Polyethylenimine: An Intranasal Adjuvant for Liposomal Peptide-Based Subunit Vaccine against Group A *Streptococcus*

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ABSTRACT

Group A *Streptococcus* (GAS) and GAS-related infections are a worldwide challenge, with no commercial GAS vaccine available. Polyethylenimine (PEI) attaches to the cells surface and delivers cargo into endosomal and cytosolic compartments. We hypothesized that this will confer mucosal adjuvant properties for peptide antigens against group A *Streptococcus* (GAS). In this study, we successfully demonstrated the development of PEI incorporated liposomes for the delivery of a lipopeptide-based vaccine (LCP-1) against GAS. Outbred mice were administrated with the vaccine formulations intranasally, and immunological investigation showed the PEI liposomes elicited significant mucosal and systemic immunity with the production of IgA and IgG antibodies. Antibodies were shown to effectively opsonize multiple isolates of clinically isolated GAS. This proof-of-concept study showed the capability for PEI liposomes to act as a safe vehicle for the delivery of GAS peptide antigens to elicit immune responses against GAS infection, making PEI a promising addition to liposomal mucosal vaccines.

KEYWORDS

Liposome; Vaccine Delivery System; Group A *Streptococcus*; Subunit vaccine; Polyethylenimine; Peptide.

Adjuvant discovery remains an important area of vaccine research, and the requirement to demonstrate the safety and importance of new adjuvants has hampered their acceptance, resulting in limited licensing of vaccines containing novel adjuvants.¹⁻² Subunit vaccines are generally made up of small fragments of pathogen-derived proteins or small peptide antigens.³ The use of these small selected antigens/peptides with the exclusion of unnecessary elements enhances the safety profile of a subunit vaccine; however, this is at the cost of significantly reduced immunogenicity.³ Compared with live attenuated or killed pathogen-based vaccines, subunit vaccines usually require adjuvants (immune stimulants) to improve their performance.⁴⁻⁵ Vaccines have many routes of administration, including subcutaneous, intramuscular and mucosal delivery. From these, intranasal mucosal delivery has many benefits, including a highly vascularised mucosal surface; needle free and non-invasive administration; the requirement for a small antigenic dose; free from enzyme degradation in the digestive system; and induction of the systemic and mucosal immunity inducing a rapid local and protective immune response in the lungs and upper respiratory tract (important for pathogens that infect through the respiratory tract mucosa).⁶

Despite these benefits, to date no mucosal adjuvant formulations have been licensed for use in humans. This is largely due to safety concerns linked to local or systemic toxicity or reactogenicity, and the development of mucosal vaccines was hindered by the empiric trials and non-comprehensive methods in the safety evaluation stages.⁷ To date, only limited experimental mucosal adjuvants are currently used, including cholera toxin and its B subunit, which is not suitable for use in humans.^{1, 8-9}

Polyethylenimine (PEI) represents a family of organic, hydrophilic cationic polymers with linear or branched topologies (Figure 1). PEI has a strong positive-charge enabling *in vitro* and *in vivo*

condensation of negatively-charge particles (e.g. DNA) via electrostatic interactions to form nanoscale ‘polyplexes’.¹⁰ PEI also contains a powerful network of primary, secondary and tertiary amine groups enabling numerous possibilities for structural modification.¹¹⁻¹² As a result, PEI has been widely used in broad fields of application, including effluent treatments, carbon dioxide absorption, and the separation and purification of proteins. PEI, as a cationic polymer, can also promote the maturation of macrophages via TLR-4 to enhance an immune response.¹² However, since the 1995 discovery of DNA transfer into nerve cells by PEI/DNA nanoparticles by Boussif and co-workers, PEI has drawn more attention in the biomedical field, principally as drug carriers, biological labels, and vaccine adjuvants.¹³

Figure 1. Branched and linear PEI. PEI 600 used in this study is branched and has an approximate ratio of 25:50:25 (primary:secondary:tertiary) amino groups.

PEI-coated nanoparticles, used as drug delivery systems, have been shown to promote cellular uptake, improving an immune response.¹⁴⁻¹⁶ Delivery systems containing PEI have been shown to be taken up into cells expressing heparin sulphate proteoglycans, including antigen-presenting cells, which are critical in immunological vaccine delivery.¹ In a series of recent studies, vaccines composed of PEI as the immune stimulant have been successful in treating infections or tumors.¹⁷⁻

