Biomimetic Multilayered Lipid Nanovesicles for Potent Protein Vaccination

Bon Il Koo, Dong Jae Lee, Rafia Tasnim Rahman, and Yoon Sung Nam*

Lipid vesicles are widely used for drug and gene delivery, but their structural instability reduces in vivo efficacy and requires specialized handling. To address these limitations, strategies like lipid cross-linking and polymer–lipid conjugation are suggested to enhance stability and biological efficacy. However, the in vivo metabolism of these altered lipids remains unclear, necessitating further studies. A new stabilization technique without chemical modification is urgently needed. Here, a bio-mimetic approach for fabricating robust multilamellar lipid vesicles to enhance in vivo delivery and stabilization of protein antigens is presented. This method leverages 1-O-acylceramide, a natural skin lipid, to facilitate the self-assembly of lipid nanovesicles. Incorporating 1-O-acylceramide, anchoring lipid bilayers akin to its role in the stratum corneum, provides excellent stability under environmental stresses, including freeze–thaw cycles. Encapsulating ovalbumin as a model antigen and the adjuvant monophosphoryllipid A demonstrates the vesicle's potential as a nanovaccine platform. In vitro studies show enhanced immune responses with both unilamellar and multilamellar vesicles, but in vivo analyses highlight the superior efficiency of multilamellar vesicles in inducing higher antibody and cytokine levels. This work suggests ceramide-induced multilamellar lipid vesicles as an effective nanovaccine platform for enhanced antigen delivery and stability.

1. Introduction

The impressive efficacy of COVID-19 vaccines has rekindled interest in lipid nanoparticles (LNPs) as drug and gene delivery carriers. Their biocompatibility and biodegradability, coupled with their versatility in administration, present distinctive advantages. However, they face challenges due to the structural vulnerability to environmental and physiological conditions that can disrupt their structure and result in premature release of payloads. To solve this problem, improving the structural stability of LNPs is critically important. One common strategy involves “PEGylation,” the conjugation of polyethylene glycol (PEG) to the surface, which has become standard for prolonging the circulation time of LNPs. Nevertheless, such modifications do not fundamentally alter the mechanical instability of LNPs. For reinforcing structural integrity, constructing multilayered lipid structures has emerged as a solution. Achieving this involves the sophisticated control of interlayer forces to promote the self-assembly of multiple lipid bilayers. In situ, cross-linking and polymerization techniques have been proposed to enhance the structural integrity of lipid-based particles. For instance, the lipid vesicles crosslinked by polymerizable lipids exhibit structural robustness. Furthermore, highly robust lipid–polymer hybrid particles can be prepared by incorporating hydrophobic monomers into the hydrophobic region of the lipid membrane for in situ polymerization. Additionally, employing techniques like the covalent linkage of functionalized lipids, facilitated by divalent ions, enhances electrostatic interactions with anionic lipids, showing significant potential. This approach initially forms a loosely arranged multilamellar structure using divalent ions, which is subsequently reinforced by strong chemical bonds between the lipid membranes, resulting in a tightly interconnected structure. However, the in vivo metabolic pathways of these chemically modified lipids and biomolecules remain poorly understood, presenting a barrier to their clinical application. Consequently, there is a growing demand for stabilization methods that avoid chemical modifications and rely on non-covalent means to generate a robust multilayer structure.

An alternative approach leverages the electrostatic interactions between the lipid vesicle surface and biomacromolecules with opposite charges, such as nucleic acids, chitosan, and proteins. An additional effective approach is the formation of a multilamellar structure through the electrostatic attraction between...
biomolecules and liposomes.[21–27] When mixed, these molecules self-organize with the lipid vesicles to construct multilamellar lipid vesicles (MLVs), facilitated by electrostatic attraction. This method of self-assembly not only preserves the native structures and functions of the encapsulated biomolecules but also facilitates their effective immobilization within the lipid bilayers’ interlamellar spaces.[24] Specifically, multilamellar protein–lipid vesicles have shown promise as a versatile delivery platform for topical applications and vaccine administration in vivo, attributing to their role in providing physical, chemical, and biological stabilization to proteins.[24,25] Nevertheless, its practical applications are hampered by the variability in self-assembly conditions and efficiencies, which are contingent on the proteins’ charge distributions, sizes, and conformations. Therefore, there is a significant demand for a simple method to fabricate robust multilamellar lipid nanovesicles that can be applied to a diverse range of biomolecules irrespective of their intrinsic physical and chemical properties.

