10 T-cells transgenic

that immunization with DDA:TDB leads to high levels of IFNγ secretion and low levels of interleukin-5 (IL-5) secretion by CD4⁺ T-cells, whereas aluminum hydroxide immunized mice exhibited the opposite pattern, with a negligible IFNγ secretion and higher levels of IL-5 secretion.[7] The DDA:TDB-adjuvated vaccine resulted in the same high levels of IgG1 antibody titers, as seen after immunization with the aluminum hydroxide-adjuvated vaccine, whereas the level of IgG2 antibodies were significantly higher after immunization using DDA:TDB as an adjuvant, compared with aluminum hydroxide.[7,27] Additionally, studies have shown that DDA:TDB induces a CD4⁺ T-cell population, predominantly tumor necrosis factor-α (TNF-α)⁺, IL-2⁺, IFNγ⁺, and IL-2⁺ multifunctional T-cells, which are long-lived and maintained for at least 1 year in mice.[28]

In terms of its mechanism of action, the C-type lectin receptor Mincle, expressed in macrophages, is upregulated in the presence of TDM and TDB and triggers the FcRγ-Syk-Card9 pathway. The activation of macrophages by TDB/TDM depends on FcRγ, which represents the Sky-coupling adaptor protein. Deletion of the FcRγ protein resulted in loss of transcriptional responses to TDB/TDM.[29] The Syk-Card9 pathway is crucial for antigen-presenting cell activation in vitro and for the adjuvanticity of TDB/TDM in vivo.[30,31] The glycolipids TDM and TDB are Mincle-recognized ligands. Molecular mechanisms of TDB/TDM adjuvanticity were identified to be strictly Mincle-dependent and strong IL-17 production post immunization controls the generation of cellular immunity.[31,32] Desel et al.[32] showed that adjuvanticity of TDM/TDB not only depends on Mincle, but also necessitates MyD88. Whereas in vitro studies identified MyD88 independence, in vivo adjuvanticity was strongly dependent on MyD88 signaling. Immunization experiments included DDA/TDB and H1 subunit vaccine for TB, composed of Ag85B and ESAT-6. Mincle and MyD88 were shown to be indispensable prerequisite for antigen-specific Th1 and Th17 immune responses in vivo supported by IL-1/IL-1R1 signaling.[32]

Systematically Investigating the Formulation Parameters Controlling the Efficacy of DDA:TDB as a Liposomal Adjuvant

Building on the demonstrated potential of the DDA:TDB system developed by the Staten Serum Institut (e.g., ref. 13), a series of collaborative studies were undertaken to investigate the principles behind the efficacy of this formulation, with one focus being consideration of physicochemical attributes. Our aim was to identify formulation attributes that could be considered to act as correlates for efficacy. These investigations were based on early studies[7] which investigated the incorporation of TDB into DDA liposomes and considering the method of liposome preparation and TDB content. These studies demonstrated that incorporation of TDB into DDA liposome bilayers had no impact on antigen loading; however, increasing the TDB concentration (0 to 20 mol%) reduced the transition temperature of the bilayers from 47 to 42°C. The TDB concentration also impacted on immune response. While all the DDA liposome formulations were able to induce high levels of IFNγ, the formulation containing 11% TDB gave the highest responses in terms of IFNγ, IgG1 and IgG2b antigen specific responses in C57Bl/6j mice. In comparison to Alum, IFNγ responses from blood lymphocytes isolated from immunized mice were significantly higher for DDA:TDB (11%), while IL-5 levels were significantly lower. In addition to potentiating the immune response of DDA liposomes, incorporation of TDB within the formulation was shown to effectively stabilize the DDA liposomes.[7] Given that this DDA:TDB combination was shown to be highly effective, this combination was also incorporated into a range of other particulate based systems including niosomes[33] and microspheres.[34,35] Incorporation of DDA and TDB within either of these systems did not produce particulates with enhanced adjuvant properties compared with the liposomal DDA:TDB liposome formulation. Therefore, the next stage was to focus on the DDA:TDB liposome formulation and consider the role of its physicochemical characteristics in it performance as an adjuvant, with particular reference to it biodistribution.
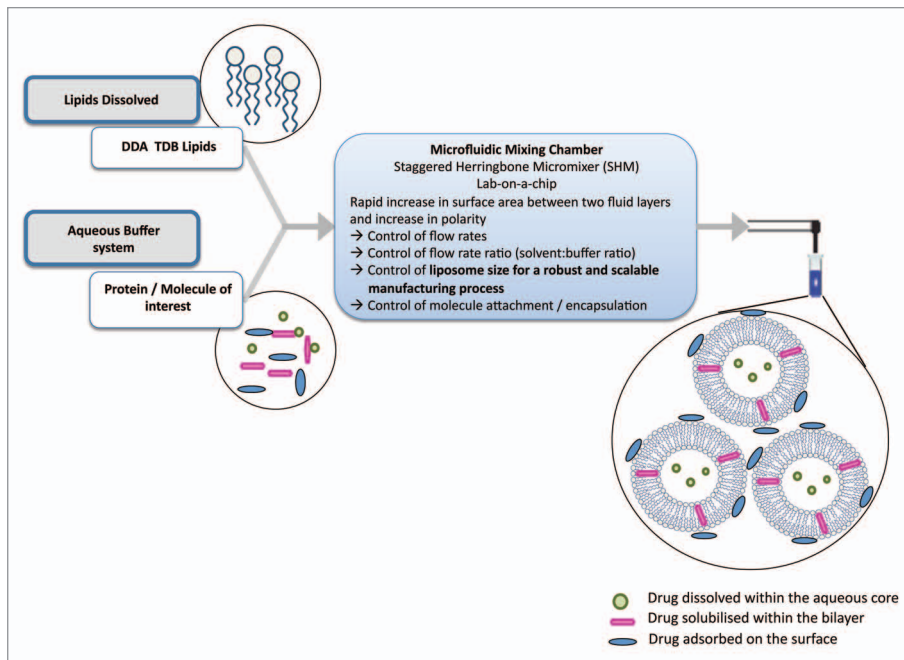