¹⁸ For example, PEI was shown to act as a versatile systemic adjuvant for induction of structure-reactive antibodies against glycoprotein antigens via the mucosal route against influenza and herpes simplex virus-2 following a single intranasal immunization.¹⁷ In addition, *Angelica sinensis* polysaccharide (ASP) encapsulated poly(lactic-co-glycolic acid) (PLGA) nanoparticles coated with PEI, (ASP-PLGA-PEI) significantly enhanced a porcine circovirus type 2-specific IgG immune response demonstrating that the positively charged ASP-PLGA-PEI nanoparticles have the potential to serve as an effective vaccine delivery and adjuvant system to induce vigorous and long-term immunity.¹⁹ The derivatives of PEI have also been broadly used in gene and vaccine delivery.²⁰ Further, PEI-based nanocomplexes were employed to deliver the human papillomavirus protein and DNA as a vaccine with moderate success, which showed the addition of PEI can improve the cellular uptake and enhance the gene knockdown effects *in vitro*.^{18, 20}

In this study, we aimed to investigate an intranasal mucosal vaccine delivery system to deliver vaccine constructs against group A *Streptococcus* (GAS). GAS is a major human pathogen that causes health implications ranging from superficial infection to life-threatening rheumatic heart disease.²¹⁻²² GAS is a worldwide problem that disproportionally affects disadvantaged populations. A recent global burden of disease study estimated that rheumatic heart disease, caused by an untreated infection with GAS, affects 34,000,000 people worldwide with around 345,000 deaths per year. The mortality rates associated with rheumatic heart disease and the invasive deaths associated with GAS infections (an additional 160,000 deaths per year) put GAS as the fifth leading cause of single-pathogen infectious diseases deaths, behind HIV, tuberculosis, malaria, and *Streptococcus pneumoniae*.²¹⁻²² The main infection route of GAS is the contact and binding of

the surface M protein to the mucosal membrane.²³ No licensed or marketed vaccine is currently available for the prevention and/or treatment of GAS infection.²⁴

Nanoparticle delivery systems have been widely investigated as new vaccines strategies to enhance the immune responses to antigens against infectious diseases. Positively charged nanoparticles could efficiently improve the immune responses due to targeting and activating antigen-presenting cells. Liposomes are a series of nanoparticles with bilayered phospholipid membrane structure.²⁵ In this study, we assessed the immunological properties of PEI-containing liposomes composed of cholesterol and dipalmitoylphosphatidylcholine (DPPC) with a self-adjuvating B and T cell lipopeptide (**LCP-1**; Figure 2). **LCP-1** is comprised of a lipid core (Ser-Ser-C₁₆-C₁₆, where C₁₆ refers to the lipid 2-amino-1-hexadecanoic acid), the B-cell antigen (J8) developed from the GAS surface M protein and the universal T helper epitope (P25) (Figure 2). We were interested to see if PEI-conjugated to a lipid (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, DOPE or palmitic acid, PAM), to help anchor PEI onto the surface of the liposomes, would enhance the immune response compared with liposomes formulated with non-lipidated PEI. To evaluate the vaccines adjuvanting properties, two positive controls were used including: the commercial adjuvant CTB in a mixture with **Pep-2** (non-lipidated GAS B and T helper peptide; Figure 2) and dimethyldioctadecylammonium bromide (DDAB) liposome incorporating **LCP-1** to compare with previous work.²⁶

Figure 2. Schematic illustration of the PEI-liposome vaccine candidates. Non-lipidated PEI liposome: PEI 600 (branched) not incorporate to DOPE or palmitic acid in the liposome formulation. Lipidated-PEI incorporated liposomes: branched PEI 600 incorporate to DOPE or palmitic acid. Experimental control liposomes: DDAB liposome (DDAB, cholesterol and DPPC) and neutral liposome (cholesterol and DPPC only). Included in each formulation is **LCP-1**: GAS B cell antigen (J8; QAEDKVKQSREAKKQVEKALKQLEDKVQ) and the universal T helper antigen (P25; KLIPNASLIENCTKAEL) conjugated to a lipid core (Ser-Ser-C₁₆-C₁₆). PEI 600 (branched) was included in **L2**, and branched PEI 600 conjugated to palmitic acid (or DOPE) was

included in **L3** (or **L4**). **Pep-2**, one of the controls in this study, the same sequence as **LCP-1** without the lipid core Ser-Ser-C₁₆-C₁₆.

RESULTS AND DISCUSSION

Design and synthesis of peptides. Vaccines have many routes of administration, including subcutaneous and intramuscular injection, or intranasal and oral delivery. Intranasal delivery has many benefits, including decreased pain upon administration and increased patient compliance.²⁷ Intranasal vaccine delivery is suitable for prevent against the pathogens infected via respiratory tract (e.g. GAS infection via pharynx), where intranasal vaccines are able to elicit both mucosal secretory IgA and systemic IgG antibodies production to reduce GAS colonization in the throat.²⁸ However, peptide-based mucosal vaccines lack effective delivery and adjuvanting systems that ensures the peptide epitopes are protected from degradation and induces strong mucosal and systemic immune responses.²⁹⁻³⁰ In this study, PEI-adjuvanted liposomal delivery systems have been applied to lipo-peptide-based vaccine constructs to enhance their *in vivo* immunity.