The self-assembly via hydrophobic interactions presents an alternative method that avoids the complexities of chemical reactions and remains relatively unaffected by variations in solution conditions, such as ionic strength and pH. This method involves the integration of graft-type copolymers, modified to carry hydrophobic molecules, into the lipid bilayer.[28–30] This technique mirrors the natural architecture in the skin’s stratum corneum, where corneocytes and intercellular lipids collaborate to form a “brick and mortar” multilamellar structure, shielding the body from external factors.[31,32] A pivotal component in this protective structure is acylceramide, a ceramide family member that reinforces skin integrity through its role in inter-lipid and lipid-corneocyte connections.[33] Notably, 1-O-acylceramide, a newly categorized and successfully synthesized compound, is naturally present in human skin and plays a significant role in enhancing the skin’s barrier function.[34]

In this study, we present the development of robust ceramide-induced MLVs, stabilized by ceramide linkers, which demonstrate potential as an efficient vaccine delivery system. The vesicles are constituted of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-O-acylceramide, and cholesterol, with ovalbumin (OVA) as the antigen and monophosphoryl lipid A (MPLA) as the adjuvant. In particular, 1-O-acylceramide physically connects lipid bilayers and increases the mechanical properties of the vesicles. 1-O-acylceramide contains a long acyl chain with 17 carbons, in addition to other long oleoyl and sphingosine chains. 1-O-acylceramide contains long acyl chains in both the N- and 1-O-positions, containing more polar functional groups in the lipid head.[35] The polar head groups of the lipids form hydrogen bonds, contributing to the formation of the lipid matrix.[36,37] The sphingosine groups can act as both donors and acceptors of the hydrogen bonds. The EcoCeramide ENP used in our study contains six sites for the donor/acceptor of hydrogen bonds in the head group. As such, the ceramides are expected to form multiple hydrogen bonds with the neighboring lipids (DPPC and cholesterol), which stabilizes the membrane structure and in turn forms the multilamellar layers. The impact of 1-O-acylceramide on the morphology, stability, and molecular organization of the lipid vesicles was evaluated. To assess the immunogenic potential of the vesicles, we conducted a comprehensive analysis of both humoral and cellular immune responses. This included evaluating cellular uptake, cytokine secretion, the expression of immune surface markers, the production of antigen-specific antibodies, and the accumulation of intracellular cytokines.

2. Results and Discussion

2.1. Preparation and Characterization of Lipid Vesicles

Ceramide-induced MLVs were formulated with DPPC, EcoCeramide ENP, and cholesterol in varying weight ratios (WR), as outlined in Table S1 (Supporting Information). EcoCeramide ENP comprises of 16.3% ceramide NP (oley), 73% 1-O-acylceramide, and 8.8% stearic acid. Here, 1-O-acylceramide’s linear carbon chains interlink the lipid bilayers, acting as a physical connector (Figure 1A). We adopted the thin-film hydration-extrusion technique to prepare all vesicles (Figure S1, Supporting Information). Specifically, multi-layered lipid thin films were prepared by drying the solution mixture of EcoCeramide ENP and lipids dissolved in chloroform. The tightly-stacked lipid thin film layers formed by 1-O-acylceramide in EcoCeramide ENP directly fabricated multilamellar lipid particles rather than mono-layered particles or vesicle-in-vesicle structures during the hydration step. The multilamellar structure was maintained after the extrusion step since the 1-O-acylceramide aids in retaining the robustness of the structure. The optimal ratio of DPPC, EcoCeramide ENP, and cholesterol was determined to be 80:5:15, respectively. This ratio effectively prevented the formation of micron-sized particles, as verified by particle size analysis (Table S2, Figures S2–S12, Supporting Information). The results show that the stability of the colloidal particles is significantly influenced by the composition of the lipids. Adjusting the ratios of DPPC, EcoCeramide ENP, and cholesterol allowed for fine-tuning of particle size and stability. If cholesterol was not added or added in too large amounts, particles were not formed. Thus, the cholesterol levels were adjusted to between 5% and 20%. For EcoCeramide ENP, using more than 7.5% prevented the formation of a stable, small colloidal dispersion. The most stable particles were formed when LUVs contained 15% cholesterol. In samples with EcoCeramide ENP, adding 5% EcoCeramide ENP created stable colloids, especially when paired with 15% cholesterol, resulting in relatively small particles. Similar results were observed when making LUVs, suggesting that 5% EcoCeramide ENP does not alter the overall structures of the lipid layers. Our experiments confirmed that a DPPC: EcoCeramide ENP: cholesterol ratio of 80:5:15 produces stable colloidal particles with an average diameter of ≈210–230 nm. This optimal condition was used in further experiments to ensure consistent and reliable results.