Figure 2. Summary of the advantages and disadvantages of some of the common processes used in the manufacture of liposomes.

Figure 3. Schematic outline of the principles involved in the preparation of liposomes using microfluidics.

The Impact of Lipid Choice— The Controlling Role of Charge

The highly cationic nature of the DDA:TDB liposome formulation offers the advantage of these systems being able to electrostatically bind a range of antigens; however, we also proposed the hypothesis that this cationic charge would influence the biodistribution of the vesicles and their adsorbed antigen. Therefore, we undertook a range of studies applying a relatively simple, effective and reproducible method to follow the biodistribution of the liposomes and their associated antigen using a dual-radiolabeling method developed within our laboratories, where the liposomes are labeled with ^{3}H and the antigen with ^{125}I.[36] Using this method we were able to demonstrate that while antigen delivered without liposomes were removed quickly from the body, liposomes based on DDA promoted a depot at the injection site (both after subcutaneous or intramuscular injection) and that TDB did not significantly influence this deport effect.[37] However, the presence of TDB in the DDA liposomes increased the influx of monocytes to the site of injection, and the subsequent draining of the liposomal adjuvant to the popliteal lymph nodes, in addition to inducing a powerful Th1 response.[37]

The impact on vesicle charge on the deposition of antigen at the injection site was further considered by comparing cationic DDA:TDB liposomes with a comparable near-neutral liposome formulation composed of Distearoylphosphatidylcholine (DSPC) and TDB (11 mol%).[38] This study demonstrates that the cationic nature of the vesicles promotes the retention of the liposomal components at the site of injection, with the DSPC:TDB formulation being more rapidly cleared. Furthermore, the electrostatic adsorption of antigen to the vesicles was demonstrated to be a key requirement for antigen retention. Not only did the neutral liposomes give poor antigen absorption and retention at the injection site, cationic liposomes which were unable to absorb a cationic model antigen (as would be expected) also failed to promote an antigen depot, despite the liposomes being retained at the injection site.[38] However, distearoylphosphatidylcholine-based liposomes entrapping antigen within the vesicles were shown to offer a potential alternative to cationic-based systems, especially for the delivery of zwitterionic or cationic molecules.[39]