To compare the formulations of the PEI-adjuvanted liposomes, in this study we investigated different PEI-adjuvanted liposome delivery systems for GAS vaccine constructs (Figure 2). Due to the cross-reactivity of the GAS M protein with human cardiac cells, GAS vaccine constructs have focused on using small peptide antigens derived from the GAS M protein to provide protection against a broad spectrum of GAS strains, while free from any autoimmune responses. In this study, we used the GAS J8 epitope as the B cell epitope. J8 was identified from the conserved C-terminal of the GAS M protein and has been flanked by a GCN4 yeast peptide to maintain the epitopes native α -helix structure. J8 has been shown to be capable of stimulating

humoral immunity while not stimulating T cell responses.³¹ However, the J8 antigen lacks the ability to activate the CD4⁺ T helper cells to efficiently deliver the antigen to B cells. Therefore, the addition of a safe foreign T helper epitope for enhanced major histocompatibility complex binding was necessary for vaccine development. The P25 epitope is a universal T helper epitope derived from the fusion protein of canine distemper virus and is essential for binding to the peripheral blood mononuclear cells, especially the CD4⁺ T helper cells, which are critical to activate B cells.

The lipid core sequence (Ser-Ser-C₁₆-C₁₆) has been shown to be a TLR-2 targeting moiety that improves antigen recognition and enhance antigen presenting cell uptake.^{11, 32-33} TLR-2 primarily recognizes bacterial cell wall components, including lipoproteins and peptidoglycans, and is critical in the immune response associated with pathogen associated molecular patterns, inducing the CD4⁺ T helper cell response. Vaccines containing lipid adjuvants have been shown to stabilize peptide antigens from degradation and have increased stability during storage. The lipopeptide tails are hydrophobic and allow insertion into the liposome lipid bilayers, directing the peptide antigens both outside and inside the liposome. In this study, a peptide without a lipid core (**Pep-2**), formulating with a traditional commercial adjuvant (CTB), was designed to compare the immunological response with the lipid core peptide (**LCP-1**) (Figure 2). Both peptides were successfully synthesised, purified and characterised as previously reported.³⁴

Liposome formulation. To date, liposomes are widely used in the pharmaceutical industry as a novel biocompatible and biodegradable carrier to enhance the potency, improve the water solubility and reduce the toxicity of chemicals. Cationic liposomes were used in this study and

have been formulated with DPPC and cholesterol using the lipid film hydration method previously reported (Scheme 1).²⁶ Notably, DPPC is a zwitterion lipid which is neutrally charged, and DDAB is positively charged. LCP-1 has a 10:8 positive to negative amino acid charge ratio. Cholesterol is a neutral lipid, and the addition of cholesterol reduces the overall charge of the liposome by decreasing the sodium ion binding capacity onto the liposomes surface.³⁵ Liposomes were formulated to be 100 nm – 200 nm in size with a low polydispersity index (PDI) (Table 1 and Supporting Information Figures S1 – S5). A PDI between 0.1 and 0.4 suggests the sample is moderately polydisperse where, ideally, a lower PDI is better in vaccine development showing uniformity.³⁶ Liposomes with these characteristics are in the similar size to viruses and *mycoplasma*, and liposomes have been shown to be able to travel to lymph nodes and endocytosed by dendritic cells, important for the antigen up take by the antigen-presenting cells to enhance the immunity and induce the local and systemic immune response.³⁷ Specifically, **L1-L5** liposomes were positively charged with a Zeta potential around 40 mV (Table 1), with **L3** and **L4** showing a higher charge than other groups. This positive charge can be associated with the inclusion of the positively charged **LCP-1** lipopeptide, with the addition of a cationic polymer (PEI) or lipid (DDAB) into the formulation being shown to increase the charge (**L2-L5**, Table 1). Further, this small increase in charge also suggested that both PEI and DDAB were likely incorporated into the inner surface of the liposome.

Scheme 1. Liposome formulation using the lipid film hydration methods: (A) Formulation of **L2**.
(B) Formulation of **L3**.

Table 1. Physicochemical characterisation of liposomal constructs.