Vesicles incorporating 1-O-acylceramide manifested multilamellar structures with a measured membrane thickness of 3.9 ± 0.5 nm, as observed from cryo-TEM (Figure 1B). The cryo-TEM image shows a mixture of LUVs and MLVs, with the majority being the MLVs (≈70%). Obtaining a lipid vesicle solution with a pure population of MLVs is expected to be quite a challenge. However, from the cryo-TEM images, the lipid vesicle solution used for this study mainly consists of the MLVs. TEM images of MLVs revealed condensed multilayers, suggesting the vesicles were in a solid state (indicated by an orange triangle in
relative to LUVs, indicating an ordered structure. The exhibited peak positions at integer ratios and higher intensities X-ray scattering (SAXS) (Figure 1C). The SAXS profile of MLVs for LUVs and MLVs was also conducted using small-angle distance. A comparative analysis of the membrane structure lipid membranes, which in turn yields a shortened inter-bilayer three long chains containing several sites for hydrogen bonds, intermembrane distance (O−acylceramide). As the cholesterol concentration increased, the membrane fluidity decreased, producing larger particle sizes. Furthermore, an elevated ratio of 1-O-acylceramide was also associated with the generation of larger particles. The MLVs showed a mean diameter of 265.8 ± 3.3 nm, coupled with a zeta potential of −1.1 ± 1.8 mV, under an optimal WR. The MLVs were significantly larger than the LUVs, which had a diameter of 228.3 ± 11.3 nm. These observations suggest that the integration of 1-O-acylceramide into the hydrophobic region of the lipid membrane is a spontaneous process that contributes to the formation of MLVs.

2.2. Dispersion Stability of Lipid Vesicles

The hydrodynamic size changes of lipid vesicles were systematically characterized through particle size analysis under a range of pH levels, salt concentrations, and temperature conditions, as illustrated in Figure 2. MLVs exhibited enhanced dispersion stability relative to LUVs in neutral pH environments. However, all the vesicles were aggregated in acidic and basic solutions, attributable to diminished surface charge. MLVs maintained dispersion stability for 7 days in 70 and 140 mM NaCl, while LUVs showed a marked increase in diameter, surpassing 1 μm by the third day in 140 mM NaCl. At a higher salt concentration, a pronounced aggregation of all lipid vesicles occurred, likely due to the increased Debye screening effects at elevated ionic strengths. Remarkably, MLVs sustained dispersion stability over seven days across the entire temperature range examined. Additionally, the long-term dispersion stability of MLVs at 4, 25, and 37 °C was confirmed by measuring hydrodynamic size and turbidity, as shown in Figure S14 (Supporting Information). However, at 60 °C, above the phase transition temperature of DPPC at 41.3 °C, both size and turbidity began to decrease, indicating increased lipid fluidity that can induce greater structural fluctuations. These findings support the potential of MLVs as a durable lipid-based structure capable of retaining structural integrity under diverse environmental challenges.

2.3. Improved Robustness of Lipid Vesicles by Linker Ceramides

Förster resonance energy transfer (FRET) analysis was employed to elucidate the structural association and dissociation of LUVs and MLVs, as shown in Figure 3A. The lipid vesicles were labeled with 1 wt% of fluorescein-DHPE or TRITC-DHPE, which has fluorescence emission at 519 and 580 nm with excitation at 496 and 540 nm, respectively. These fluorescently labeled vesicles are referred to as f-LUVs or f-MLVs for fluorescein, and t-LUVs or t-MLVs for TRITC. A mixture of f-LUVs with t-LUVs...
and f-MLVs with t-MLVs was prepared to assess FRET efficiency. Over the seven-day incubation, the FRET efficiency of LUVs increased ≈1.54-fold, from 22.4 ± 1.1% to 34.5 ± 3.6% (Figure S15, Supporting Information), while MLVs showed a lower FRET efficiency compared to LUVs. Moreover, MLVs exhibited a slower initial rate of increase in FRET efficiency over the same period. These observations indicate that MLVs engage in fewer particle-to-particle interactions, such as fusion and rupture events, than LUVs, likely owing to their inherently stable and robust multilamellar structures.

Membrane fluidity within lipid vesicles was quantified by measuring the fluorescence anisotropy at varying concentrations of fetal bovine serum (FBS), as presented in Figure 3B. As a hydrophobic polarization probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) was integrated into the lipid membranes of pre-formed vesicles. It was observed that MLVs consistently exhibited lower fluidity compared to LUVs across all tested conditions, suggesting that the multilamellar architecture contributes to reduced membrane fluidity. In contrast, LUVs demonstrated an increase in fluidity with increasing FBS concentrations, implying a relative instability of the unilamellar membrane under these conditions. By contrast, the fluidity of MLVs remained relatively stable within the range of 10–50% FBS. This stability is ascribed to the multilamellar structures, which mitigate the vesicles' deformation in response to interactions with external biomolecules that can cause significant alterations in LUVs. [25,41] These findings underscore the robustness of MLVs against environmental changes, in contrast to the more sensitive LUVs.