Choosing the cationic lipid component. Given that we had demonstrated the controlling role of the cationic lipid in the promotion of a depot at the site of injection, the next consideration addressed whether this was applicable to a range of cationic formulations. Therefore the ability of 3β-[N-(N',N'-dimethylaminoethane) carbomyl] cholesterol (DC-Chol), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), or DDA liposomes incorporating immunomodulating trehalose dibehenate (TDB) to form an antigen depot at the site of injection (SOI) and to induce immunological recall responses against co-administered tuberculosis vaccine antigen was investigated.[40] Despite all three formulations being cationic in nature, differences in the biodistribution of these vesicles were noted, with the DOTAP formulation draining more rapidly from the injection site compared with the DDA or DC-Chol formulations. This was reflected in a slower drainage to the local lymphoid tissue. This was attributed to the DOTAP liposomes having a lower transition temperature and therefore more fluid in nature, thus promoting easier drainage from the injection site. This was confirmed by comparing liposomes prepared using DDA with liposomes prepared with its unsaturated analog dimethyldioleoylammonium bromide (DODA), which contained one unsaturated C = C bound in each of the lipophilic acyl chains. By comparing the delivery properties of liposomes prepared using these two lipids, liposomes which were rigid (DDA:TDB liposomes) or fluid (DODA:TDB liposomes) in nature at physiological temperatures could be considered.[41] Through a series of studies it was shown that these two different formulations showed major differences in their ability to drive a Th1 immune response. The rigid DDA-based liposomes retained higher levels of antigen at the injection site, resulting in a continuous attraction of antigen-presenting cells that expressed elevated levels of the co-stimulatory molecules CD40 and CD86.[41] Overall the rigid, DDA-liposomes induced 100-fold higher Th1 responses than the fluid DODA liposome counterparts, confirming that a range of physicochemical properties have a major influence on the efficacy of liposomal adjuvants.

Enhancing delivery of DDA:TDB to the target site. While all the studies undertaken with the DDA:TDB had proved it

gave strong Th1 responses and formed a strong depot at the site of injection, this did not necessarily demonstrate a direct correlation between the these two attributes, and enhancing the delivery of DDA:TDB+ antigen to APC should further boost its potency. This was nicely demonstrated by a study led by Johansen's group,[42] where the immune responses achieved by DDA:TDB when administered via the sub-cutaneous, intradermal, intra-muscular or intra-lymphatic routes. This study showed that the route of administration promoted no notable differences in IgG1. However, the administration route had a major influence on Th1 responses; intra-lymphatic injection gave strong early IgG2a responses and significantly higher IFNγ secretion from spleno-cytes collected from immunized mice via this route compared with the other routes.[42] This suggested that while the highly effective DDA:TDB liposome formulation formed a strong depot

DDA - Dimethyldioctadecylammonium (Bromide Salt)

$C_{38}H_{80}NBr$

Synthetic amphiphile
- Hydrophobic tail: quaternary ammonium group with two 18-carbon-long alkyl chains
- Polar head group: and two methyl groups, which together with the ammonium group

DDA:TDB Liposomes
→ Efficient adjuvant for TB subunit vaccines,
→ Strong gamma interferon (IFNγ) response
→ Induction of a Th_1 immune response
→ Essential for effective anti-mycobacterial immunity

TDB
D-(+)-trehalose 6,6'-dibehenate

$C_{56}H_{106}O_{13}$

Enhance DDA liposomes potency
- Hydrophobic tail: two saturated fatty-acid chains (behenyl)
- Polar head group: glucopyranose rings

Figure 4. Liposomal adjuvants can be prepared from a range of lipids, for example the cationic lipid Dimethyldioctadecylammonium bromide and D-(+)-trehalose 6,6'-dibehenate. Combined together, these lipids from liposomes with strong adjuvant properties.

effect at the injection site, trafficking of this formulation to the draining lymphatics would enhance vaccine efficacy.

The role of particulate size in the biodistribution of DDA:TDB liposomal adjuvants. When considering the biodistribution of liposomes, size is often shown to have a significant impact. Hence, liposome size was also an important attribute to consider. In the above studies the DDA:TDB liposomes studied were around 500–600 nm in size, therefore consideration of the impact of the size of the DDA:TDB vesicles on their role as adjuvants was also investigated.[43] DDA:TDB liposomes were prepared in the following size ranges: small (< 200 nm), medium (500–600 nm), and large (~1500 nm). No significant difference in the drainage of the liposomes or their adsorbed antigen from the site of injection was seen between the different sized liposomes. However, significantly higher levels (but still relatively low amounts of the total dose) of the smaller liposomes were noted at the popliteal lymph node, 6 h after injection.[43] This was shown to be independent to cellular phagocytosis, as macrophage uptake of these various liposomes was not shown to be size-dependent.[43] This would suggest that due to their cationic nature, and independent of their size, the vesicles aggregate after administration, due to interaction with interstitial proteins which are generally anionic in nature, thus prohibiting their clearance from the site of injection.

Retaining the DDA component but masking its cationic nature—pegylated DDA:TDB. Given that the depot effect had been shown to be primarily due to the cationic nature of the vesicles, which results in electrostatic adsorption of the antigen and aggregation of the vesicles at the site of injection, a second method to promote drainage (yet retain the DDA content
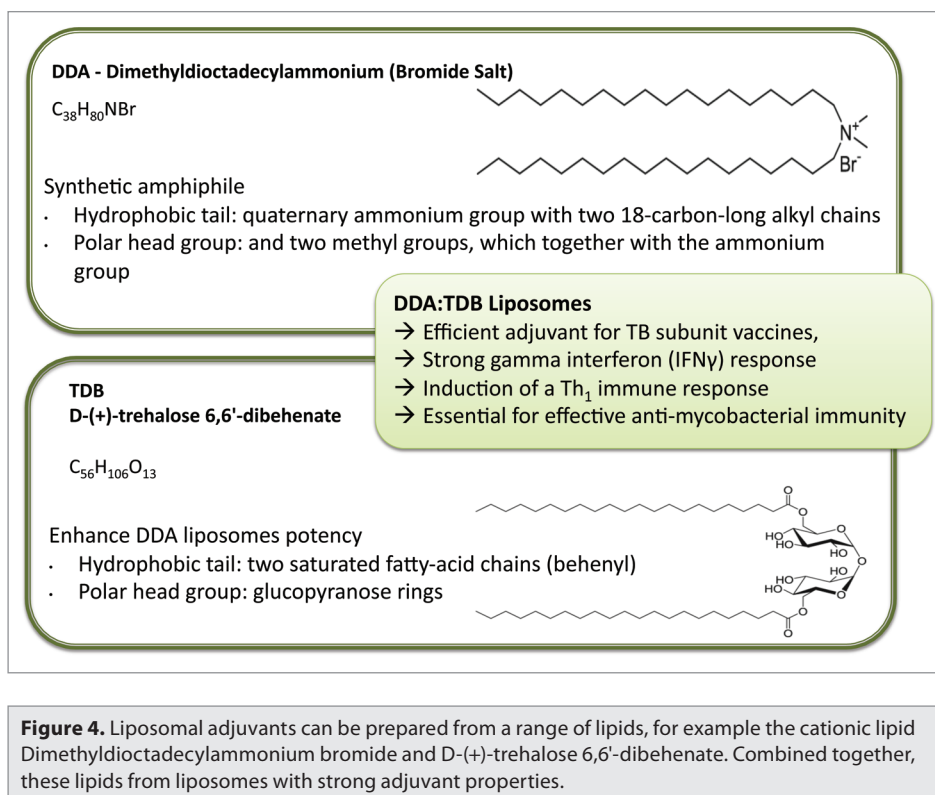
of the DDA:TDB:antigen formulation) to the lymphatics was considered. A series of studies were undertaken where the cationic DDA component of the liposomes was retained, yet the cationic nature masked with polyethylene glycol. Polyethylene glycol (PEG) is the most widely used hydrophilic polymer for the steric stabilization of liposome drug delivery systems. It is a linear polyether diol with many useful properties, including the ability to be conjugated to a liposomal surface to create a steric, hydrophilic barrier which can enhance increasing half-life of the liposomes, through steric stabilization.[44] Coating of liposomes with PEG (often referred to as PEGylation), is exploited in therapeutic products such as Caelyx, which is a PEGylated liposomal delivery system for doxorubicin. PEG can be incorporated onto the surface of liposomes in different ways, but the most widely used method at present is to anchor the polymer in the liposomal membrane via a cross-linked lipid (i.e., PEG-distearoylphosphatidylethanolamine [DSPE]).

DDA:TDB liposomes were prepared with increasing PEG concentrations (0 to 25 mol%). This resulted in the cationic zeta potential of the vesicles dropping from ~55 mV for DDA:TDB down to 39 mV with the addition of 10% PEG, and to 3 mV when 25% PEG was added.[45] This drop in zeta potential results in a reduction in both the liposome and antigen depot at the injection site, and reduced monocyte influx to the injection site. However, while PEGylation of DDA:TDB tended to promote an increased drainage of liposomes to the local lymph node, this did not translate to an increased antigen delivery to the draining lymph nodes.[45] In terms of immune responses, while increasing PEG concentrations in the DDA:TDB liposomal adjuvant made no significant difference in IgG1 responses, both IgG2b
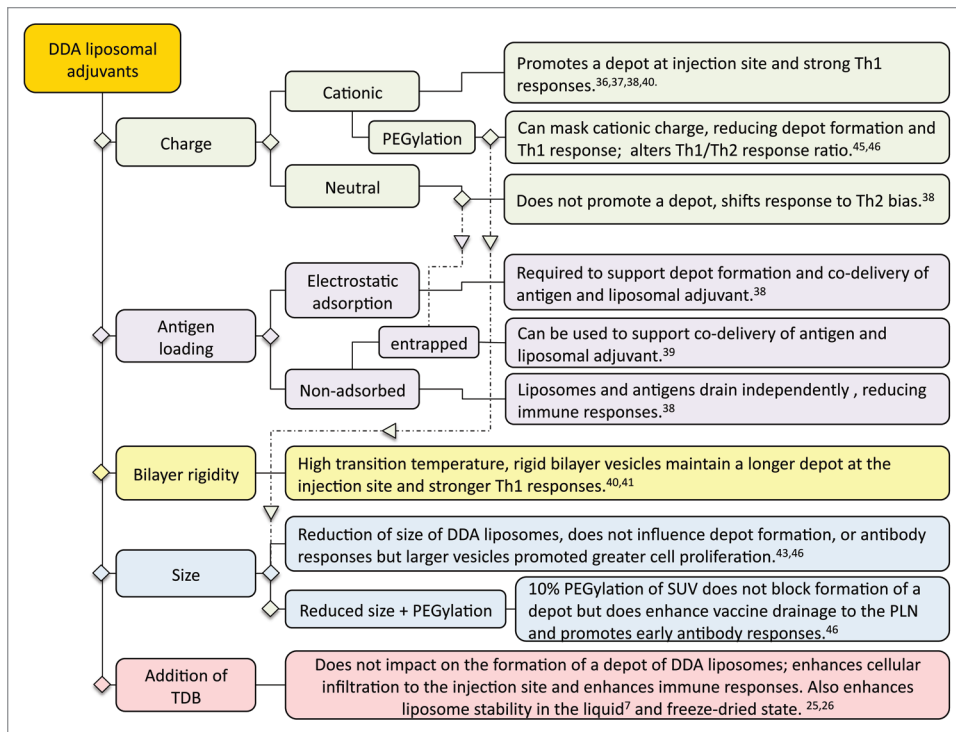
Figure 5. Formulation parameters shown to impact on the formation of a DDA:TDB liposome depot at the injection site and a summary of the impact this has on immune responses.

and IFNγ responses reduced with increasing PEG concentrations.[45] This suggested that PEGylation was able to block the formation at the site of injection, yet the PEGylated liposomes were less able to carry the antigen with them to the draining lymph nodes.

In an attempt to address this and improve co-delivery of the liposomes and antigen to the draining lymph nodes, a second series of PEGylated liposomes were prepared where the antigen was incorporated within the liposomes rather than surface adsorbed.[45] However, this did not significantly enhance antigen delivery to the local lymph node, nor change the immune-responses compared with the DDA:TDB formulations with the surface-adsorbed antigen.[45] To consider if a combination of size reduction and PEGylation of the vesicles could further modify the clearance kinetics of these liposomal adjuvants, DDA:TDB liposomes with and without PEGylation were also prepared as small unilamellar vesicles (SUV).[46] By using DDA:TDB:10% PEG vesicles which were ~150 nm in size, both liposome and antigen dose at the popliteal lymph node 4 days after injection was increased and earlier antibody responses noted.[46]

The ability of PEGylation of small cationic liposomes to enhance drainage was also demonstrated with DNA vaccine carriers. Liposomes composed of phosphtidylcholine,

dioleoylphosphatidylethanolamine and the cationic lipid DOTAP were prepared with entrapped OVA-encoding plasmid DNA; the introduction of PEG onto the surface of these small cationic vesicles resulted in enhanced lymphatic drainage after sub-cutaneous injection, but the immune responses measured were not improved when compared with non-PEGylated liposomes.[47]

The controlling role of the adjuvant depot on Th1 but not Th2 responses. A particular reference point in the above outlined studies was the ability of the liposomal formulation to form a depot at the site of injection and the overall design factors identified to control the formation of a depot at the site of injection for liposomal adjuvants are summarized in **Figure 5**. Until recently, it had been widely accepted that the activity of alum-based adjuvants was attributed to its ability to retain the antigen at the site of injection.[48] This theory was recently put into question through various studies which demonstrated that alum with non-adsorbed or adsorbed antigen gave rise to similar antibody responses[49,50] and indeed removal of the alum injection site did not influence the subsequent immune response.[51]

Through the above series of studies, we have systematically considered the correlation between the induction of immune responses and the ability of the liposomal formulation to promote depot formation. These studies demonstrate that (1) liposomes that promote a strong depot effect also potentiate a strong Th1 response, and (2) Th2 responses generated by these liposomal adjuvants were not influenced by/reliant upon the depot formation. These findings are in line with the more recent understanding of Alum's adjuvant action, which predominately drives a Th2 response and appears to act independent of a depot.[49-51]

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