Liposome	Diameter (nm) $\pm$ STD	Polydispersity Index (PDI) $\pm$ STD	Charge (mV) $\pm$ STD
L1	135 $\pm$ 1	0.114 $\pm$ 0.004	+38 $\pm$ 2
L2	138 $\pm$ 3	0.09 $\pm$ 0.02	+39 $\pm$ 1
L3	137 $\pm$ 2	0.159 $\pm$ 0.005	+41 $\pm$ 1
L4	133 $\pm$ 1	0.11 $\pm$ 0.01	+43 $\pm$ 1
L5	148 $\pm$ 2	0.080 $\pm$ 0.009	+39.7 $\pm$ 0.3

TEM images (Figure 3) indicated that **L2** formed typical spherical structures often seen with liposomes, uniform in size in the solution, which were a similar size to that observed by DLS.

Figure 3. Transmission electron micrograph (TEM) of **L2** stained with 2% ammonium molybdate.

Cytotoxic study. Cytotoxic evaluation was performed on **LCP-1**, **LCP-1+PAM-PEI** and **L1-L5** against two types of cell lines, HEK293 (human embryonic kidney cells) and SW620 (human colorectal cancer cells). In this assay, the commercial chemical doxorubicin was used as a positive control, and 1% DMSO in PBS was used as the negative control. In the cytotoxicity assay, the alive cells reduce the chemical dye, MTT, to a dark-blue formazan crystal³⁸. The percentage of alive cells after treatment could then be estimated by measuring the absorbance of the formazan crystal at 600 nm.

The cytotoxicity study was conducted at two different concentrations for each vaccine construct, 2 $\mu\text{g}/\mu\text{L}$ (the same as the concentration used *in vivo*) and half of the *in vivo* concentration (1 $\mu\text{g}/\mu\text{L}$) on both cell lines (Supporting Information, Figure S6). Interestingly, two vaccine groups, **LCP-1+PAM-PEI** and **L3** showed a substantial toxicity against both cell lines tested, while the rest of

vaccine groups showed a mild toxicity (Supporting Information, Figure S6). The results suggested that the incorporation of PEI to palmitic acid increased the cytotoxicity of the tested formulations, while the introduction of liposomes to **LCP-1** did not alter the vaccines cytotoxicity. The addition of DOPE also did not alter the liposomes cytotoxicity, and when compared to the DDAB liposomes (**L5**, a published formulation used as an experimental control in this study²⁶), the results suggested that both PEI and DDAB showed a similar cytotoxicity in our formulations.

In vivo immunological assessment. *In vivo* immunological evaluation was performed in outbred female ARC Swiss mice (Figure 4A). All mice received four intranasal immunizations (a single primary immunization and three boosts) on days 21, 42 and 63 under anesthesia with isoflurane. Mice (5 mice per group, 7-8 weeks old, female) were intranasally administered (15 μ L /nostril) on days 0, 21, 42, and 63 with vaccine constructs (**LCP-1**, **LCP-1+PAM-PEI**, **L1-L5**; Figure 4A). The positive control group were administered **Pep-2** (60 μ g in 30 μ L sterile Milli-Q water; 15 μ L /nostril) co-administered with commercial CTB adjuvant (10 μ g). The negative control group received sterile Milli-Q water (15 μ L /nostril). Blood was collected from the tail tip one day prior to each immunization and 14-days following the final boost. Sera was analyzed using ELISA for total antigen-specific IgG antibodies. Saliva was collected 7-days post each immunisation and analyzed by ELISA for antigen-specific IgA antibodies.

Following the third boost, sera from mice that received the **L2** vaccine showed a significant saliva IgA antibody titre when compared to sera of mice from the negative control group ($p < 0.01$, negative control: sterile Milli-Q water) and positive control (**Pep-2+CTB**) (Figure 4B). Sera of mice from vaccine groups **LCP-1+PAM-PEI** and **L1** also showed a significant IgA antibody titre when compared to the negative control, water ($p < 0.05$). Sera IgG antibody titres were similar to

those observed for IgA with L2 showing a significant IgG antibody titre when compared with the negative control group, water ($p < 0.01$) (Figure 4C). All other vaccine groups showed no significant difference in the development of antibodies when compared to the negative control group. Interestingly, the variation in antibody titres for all groups, excluding the water and CTB controls, showed increased variation (Figure 4B and 4C). The outbred mice used in this study were a heterogeneous population. Like the human population, their genetical and phenotypical types were different within the population of mice used in this study. As a result, a larger variation of IgG and IgA titers are observed with a reduction in IgG and IgA responses when comparing to the previously published result for L4 group.²⁶ This may also be attributed to the different GAS antigens used, J8 epitope was used in this study versus the GAS J14 epitope in a previous report.