The sustained release profile of cargo encapsulated within lipid vesicles was determined by incorporating fluorescein isothiocyanate-dextran (FITC-dextran) into the vesicles, followed by incubation within a dialysis membrane to monitor the diffusion of FITC-dextran into the dialysate (Figure 3C). Within the lipid vesicles, the actual loading yields of FITC-dextran in LUVs and MLVs were ≈0.28 and ≈0.54 wt%, respectively. During the first 24 h at 37 °C, the rate of FITC-dextran release from LUVs was ≈1.5 times greater than that from MLVs. After a week-long incubation, the cumulative release of FITC-dextran from LUVs was 34.5 ± 3.6%, whereas for MLVs, it was much lower at 22.9 ± 2.7%. The results support that MLVs possess a durable multilamellar architecture that effectively protects the entrapped cargo from external factors, facilitating release in a controlled manner, presumably upon reaching the targeted delivery site. Hence, these findings indicate that MLVs offer a viable and efficient delivery mechanism for the stable and controlled encapsulation of biomolecules.

2.4. Characterization of Multilamellar Nanovaccines

MLVs were further investigated as a nanovaccine platform incorporating OVA as the antigen and MPLA as the adjuvant (Figure 4A).
properties, MPLA was incorporated at 0.5 wt% into both LUVs and MLVs, henceforth referred to as mLUVs and mMLVs, respectively. Cryo-TEM confirmed the unilamellar and multilamellar structures of mLUVs and mMLVs, respectively (Figure S16, Supporting Information). The integration of MPLA into the vesicles led to an increase in membrane thickness. The mean diameter of the vesicles expanded from $238.1 \pm 23.5$ nm in mLUVs to $296.8 \pm 9.0$ nm in mMLVs, with the increased hydrodynamic size of mMLVs attributed to the additional lipid bilayers present as compared to mLUVs. mLUVs exhibited a negative surface charge of $-4.8 \pm 0.9$ mV, indicating their potential compatibility for in vivo applications. MPLA also contributed to membrane rigidity, resulting in reduced fluidity as evidenced in Figure S17 (Supporting Information). Next, OVA was employed as a model antigen. The resulting OVA-incorporated mLUVs and mMLVs were designated as mO-LUVs and mO-MLVs, respectively. Encapsulation efficiency for OVA was $65.3 \pm 6.0\%$ for mO-LUVs and $71.3 \pm 15.1\%$ for mO-MLVs (Figure S18, Supporting Information). Within the MLVs, the loading yield of MPLA and OVA were measured to be $\approx 0.20$ and $0.28$ wt%, respectively. The long-term colloidal stability of mO-MLVs at high FBS concentrations was confirmed (Figure 4B), and the fluorescence anisotropy analysis also exhibited lower membrane fluidity (Figure S19, Supporting Information), which corroborated the robustness of mO-MLVs.

Each vaccine formulation has specific storage requirements: the majority of vaccines need to be refrigerated, while some vaccines must be frozen until use. To maintain their potency and avoid side effects, it is critically important to keep vaccines within a proper temperature range. However, equipment failure or mishandling may lead to accidental freezing, thawing, and heating, which can cause irreversible damage and instability issues, such as denaturation and degradation of active compounds, aggregation and sedimentation of particles, and even bacteria growth. The operational sustainability of mO-MLVs under such conditions was investigated by assessing their colloidal stability across repeated freeze–thaw cycles. Remarkably, mO-MLVs retained their original hydrodynamic size after undergoing five cycles, while mO-LUVs exhibited the immediate formation of aggregates after the first cycle (Figure 4C). Our findings suggest that mO-MLVs present a highly stable vaccine formulation capable of withstanding large temperature variations.

Efficient antigen release from lipid vesicles in endolysosomal conditions is crucial for vaccine efficacy. We investigated this by analyzing the role of phospholipase A2 (PLA2) in Figure 4D, a key enzyme in phospholipid hydrolysis. Without PLA2, OVA release from the vesicles was notably poor under both neutral and acidic (pH 5.0) conditions. In contrast, PLA2 presence significantly enhanced OVA release, even though a minor decrease at low pH was observed, likely due to OVA’s reduced solubility near its iso-
electric point. This result suggests that MLVs when administered in vivo, could effectively release immunogenic antigens within dendritic cells under endolysosomal conditions.