Figure 4. (A) Immunization schedule with the immunization, saliva and serum collection points in days. (B) J8 antigen-specific IgA responses obtained in mice saliva on day 77. (C) J8 antigen-specific IgG responses obtained in mice sera on day 70. **L1:** liposome/**LCP-1**; **L2:** non-lipidated PEI liposome/**LCP-1**; **L3:** PAM-PEI incorporated liposome/**LCP-1**; **L4:** DOPE-PEI incorporated liposome/**LCP-1**; **L5:** DDAB incorporated liposome/**LCP-1**. Geometric mean of antigen-specific IgG titres are represented as a horizontal bar for each group of five mouse. Statistical analysis was performed by one-way ANOVA followed by Tukey post hoc test where, probability value of $p < 0.05$ was considered statistically significant (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Liposomal intranasal vaccine administrations are able to induce both systemic and mucosal immunities. More specifically, the adaptive humoral immune defense in mucosa is to a large extent mediated by secretory IgA antibodies, which has been shown to be important in the early stage of infection to prevent the GAS binding to the mucosal cells. A stronger systemic immunity, mediated by sera IgG, has been shown to be critical for protection against an invasive infection.²⁶ Pandey and co-workers showed the importance of IgA in controlling GAS infection in a mice model, where antigen-specific IgA contributed to the survival of mice following GAS infection, even without significant IgG production.³⁹ Overall, the highest IgA and IgG antibody titers can be seen in sera obtained from the mice vaccinated with **L2**. **L5**, which are positively charged liposomes with DDAB. In the study, the sera collected from the mice that received the **L5** vaccine showed both lower IgG and IgA antibody titres than **L2**, which suggested the importance of PEI in liposome vaccine delivery (Figure 4B and 4C). **L3** and **L4**, containing PAM-PEI and DOPE-PEI respectively. The sera collected from mice vaccine groups, **L3** and **L4**, showed lower IgA antibody

titers than **L2**, suggesting the importance of the use of free PEI rather than the lipidated derivatives (Figure 4C).

***In vitro* opsonization study.** To test the opsonic activity of the IgG antibodies elicited by the vaccine candidates and formulations, we used five clinical GAS isolates from Australian patients (Figure 5). The pooled sera obtained from the mice immunized with **L2** (non-lipidated PEI liposome/LCP-1) showed significantly higher levels of opsonic activity against all five tested GAS isolates compared to the negative control (water), **LCP-1**, **LCP-1+PAM-PEI**, **L1**, **L3** and **L4** (Figure 5). No significant difference in opsonic activity between **L2** and the positive control (CTB group) was observed (Figure 5). The average opsonic activities of serum collected from mice treated with **L2** ranged from 60 % to 75 %, while the opsonic activities of serum collected from the positive control (CTB) mice ranged from 50 % to 71 %. However, the pooled sera from mice treated with **LCP-1**, **LCP-1+PAM-PEI** and **L3** did not have any significant opsonic activities against all isolates compared to the negative control (water). The opsonization results suggested that the sera from mice vaccinated with **L2** showed significant protection against GAS infection, and the sera from the mice vaccinated with **L2** showed higher opsonic rate than the positive control (CTB) group against three of five GAS isolates. The low opsonic rates of **L3** and **LCP-1+PAM-PEI** groups are related to the low average sera IgG antibody titres (Figure 4). This also suggested the importance of the PEI-adjuvanted liposomal delivery system (**L2**) in the opsonic study when comparing with **LCP-1** and **LCP-1+PAM-PEI** groups.

Figure 5. Average opsonization (duplicate from two independent cultures) of sera obtained from mice on day 77 against five different clinical isolates (ACM-2727, ACM-2002, D2612, D3840, GC2 203) of GAS. **L1:** liposome/**LCP-1**; **L2:** non-lipidated PEI liposome/**LCP-1**; **L3:** **PAM-PEI** incorporated liposome/**LCP-1**; **L4:** **DOPE-PEI** incorporated liposome/**LCP-1**; **L5:** DDAB incorporated liposome/**LCP-1**. Negative control pooled sera from five mice received Milli-Q water only. Results are represented as opsonization percentage compared to untreated wells as a reference and error is represented as standard error of the mean (SEM). Statistical analysis was performed by one-way ANOVA followed by Tukey post hoc test where, probability value of $p < 0.05$ was considered statistically significant (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ****, $p < 0.0001$). CFU = Colony forming unit.