2.5. Immune Responses by Multilamellar Nanovaccines

To investigate the immune responses of cells in vitro, murine bone marrow-derived dendritic cells (BMDCs) were treated with PBS, soluble OVA (sO), MPLA, soluble OVA with MPLA (sO + M), mO-LUVs, and mO-MLVs. Post a 24-hour incubation, mO-MLVs yielded a fluorescent signal exceeding the sO and sO + M groups by 1.6 times (Figure 5A; Figure S20, Supporting Information) but slightly higher than mO-LUVs. Significantly, mO-MLVs elicited a 2.6 to 4.1-fold increase in tumor necrosis factor-alpha (TNF-α) secretion from BMDCs over sO and sO + M (Figure 5B), and ≈1.2-fold more than mO-LUVs, indicating a potentiated immune response.

The expression of CD40, CD80, and CD86, which are immunological surface markers on BMDCs, was examined by flow cytometry (Figure 5C–E). The cells were first gated with the CD11c surface marker to select for the BMDCs from the rest of the cells. The results show that the expression levels of immunological surface markers for the cells treated with OVA and MPLA are similar to the PBS-treated control group. Considering the amount of OVA added to the cells (3 μg per 1 × 10^5 cells), this may not have been enough to elicit a more marked difference in the surface marker expression level. However, when analyzing the number of positive cells, it can be inferred that the DC maturation occurred after treatment of OVA and MPLA (Figure S21, Supporting Information). The lipid vesicle groups (mO-LUVs and mO-MLVs) showed a ≈1.4 to twofold increase in surface marker expression...
Figure 6. Long-term immune response evaluation in C57BL/6 mice postimmunization. A) Timeline illustrating the immunization schedule, where mice were treated with PBS, sO, MPLA, sO + M, mO-LUVs, and mO-MLVs at weeks 3, 6, and 9. B) Quantitative analysis of anti-OVA IgG serum levels in the treated mice, with measurements taken at weeks 3 (white), 6 (gray), and 9 (black). C) MFI of IFN-γ MFI of IFN-in the treated mice splenocytes, accessed at week 9. ** $p < 0.01$, *** $p < 0.001$, n = 4.

compared to the sO and sO + M groups. In addition, these groups had the highest distribution of cells positive for co-stimulatory protein markers (Figure S22, Supporting Information), suggesting enhanced uptake of lipid vesicles by BMDCs. In addition, the increases in OVA uptake of BMDCs, release of cytokine, and generation of immunological surface markers are due to co-delivery by the particulation of antigens and adjuvants. Unlike soluble antigens with adjuvants, the particulate antigens and adjuvants are easily engulfed into immune cells and can simultaneously interact with MHC I and MHC II receptors. [46] Note that mO-LUVs and mO-MLVs showed comparable levels of marker expression, leading us to further explore the impact of structural differences between LUVs and MLVs through in vivo experiments.

2.6. Enhanced Cross-Presentation by mO-MLVs

In our investigation of in vivo humoral and cellular responses to multilamellar vaccines, mice received three doses of either PBS, sO, MPLA, sO + M, mO-LUVs, or mO-MLVs at three-week intervals (Figure 6A). The mO-LUV and mO-MLV groups induced significantly elevated anti-OVA IgG levels, $\approx 14.4$ and $62.6$ times greater than the soluble groups, respectively, at the end of 9 weeks (Figure 6B). Compared to the lipid vesicles, the generation of antibodies with naked OVA was limited. Previous studies have reported on this matter, revealing that in vivo delivery of the bare antigen yielded poor immunological responses. [47-48] Instead, enhancement of the immune response via co-delivery of the antigen and adjuvant in a stable delivery carrier is considered to be more meaningful. The result indicates the superiority of particulate antigens and adjuvants in enhancing immune responses. Moreover, mO-MLVs elicited $\approx 4.4$ times the OVA-specific IgG concentration compared to mO-LUVs at 9 weeks, attributing to the more stable particulate delivery of antigens by mO-MLVs in vivo.

We utilized flow cytometry to assess IFN-γ accumulation in CD8⁺ T cells from splenocytes, evaluating the cellular immune boost provided by mO-MLVs (Figure 6C). Cytokine secretion was suppressed using a protein transport inhibitor in the splenocytes of vaccinated mice, while IFN-γ was allowed to accumulate following re-stimulation with the OVA257-264 (SIINFEKL) peptide. The mO-MLVs exhibited a higher mean fluorescence intensity (MFI) for CD8⁺IFN-γ compared to both soluble groups and mO-LUVs. The mO-MLVs, due to their structural in vivo instability, failed to generate significant cellular immunity. ELISPOT analysis confirmed that IFN secretion was the highest with mO-MLVs (Figure S23, Supporting Information), suggesting that mO-MLVs are more effective at inducing both humoral and cellular responses through robust antigen cross-presentation.