CONCLUSION

GAS infection and its associated diseases are global challenges with no commercial vaccine currently available. In this study, we successfully synthesised a lipo-peptide based GAS vaccine candidate comprised of a GAS B cell epitope (J8), universal T helper epitope (P25) and lipid adjuvant (Ser-Ser-C₁₆-C₁₆), formulated into liposomes containing PEI. Following a single primary immunisation and three boosts of the liposomes to mice intranasally, mice developed both mucosal and systemic immune responses in the form of IgG and IgA antibody production, respectively. Sera collected from the mice that received the **L2** (non-lipidated PEI liposome with **LCP-1**) vaccine showed the highest IgG and IgA antibody titres when compared to sera from other experimental groups and the positive control group. The antibody developed by the mice that received the **L2** vaccine also showed significantly higher opsonic activities against all five clinical isolates of GAS tested. In the cytotoxicity study, non-lipidated PEI liposomes showed similar

cytotoxicity to DDAB liposomes (a published formulation). All the results above suggested that liposomal vaccine formulations with PEI were able to induce both mucosal and systemic immune responses with low cytotoxicity, making a promising advance in the development of mucosal vaccines. In addition, we also highlighted that liposomes containing palmitic acid lipidated with PEI (**PAM-PEI**) had a high cytotoxicity, which should be drawn to attention in future GAS vaccine development studies. In future, animal challenge studies will be applied to validate the *in vivo* efficacy and protection of the vaccine formulation, in addition to analysis of secretory IgA following nasal washing.

METHODS

General methods. All chemicals used were of analytical grade unless otherwise stated. Chromatography grade chloroform, methanol, skim milk powder, Tween-20 and other solvents were purchased from Merck (Kilsyth, VIC, Australia). PEI (MW 600, branched) were purchased from Alfa Aesar (Ward Hill, MA, United States). 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) sodium salt (NGPE), DPPC and cholesterol were purchased from Avanti (Alabaster, AL, U.S.). DDAB, CTB and SIGMAFAST™ O-phenylenediamine dihydrochloride (OPD) tablets was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Goat anti-mouse IgG (H+L)-HRP conjugate secondary antibody was purchased from Bio-Rad (Gladesville, NSW, Australia). Thermo Scientific Snakeskin® Pleated Dialysis Tube purchased from Thermo Scientific (Victoria, Australia). All other reagents were purchased from Merck or Sigma-Aldrich.

Dynamic light scattering (DLS) measurements were taken using a Zetasizer Nano ZP instrument (Malvern Instrument, UK) with Dispersion Technology software. Particle images were captured using a JEM-1010 transmission electron microscope (TEM) (JEOL Ltd., Japan). 96-Well microtest plates (flat base, polystyrene, high binding) were purchased from Sarstedt (Mawson Lakes, SA, Australia). ELISA Optical Density (O.D.) values were read by the SpectraMAX 250: 96-well plate reader purchased from Molecular Devices (San Jose, CA, USA). All data was analysed with GraphPad Prism 8.3.1 software.

Peptide synthesis. LCP-1 and Pep-2 were synthesized and purified as previously reported by Dai *et al.*³⁴

PAM-PEI and DOPE-PEI synthesis. PAM-PEI and DOPE-PEI were synthesised according to the previously published method described by Incani *et al.* and Hussein *et al.*^{20, 40} The concentrations of PEI in DOPE-PEI and PAM-PEI was determined by the 2,4,6-trinitrobenzene sulfonate (TNBS) assay. Yield: DOPE-PEI 0.826 mg (11%), PAM-PEI 13.3 mg (48%).

Non-liposomal vaccine constructs nasal dropping solution preparation. LCP-1: LCP-1 (450 µg) was added into sterile Milli-Q water (225 µL) and sonicated (2 min). LCP-1 + PAM-PEI: LCP-1 (450 µg) and PAM-PEI (250 µg) were added to sterile Milli-Q water (225 µL) and sonicated (2 min). CTB + Pep-2: Pep-2 (450 µg) and CTB (75 µg) were added to sterile Milli-Q water (225 µL) and sonicated (2 min).

Formulation of liposomal vaccine constructs into nasal dropping solution. Liposomes (**L1-L5**, Figure 2) were formulated using the lipid film hydration method previously reported.²⁶ Briefly, all lipids (DPPC, cholesterol, lipo-PEI, or DDAB) were dissolved in chloroform and LCP-1 was dissolved in methanol prior to formulation (Table 2). For each formulation (0.5 mL), lipids and **LCP-1** were added to the mixture according to the amounts described in Table 1. Solvent was evaporated slowly *in vacuo* to make a continuous lipid film (Scheme 1), which was lyophilized overnight. Milli-Q water (0.5 mL) or Milli-Q water with PEI was added to hydrate the film with vortexing (Scheme 1). The resulting mixture was extruded twice using 400 nm PC membrane followed by 100 nm PC membrane at 55 °C.

Table 2. Formulation of **L1-L5**. Each formulation is 0.5 mL in final volume.

Liposome Formulation	DPPC + Cholesterol	Lipo-PEI	DDAB	PEI	LCP-1
L1	1.25 mg + 0.25 mg	-	-	-	1 mg
L2	1.25 mg + 0.25 mg	-	-	0.25 mg	1 mg
L3	1.25 mg + 0.25 mg	PAM-PEI (0.25 mg)	-	-	1 mg
L4	1.25 mg + 0.25 mg	DOPE-PEI (0.25 mg)	-	-	1 mg
L5	1.25 mg + 0.25 mg	-	0.25 mg	-	1 mg

Liposome characterization. **L1-L5** were analyzed using DLS to measure the particles size and PDI. All samples were diluted 10 times with Milli-Q water, transferred to disposable cuvettes before measurement at 25 °C with a 173° light scattering angle.^{4,26} **L1-L5** were characterized with TEM to check the particle size and shape. All samples were diluted in Milli-Q water and applied

to glow-discharged carbon-coated copper 200 mesh grids and negative-stained with 2% ammonium molybdate. The image capturing was operated at 80kV.^{4, 26}

Cytotoxic study. A MTT cytotoxicity assay was performed on all liposomes with HEK293 and SW620 adherent cell lines following the published method.⁴¹⁻⁴² Briefly, both cell lines were cultured in RPMI 1640 medium and Dulbecco's Modified Eagles' Medium with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL), incubated at 37 °C with 5% CO₂. Cells were collected with trypsin and dispensed into the 96-well microtiter assay plates (2000 cells/well) and incubated for 18 h at 37 °C with 5% CO₂. LCP-1, LCP-1 + PAM-PEI, L1 – L5 were dissolved in PBS (v/v), and aliquots (10 µL) were tested at the final concentration of 1.5 µg/ µL (same concentration as the intranasal *in vivo* study) and 0.75 µg/µL (calculated based on LCP-1). The negative control used was untreated cells in media. The positive control used was doxorubicin (1% DMSO was added to dissolve doxorubicin). All groups were incubated for 68 h at 37 °C with 5% CO₂, and an aliquot (10 µL) of MTT in PBS (5 mg/mL) was added to each well. The plates were incubated for another 4 h and the medium was aspirated before the precipitated formazan crystals were dissolved in DMSO (100 µL/well). The plates were read by a plate reader at 600 nm absorbance.

***In vivo* immunological assessment.** All animal study procedures and protocols were approved by The University of Queensland Animal Ethics Committee (AEC) with AEC approval number SCMB/AIBN/069/017 in accordance with the National Health and Medical Research Council (NHMRC) of Australia. Outbred ARC Swiss mice (female, 7-8 weeks old, 5 mice per group; purchased from The Animal Resource Centre, Perth, WA, Australia) were housed in the Australian

Institute for Bioengineering and Nanotechnology (AIBN) Animal Facility, and all mice were allowed to acclimatize for 7 days before the experiment. All mice were anaesthetized with isoflurane/oxygen (1:4) gas stream prior the intranasal administration. Each mouse was intranasally immunized with 30 μ L (15 μ L/nare) of the vaccine constructs containing 60 μ g **LCP-1**. Each negative control mouse received sterile Milli-Q water (30 μ L). Each positive control (**CTB + Pep-2**) mouse received **Pep-2** (60 μ g) and CTB (10 μ g) in sterile Milli-Q water (30 μ L). All mice received 3 booster doses on days 21, 42 and 63 (Figure 4A).²⁶

Collection of serum and saliva. Serum was collected on days 0, 20, 41, and 62 post primary immunization (Figure 4A) to determine the level of J8-specific IgG antibodies. Blood (10 μ L) was collected via tail bleed and diluted with PBS (90 μ L). On day 77, the mice were euthanized with a CO₂ inhalation chamber and the neat blood was collected via cardiac bleed. Serum was collected following centrifugation at 2000g for 20 min and supernatant was kept at -80 °C before further analysis. Saliva was collected on days 0, 7, 28, 49, and 70 post primary immunization (Figure 4A). Mice were given pilocarpine (0.1% pilocarpine in sterile Milli-Q water, 50 μ L per mouse) intraperitoneally and left for 10 min before collecting saliva. Collected saliva was added to a tube coated with phenylmethylsulphonyl fluoride protease inhibitor and kept at -80 °C before further analysis.

Determination of antibody titers by ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed to measure J8-specific IgG and IgA antibodies.²⁶ Plates were coated with a solution of J8 peptide in carbonate coating buffer (pH=9.6, 5 μ g peptide per plate) and treated with a blocking solution (150 μ l/well) made up of 5% skim milk in PBS with 0.05% tween-20 to

reduce the non-specific binding. Serum (1:100 diluted in 0.5% skim milk/PBS-tween-20 buffer) was serially diluted 1:2 down the plate. Saliva (1:2 diluted in 0.5% skim milk/PBS-tween-20 buffer) was titrated 1:2 down the plate. To detect the mice antigen-specific antibodies, HRP-conjugated goat anti-mouse IgG or IgA antibody was added to the plates and incubated for 90 min at 37°C. Following washing, plates were treated with OPD solution (100 µl/well) and the measured the absorbance at 450 nm. Antibody titers (IgG and IgA) are defined as the lowest dilution with an optical density more than three times the standard deviation greater than the average value of the optical density of the sera or saliva collected from naïve mice.^{26, 34, 43} Statistical significances were calculated with one-way ANOVA followed by Tukey's post-hoc test with GraphPad Prism software.

Determination of opsonization activity of sera. An opsonization assay was performed as previously reported.^{4, 44-45} The bactericidal activity of immune sera IgG were tested against five GAS clinical isolates, including: D3840 (nasopharynx swabs); GC2 203 (wound swab); ACM-2727 (Royal Brisbane Hospital); ACM-2002 (Royal Brisbane Hospital, human abscess – lymph gland); and D2612 (nasopharynx swabs). Bacteria were prepared by streaking on Todd-Hewitt broth agar plates with 5% yeast extract, then incubated at 37 °C for 24 h. A single colony from the bacterium was transferred to Todd-Hewitt broth (5 mL) with 5% yeast extracts and incubated at 37 °C for 24 h to get the approximately 10⁷ colony forming units (CFU)/mL. The culture was serially diluted to 100 times in PBS and an aliquot (10 µL) was mixed with heat-inactivated (50 °C, 10 min) sera (10 µL) collected on day 77 and horse blood (80 µL). The assay was performed in duplicate on three cultures. Bacteria were incubated in a 96-well plate with sera, before 10 µL aliquot was analyzed based on the CFU enumerated from the plates. The opsonic activity was

calculated as percentage reduction of CFU when compared to the negative control (the mice received Mill-Q water) wells as previously published. Opsonic activity was quantified using the following Equation 1.

$$\left(1 - \frac{\text{CFU in the presence of serum}}{\text{Mean CFU in the presence of media}}\right) \times 100\%$$

Equation 1. Quantification of opsonic activity where CFU is colony forming units.⁴

ANCILLARY INFORMATION

This information is available free of charge on the ACS Publications website.

Supporting Information. Figure S1: DLS spectra of particle **L1** size distributions by intensity; **Figure S2:** DLS spectra of particle **L2** size distributions by intensity; **Figure S3:** DLS spectra of particle **L3** size distributions by intensity; **Figure S4:** DLS spectra of particle **L4** size distributions by intensity; **Figure S5:** DLS spectra of particle **L5** size distributions by intensity. **Figure S6:** Cytotoxicity results for assessment of **LCP-1**, **LCP-1 + PAM-PEI** and **L1 – L5** in HEK293 and SW620 cell lines.

Author Contributions

I. T., W. M. H. and R. J. S designed the project. C. C. D. performed the experiments and wrote the first draft of the manuscript. C. C. D. and W. M. H. synthesized the lipidated PEIs. J. Y. and L. Z. assisted with the animal experiment. X. W. assisted in peptide synthesis. J. Y. performed the TEM analysis. Z. G. K., W. M. H. and R. J. C. performed the opsonization study, cytotoxic study and

analyzed the data. I. T., W. M. H. and R. J. S reviewed and edited the manuscript. All authors have given approval to the final version of the manuscript.

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Abbreviations Used

CD4, cluster of differentiation 4; CFU, colony forming units; DDAB, didodecyldimethylammonium bromide; DLS, dynamic light scattering; PEI, polyethylenimine; DNA, deoxyribonucleic acid; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DPPC, dipalmitoylphosphatidylcholine; ELISA, enzyme-linked immunosorbent assay; GAS, group A *Streptococcus*; GCN4, General Control Nondepressible 4; HRP, horseradish peroxidase; IgA, immunoglobulin A; IgG, immunoglobulin G; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ; PAM, palmitic acid; PBS, phosphate-buffered saline, PDI, polydispersity index; TEM, transmission electron microscope; TLR, toll-like receptor.

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ACCEPTED MANUSCRIPT

Polyethylenimine: An Intranasal Adjuvant for Liposomal Peptide-Based Subunit Vaccine against Group A *Streptococcus*

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