3. Conclusion

This work established 1-O-acylceramide, a natural component reinforcing the skin structure, as an effective physical linker for generating robust self-assembling multilamellar lipid vesicles, tailored for protein antigen delivery. These vesicles demonstrated high antigen encapsulation efficiency and maintained structural integrity under challenging conditions, including serum-rich solutions and through freeze-thaw cycles. Nevertheless, antigen release from the multilayered structure was promoted in
endolysosomal environments. In vitro, studies showed that unilamellar and multilamellar nanovaccines both effectively induced high expression of TNF-α and key immunological surface markers (CD40, CD80, and CD86) in dendritic cells. However, our multilamellar vesicles outperformed in vivo--they bolstered the production of antigen-specific IgG and IFN-g in CD8+ T cells by simultaneously stimulating humoral and cellular immune responses due to efficient antigen cross-presentation. The enhanced in vivo efficacy can be attributed to the enhanced structural stability of the MLVs after in vivo injection compared to LUVs. The MLVs shield the protein antigen from degradation by plasma proteins, and allow for the efficient co-delivery of the antigen and adjuvant to the immune cells, leading to a more profound immune response. Although the interlinking role of 1-O-acylceramide mediates the formation of the multilamellar membrane structure, it is very challenging to control the number of formed layers. It is expected that the concentration of 1-O-acylceramide is a critical factor in determining the number of layers. Further studies on controlling the number of lipid layers will be undertaken. As for the choice of antigen, OVA has been widely employed as a model antigen in previously reported works. However, this work focuses on the design and fabrication of robust MLVs using a physical linker and their immunological effects in general. We believe that our approach can be applied to various antigens with adjuvants that will require in vivo infection studies. More specific in vivo studies will be designed for clinically important antigens and adjuvants. Our findings highlight the critical role of sophisticated molecular design in optimizing delivery systems for vaccine efficacy.

4. Experimental Section

Materials: EcoCeramide ENP (73% 1-O-acylceramide, 16.3% ceramide NP (oyel), and 8.8% stearic acid) was provided from LCS Biotech (Suwon-si, Gyeonggi-do, Republic of Korea). MPLA and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Chloroform was bought from Junsei Chemical Co., Ltd. (Tokyo, Japan). Cholesterol, fluorescein isothiocyanate (FITC-dextran, 1,6-diphenyl-1,3,5-hexatriene (DPH), phospholipase A2, Triton-X, and uranyl acetate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate (SOS) was purchased from BIO-RAD Laboratories, Inc. ( Hercules, CA, USA). AF647-OVA, fluorescein-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (flourescein-DHPE), LysoTracker Green DND-26, and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). VECTASHIELD Vibrance Antifade Mounting Medium with DAPI was obtained from Vector Laboratories (Newark, CA, USA). Ovalbumin (OVA) was bought from Worthington Biochemical Corp. (Lakewood, NJ, USA). N-tetramethylrhodamine-6-isothiocarbamoyl—1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (TRITC-DHPE) was purchased from AAT Bioquest Inc. ( Sunnyvale, CA, USA). Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4 was obtained from GeneAll Biotechnology (Seoul, Republic of Korea). FV5780, FITC-CD11, BV421-CD40, APC-CD80, BV510-CD86, BV786-CD8, and AF647-IFN-g were purchased from BD Biosciences (Franklin Lakes, NJ, USA)

Preparation of Lipid Vesicles: DPPC, EcoCeramide ENP, and cholesterol were dissolved in chloroform at a concentration of 25 mg mL⁻¹. A lipid thin film was prepared by vacuum drying the chloroform for 16 h. The dried lipid thin film was hydrated with 1x PBS and incubated at 60 °C for 1 h. The hydrated lipid vesicles were downsized using a mini extruder kit (Avanti Polar Lipids Inc.) according to the manufacturer’s instructions.

Lipid vesicles formulated with EcoCeramide ENPs formed MLVs, whereas vesicles formulated without EcoCeramide ENPs formed unilamellar lipid vesicles (LUVs). The mean hydrodynamic diameter and zeta potential of lipid particles were measured using a zeta potential and particle size analyzer (ELS-7000, Otsuka Electronics, Osaka, Japan).

TEM and Cryogenic TEM: The lipid vesicle solutions were dropped on coverslips and grids and incubated for 1 min, followed by drying with filter paper. A drop of 2% uranyl acetate solution was added to the grids and incubated for 1 min for contrast. The negatively stained vesicles were observed with a TEM (Tecnai F20, FEI Company, Hillsboro, OR, USA) operating at an acceleration voltage of 200 kV. To obtain more clear images, cryogenic TEM (cryo-TEM) observation was carried out. Cryo-TEM samples were prepared at 8 °C with a humidity of 100% using an automated vitrification device (Vitrobot Mark IV, Thermo Fisher Scientific). Lipid vesicle solution (3.2 µl) was applied onto glow discharge-treated lacey carbon-supported copper grids and blotted with a filter paper, followed by plunge-freezing in liquid ethane. The prepared samples were analyzed using a cryo-TEM (Glacios, Thermo Fisher Scientific) operating at an acceleration voltage of 200 kV. The images were recorded and investigated using Smart EPU Software (Thermo Fisher Scientific).

Small Angle X-Ray Scattering (SAXS): To investigate lamellar structures of lipid vesicles, SAXS analysis was performed at the 4C SAXS II beamline at the Pohang Accelerator Laboratory (Pohang, Republic of Korea). The measurement was repeated six times and averaged, with each measurement consisting of 5 s exposure at 25 °C, 1 m of sample-to-detector distance, and 0.734 Å of X-ray beam wavelength. Bovine serum albumin was used as a reference material to adjust the scattering angle (θ) between 0.01 and 1.0 Å⁻¹ (interlayer distance = 2e/a).

Fluidity of Lipid Vesicles: Membrane fluidity was calculated from the fluorescence polarization of DPH in lipid vesicles. DPH was incorporated into pre-formed lipid vesicles with a lipid-to-DPH weight ratio of 104. The lipid vesicles with DPH were incubated for 1 h at room temperature before mixing with FITC- and TRITC-labeled lipid vesicles. The fluorescence polarization of the lipid vesicles was measured by a fluorescence spectrophotometer (F-7000, Hitachi Ltd., Tokyo, Japan). The lipid vesicles were excited with vertically polarized light (360 nm) and the emission (430 nm) was observed at both parallel and perpendicular positions.

FITC-Dextran Release from Lipid Vesicles: To check the release of encapsulated material of the MLVs, PBS containing FITC-dextran was used to encapsulate the fluorescent dye during lipid thin film hydration. Unencapsulated FITC-dextran molecules were separated by pelleting the MLVs with ultracentrifugation at 84 000 rpm (193 617 × g) at 4 °C for 2 h (Optima TLX Ultracentrifuge, Beckman Coulter, Pasadena, CA, USA). The unencapsulated FITC-dextran was quantified using a microplate reader (CLARION, BMB Labtech International, Germany, and Tecan, Germany).

FRET Analysis: Fluorescein-DHPE or TRITC-DHPE were dissolved in chloroform with other lipids at 2.5 wt%. Fluorescent-labeled lipid vesicles were prepared using the same procedures as unlabelled lipid vesicles. FRET efficiencies were determined with a microplate reader.

Encapsulation Efficiency of OVA: During the lipid thin film hydration step, PBS containing OVA was used to encapsulate the OVA in the MLVs. To check the encapsulation efficiency, unencapsulated OVA molecules were separated by pelleting the MLVs with ultracentrifugation at 84 000 rpm (193 617 × g) at 4 °C for 2 h. The OVA in the supernatant was quantified using a BCA protein assay kit (Thermo Fisher Scientific).

OVA Release from Lipid Vesicles: Fluorescently labeled OVA (AF647-OVA) was used to measure the OVA release from lipid vesicles. Soluble AF647-OVA, AF647-OVA-encapsulated mLUVs, and AF647-OVA-encapsulated mMLVs were incubated in a dialysis membrane with a molecular weight cut-off of 100 kDa (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) at 37 °C for 24 h in 50 mM sodium citrate buffer (pH 5.0) containing 0.5 µL mL⁻¹ phospholipase A2 and 140 mM NaCl. The fluorescence intensity of the released OVA from lipid vesicles was examined by a microplate reader.

Isolation of BMDCs: Bone marrow-derived dendritic cells (BMDCs) were obtained from the femur and tibia of 6-week-old male C57BL/6 mice (Koatech, Pyeongtaek-si, Gyeonggi-do, Republic of Korea). The extracted BMDCs were suspended in 20 mL of HBSS buffer and washed...
twice at 250 x g for 8 min. The collected cells were counted and diluted to a final concentration of 1.0 x 10^7 cells mL^-1 in RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin. For BMDC maturation, 0.2 mL of the cell stock was added to 9.6 mL of the RPMI-1640 medium and stimulated with 0.2 mL of 1 μg mL^-1 recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF, R&D Systems, McKinley Place NE, MN, USA) in a CO_2 incubator (37 °C, 5% CO_2, 95% humidity) for 6 days. The cultured cells were collected through centrifugation at 250 x g for 8 min.

**Cellular Biochemical Analysis of Dendritic Cells:** The cultured BMDCs were seeded to 24-well plates at a cell density of 1.0 x 10^5 cells well^-1. The cells were treated with PBS, OVA in buffer (so), MPLA, OVA and MPLA mixture (so + M), MPLA and OVA-loaded LUVs (mO-LUVs), and MPLA and OVA-loaded MLVs (mO-MLVs) and incubated in a CO_2 incubator (37 °C, 5% CO_2, 95% humidity) for 24 h. The cells were fluorescently stained with FITC-780 dye for 15 min to discriminate live cells from dead cells during flow cytometry analyses. The cells were washed twice and stained with DAPI and LysoTracker Green DND-26 for confocal microscopy analysis. The TNF-α cytokine released from immunized BMDCs was calculated using a mouse TNF-α ELISA kit (Mouse TNF alfa ELISA Kit, Abcam, Cambridge, UK) according to the manufacturer’s instructions.

**Long-Term Vaccination:** The protocol for the animal studies was approved by the KAIST Institutional Animal Care and Use Committee (KA2024-037-v1). Immunization of C57BL/6 mice (6-weeks-old, male) was performed with PBS, OVA in buffer (so), MPLA, OVA and MPLA mixture (so + M), MPLA and OVA-loaded LUVs (mO-LUVs), and MPLA and OVA-loaded MLVs (mO-MLVs) and incubated in a CO_2 incubator (37 °C, 5% CO_2, 95% humidity) for 24 h. The collected cell suspensions were labeled with fluorescein isothiocyanate (FITC-CD11, BV421-CD40, BV510-CD86) and Alexa Fluor 780 dye (mO-MLVs, mO-M, OVA, and MPLA) for 15 min to discriminate live cells from dead cells during flow cytometry analyses. The cells were washed twice and incubated for 30 min to discriminate live cells from dead cells during flow cytometry analyses. The cells were washed twice and suspended in a Stain Buffer. Compensation beads (BD Biosciences) were used for the single-stained control samples. Finally, the cells were transferred to 5 mL round bottom tubes with a cell strainer snap cap and analyzed using flow cytometry (LSRFortessa, BD Biosciences) with a 530-nm excitation filter for FITC and a 670-nm band-pass filter for BV421. The BMDCs were calculated using a mouse TNF-α ELISA kit (Mouse TNF alfa ELISA Kit, Abcam, Cambridge, UK) according to the manufacturer’s instructions. To quantify OVA-specific IgG in the serum, a Mouse Anti-OVA IgG kit (Chondrex, Inc., Redmond, WA, USA) was used. After serum collection from the tail veins of the immunized mice on weeks 3, 6, and 9, the serum was incubated with 10 μg of OVA and 1.25 μg of MPLA by subcutaneous injection in the loose skin over the neck three times (0, 3, and 6 weeks). Blood samples were collected from the tail veins of the immunized mice on weeks 3, 6, and 9 and were incubated for 30 min at room temperature to clot the red blood cells, followed by centrifugation (1500 x g for 10 min at 4 °C) to obtain the serum. To quantify OVA-specific IgG in the serum, a Mouse Anti-OVA IgG Antibody Assay kit (Chondrex, Inc., Redmond, WA, USA) was used. After 9 weeks, the mice were euthanized and the spleenocytes were isolated from the spleen using a cell strainer. The isolated spleenocytes were counted and seeded onto 24-well plates at a concentration of 1.0 x 10^6 cells well^-1. To investigate the accumulation of IFN-g cytokine in CD8^+ T cells, the cells were stimulated with 10 μg mL^-1 of OVA257-264 (SIINFEKL) peptide for 24 h and processed with BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences). The fixed and permeabilized cells were stained with BV786-CD8 and AF647-IFN-g antibodies. Mouse IFN-gamma ELISPot Kit (R&D Systems) was used to quantify the secretion of IFN-g from CD8^+ T cells according to the manufacturer’s manual.

**Statistical Analysis:** The raw data in all experiments were obtained more than three times and calculated as the mean ± standard deviation. To evaluate the statistical significance using the p-value, the Student’s t-test was performed.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

**Acknowledgements**

B.I.K. and D.J.L. contributed equally to this work. This research was supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HV22C0209 and HP20C0018). Experiments at PLS-II were supported in part by MSIP and POSTECH.

**Conflict of Interest**

No. The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords**

1-O-acylceramide, adjuvants, antigens, multilamellar vesicles, vaccine delivery

Received: November 22, 2023
Revised: May 15, 2024

Published online: