

Polyethylenimine quantity and molecular weight influence its adjuvanting properties in liposomal peptide vaccines

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ABSTRACT

We recently reported that polyethylenimine (PEI; molecular weight of 600 Da) acted as a vaccine adjuvant for liposomal group A *Streptococcus* (GAS) vaccines, eliciting immune responses *in vivo* with IgG antibodies giving opsonic activity against five Australian GAS clinical isolates. However, to date, no investigation comparing the structure-activity relationship between the molecular weight of PEI and its adjuvanting activity in vaccine development has been performed. We hypothesized that the molecular weight and quantity of PEI in a liposomal vaccine will impact its adjuvant properties. In this study, we successfully formulated liposomes containing different molecular weights of PEI (600, 1800, 10k and 25k Da) and equivalents of PEI (0.5, 1 and 2) of branched PEI. Outbred mice were administered the vaccine formulations intranasally, and the mice that received a high ratio of PEI 600 reported a stronger immune response than the mice that received a lower ratio of PEI 600. Interestingly, mice that received the same quantity of PEI 600, PEI 10k and PEI 25k showed similar immune responses *in vivo* and *in vitro*. This comparative study highlights the ratio of PEI present in the liposome vaccines impacts adjuvant activity, however, PEI molecular weight did not significantly enhance its adjuvant properties. We also report that the stability of PEI liposomes is critical for vaccines to elicit the desired immune response.

KEYWORDS

Polyethylenimine; vaccine; liposome; group A *Streptococcus*; adjuvant

Vaccine immunization is a significant tool in the protection and/or treatment of human health from infectious diseases and cancer.¹⁻² Subunit vaccines play an important part in the development of modern-day vaccines, as they have been shown to reduce the risk of infection, avoid autoimmune responses, and simplify the manufacture and storage process when compared with traditional vaccines.³⁻⁴ However, subunit vaccines (e.g. peptide-based subunit vaccines) lack the complementary immune stimulants from pathogens (adjuvants) and effective delivery systems to elicit the necessary immune responses to be an effective vaccine.⁵

Traditionally, adjuvants are mineral salts (e.g. alum), mineral oils (e.g. MF59) or components derived from pathogens (e.g. polysaccharides and toxins) which activate both innate and adaptive immunological responses.⁶⁻⁷ Polymers, including polyphosphazenes,⁸⁻⁹ poly(*tert*-butyl acrylate),¹⁰⁻¹² chitosan¹³⁻¹⁵ and polyethylenimine (PEI)¹⁶⁻¹⁸ have also been used as vaccine adjuvants. PEI is a commercially available, cationic polyelectrolyte (linear or branched) with a molecular weight ranging from 200 Da to 800 kDa.¹⁹ Vaccines containing PEI have shown significant improvement in their efficiency against a variety of infections and tumors through maturation enhancement of antigen presenting cells (APCs), increased proliferation of effector cells and enhanced production of antibodies.²⁰

To deliver vaccine antigen(s) and adjuvant to the desired location in the body to maximize a vaccine's efficacy, the selection of a delivery system is crucial. Liposomes have been widely explored in recent decades as potential vaccine delivery systems, which benefit from reduced aggregation of vaccine components, providing an enhanced depot effect for vaccine antigens and enabling slow-release technology for improved antigen uptake.²¹⁻²³ In addition, liposomes as an intranasal delivery system have shown improved immunogenicity for subunit vaccines.²⁴ However, to date, licensed vaccines for humans are still limited to the prevention of infections for a small range of bacteria and viruses, and the prevention and treatment of cancer (e.g. cervical cancer and prostate cancer).²⁵

Group A *Streptococcus* (GAS) is a gram-positive bacterial etiologic agent resulting in a wide range of human diseases, including mild ailments (e.g. throat irritations and dermis infections), to fatal invasive illnesses (e.g. toxic shock syndrome, necrotizing fasciitis) and post-infectious complications (e.g. rheumatic fever and rheumatic heart disease).²⁶ In 2017, Watkins *et al.* reported that owing to no licensed GAS vaccine and effective therapeutic measures except for antibiotic treatment in early infectious stage, rheumatic fever and rheumatic heart disease related to reoccurring and/or untreated GAS infection was primarily responsible for more than

320,000 deaths worldwide each year.²⁷ Consequently, GAS has been listed as one of the top 10 pathogens worldwide and represents a heavy global health burden.²⁸

To enhance the efficacy of experimental vaccines against these awful diseases, including GAS, researchers have focused their efforts towards vaccine delivery systems and/or adjuvant development. We recently reported that unmodified-PEI 600 and lipidated-PEI 600 as vaccine adjuvants for liposomal (formulated from cholesterol and dipalmitoylphosphatidylcholine [DPPC]) vaccine delivery systems for the mucosal delivery of a GAS B cell and universal T helper peptide epitopes were proven to elicit both mucosal and systemic immune responses *in vivo* (mice).¹⁶ Further, these IgG antibodies showed significantly strong opsonic activities against five GAS clinical isolates derived from Australian patients.¹⁶

PEI has been used in vaccine delivery for a few decades, and before this, PEI was most widely used in gene delivery applications where researchers delivered polynucleotides (e.g. DNA and siRNA) to cells.²⁰ There is a relationship between the molecular weight of PEI and PEI's ability to deliver these complexed polynucleotides, where higher molecular weight PEI (e.g. 25k Da) demonstrated enhanced delivery compared to lower molecular weight PEI (e.g. 600 Da).²⁹ However, to date, the investigation of PEI molecular weight has been limited to gene delivery applications, and to the top of our knowledge, no one has reported the relationship between PEI molecular weight and its adjuvant activity in vaccine development.

In this study, we investigated the balance between the adjuvant efficacy of 1) molecular weight of PEI (e.g. **G1**, PEI 600; **G4**, PEI 1800, **G5**, PEI 10k; **G6**, PEI 25k) on vaccine adjuvant activity, and 2) the ratio of PEI 600 in the vaccine formulations (e.g. **G1**: 1 equivalent; **G2**: 0.5 equivalent; **G3**: 2 equivalents) (Figure 1 and Table 1). Also, the concentrations of different PEI were calculated based on the monomers (Table 1). To formulate the GAS vaccine constructs, lipopeptide **LCP-1**, composed of the GAS B cell epitope (J8) conjugated to a universal T helper epitope (P25) and a lipid core sequence (ser-ser-C₁₆-C₁₆) was used. J8, identified from the conserved C-repeating region of the GAS M protein, has been shown to elicit a humoral immune response with an opsonic antibody protection.³⁰ The GAS M protein is a major virulence factor on the cell wall of GAS bacteria, but the cross-reactivity between this M protein and the human heart leads to an autoimmune response.³¹ J8 was designed to provide the necessary protection required as a B cell epitope and avoid the T cell-mediated autoimmune responses.³² However, T helper cell activation is critical in major histocompatibility complex-II (MHC-II) molecule recognition in the adaptive humoral immunity.³³ To elicit the necessary

109 T cell responses, a universal T helper epitope (P25) was included in the vaccine constructs.^{16,}
110 ³⁴⁻³⁶ The lipid core sequence (ser-ser-C₁₆-C₁₆; Figure 1) in this vaccine has been shown to target
111 toll like receptor 2 (TLR 2), enhancing APC uptake, where the long C₁₆ hydrophobic tails
112 enable incorporation of the P25/J8 epitopes into the vaccine liposomes.^{3, 34, 37-39} To compare
113 the adjuvant properties of these vaccines, the positive control used was **Pep-2** (P25-J8 without
114 lipid, Figure 1) co-administered with commercial adjuvant, cholera toxin subunit B (CTB),
115 which showed promising antibodies production and high opsonic rate in the *in vitro*
116 immunological assays.^{30, 40}

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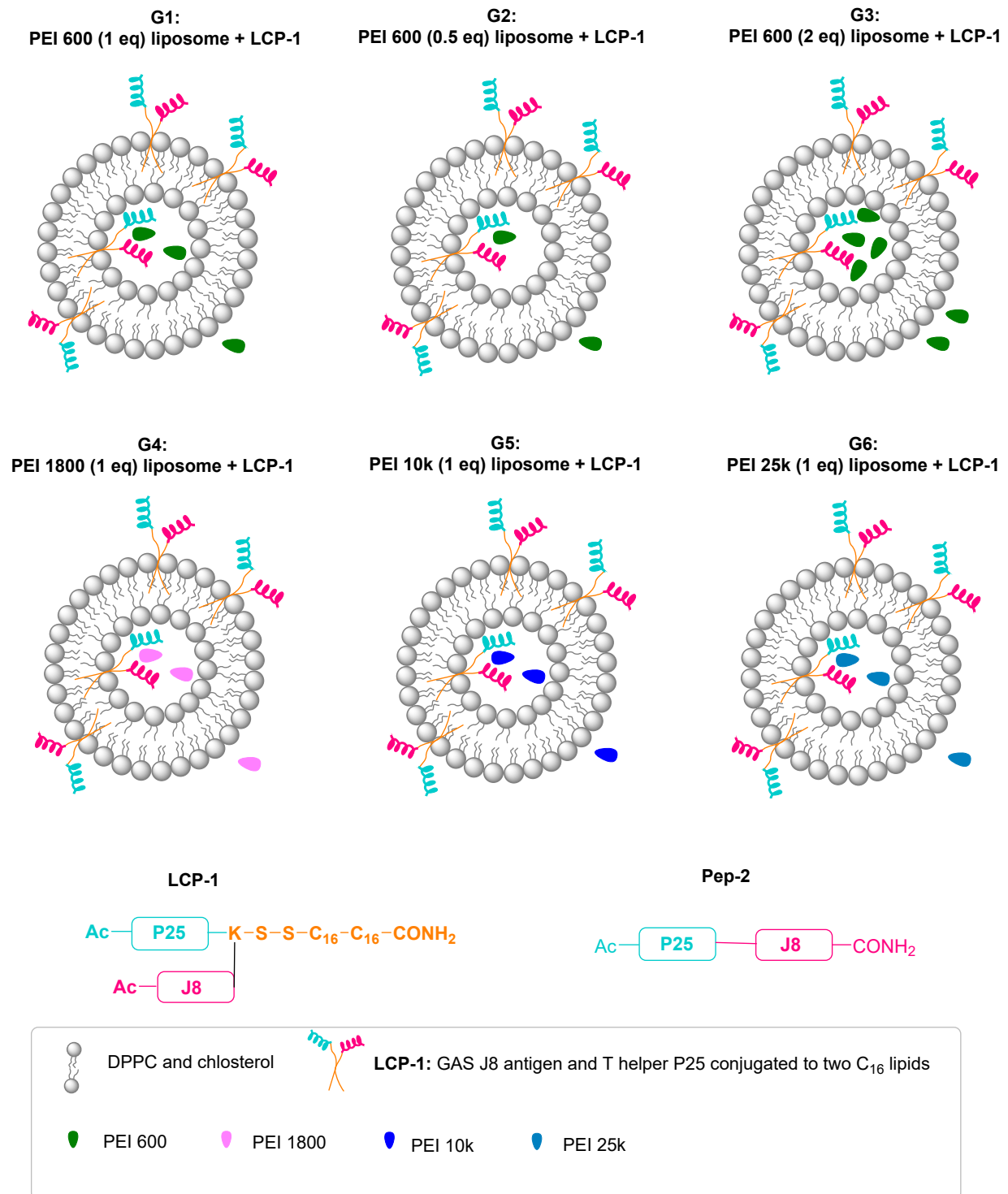


Figure 1. Schematic illustration of the PEI-liposome vaccine formulations in aqueous solution. **G1:** Liposome encapsulating **LCP-1** and branched PEI 600 Da (1 eq); **G2:** Liposome encapsulating **LCP-1** and branched PEI 600 Da (0.5 eq); **G3:** Liposome encapsulating **LCP-1** and branched PEI 600 Da (2 eq); **G4:** Liposome encapsulating **LCP-1** and branched PEI 1800 Da (1 eq); **G5:** Liposome encapsulating **LCP-1** and branched PEI 10k Da (1 eq); **G6:** Liposome encapsulating **LCP-1** and branched PEI 25k Da (1 eq). **LCP-1** is a GAS B cell

epitope (J8; QAEDKVKQSREAKKQVEKALKQLEDKVQ) conjugated to the universal T helper epitope (P25; KLIPNASLIENCTKAEL) and a lipid core (ser-ser-C₁₆-C₁₆; where 'C₁₆' refers to the lipid, 2-amino-1-hexadecanoic acid). **Pep-2**, is P25-J8 peptide that used in a mixture with CTB as a positive control in the *in vivo* immunological study.

Table 1. Formulation of **G1 – G6** vaccines

Liposome formulation*	DPPC (mg)	Cholesterol (mg)	LCP-1 (mg)	PEIs (mg)	PEI polymer concentration (mM)	PEI monomer concentration (mM)
G1	1.25	0.25	1	PEI 600 (0.25)	0.83	11.6
G2	1.25	0.25	1	PEI 600 (0.125)	0.42	5.8
G3	1.25	0.25	1	PEI 600 (0.5)	1.66	23.2
G4	1.25	0.25	1	PEI 1800 (0.25)	0.28	11.6
G5	1.25	0.25	1	PEI 10k (0.25)	0.05	11.6
G6	1.25	0.25	1	PEI 25k (0.25)	0.02	11.6

* Final volume for each formulation is 0.5 mL

Both **LCP-1** and **Pep-2** were synthesized with solid phase peptide synthesis and purified and characterized as previously reported.^{5, 16, 41-42} The yield of both peptides ranged from 7% to 17%, respectively, with a purity greater than 95% in both cases (Supporting Information, Figures S2 – S5).

Liposomes were formulated with the thin-film hydration method (Table 1 and Supporting Information, Figure S1).^{16, 34} DPPC, cholesterol (both dissolved in chloroform) and **LCP-1** (dissolved in methanol) were mixed and evaporated *in vacuo* to make the thin film on the round

bottom flask. The film was hydrated with the toxin-free Milli-Q water with PEI dissolved, then vortexed and extruded using a 100 nm membrane at 50 – 60 °C to control the liposomes size.

All liposomes were characterised with dynamic light scattering (DLS) and transmission electron microscopy (TEM) as per the method previously reported.^{16, 41} All liposomes, except **G3**, showed a positive charge around 40 mV (Table 2), which was similar to that of previous reported charges of similar PEI liposomes (Supporting Information, Figure S6 – S11).¹⁶ The positive charges are affiliated with the presence of **LCP-1** (a positively charged lipopeptide) used in their formulation. Here, **LCP-1** is anchored onto the surface of the liposome through the lipid tail providing this charge. We also noticed that in **G3**, increasing the concentration of PEI (2 eq) leads to a reduction of liposome surface charge, potentially hindering the orientation of the hydrophilic head of **LCP-1** towards the outside of the liposome.

Table 2. Physicochemical characterization of liposomal vaccines.

Liposome	Diameter	Polydispersity	Charge
	(nm) ± STD	Index (PDI) ± STD	(mV) ± STD
G1	135 ± 2	0.14 ± 0.01	37 ± 1
G2	127 ± 3	0.12 ± 0.01	38 ± 1
G3	240 ± 6	0.255 ± 0.004	23.1 ± 0.3
G4	218 ± 5	0.219 ± 0.008	38 ± 1
G5	126 ± 3	0.12 ± 0.01	40 ± 1
G6	126 ± 2	0.11 ± 0.02	40 ± 1

All vaccine liposomes, except **G3** and **G4**, showed similar sizes (approximately 130 nm) with a low polydispersity index (PDIs; Table 2 and Supporting Information Figures S6- S11). The sizes and PDIs were similar to those previously reported.¹⁶ The low PDIs suggested that all vaccine liposomes are uniform in particle size. However, **G3** and **G4** liposomes showed a larger particle size (above 200 nm) in the beginning of the stability study with a PDI above 0.2 (Table 2). Following a liposome stability test with storing the vaccine formulations at 4 °C, after one

week the size of **G3** and **G4** was significantly decreased to around 120 nm, but the surface charge of **G3** was increased to around 30 mV (Figure 2). These results suggested that liposomes **G3** and **G4** were not stable over time, but liposomes in other groups were stable for storage for at least 1 month.

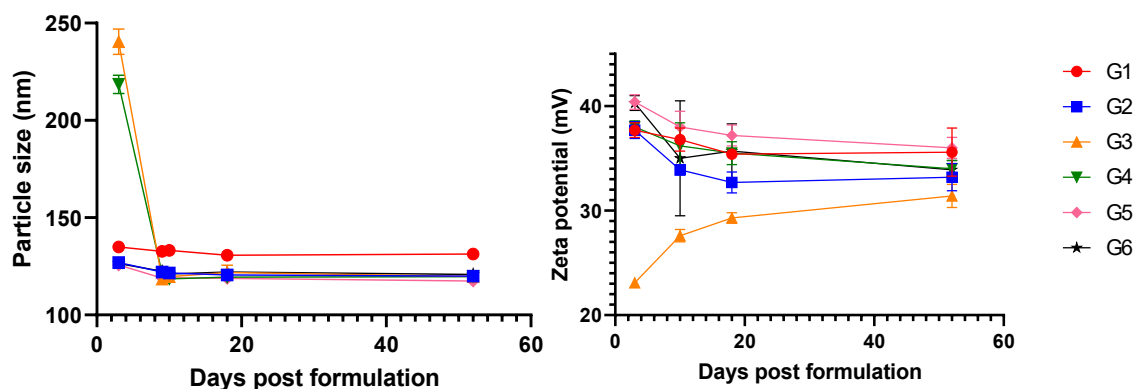


Figure 2. Long-term stability test of **G1 – G6**. Left: Particle size of **G1 – G6** over 52 days. Right: Zeta potential of **G1 – G6** over 52 days. **G1**: PEI 600 (1 eq) liposome + **LCP-1**; **G2**: PEI 600 (0.5 eq) liposome + **LCP -1**; **G3**: PEI 600 (2 eq) liposome + **LCP -1**; **G4**: PEI 1800 (1 eq) liposome + **LCP-1**; **G5**: PEI 10k (1 eq) liposome + **LCP-1**; **G6**: PEI 25k (1 eq) liposome + **LCP-1**.

TEM images (Figure 3) indicated that **G1 – G6** formed typical spherical structures often seen with liposomes, uniform in size in the solution, which were a similar size to that observed by DLS. However, the TEM showed different results from the PDI data from DLS. In DLS, all groups were with small PDIs indicating that all liposomes were in similar sizes. However, in TEM images, small fragments and broken liposomes were observed. The small fragments were identified to be from unbound, dried PEIs (Supporting Information, Figure S12 – S15), and the broken liposome is expected to be a result of the drying and dye addition processes necessary for TEM imaging (Supporting Information, Figure S16). Interestingly, after diluting the **G3** formulation with Milli-Q water and leaving for long term storage, the surface charge increased to the similar range of other formulations and the size also decreased.

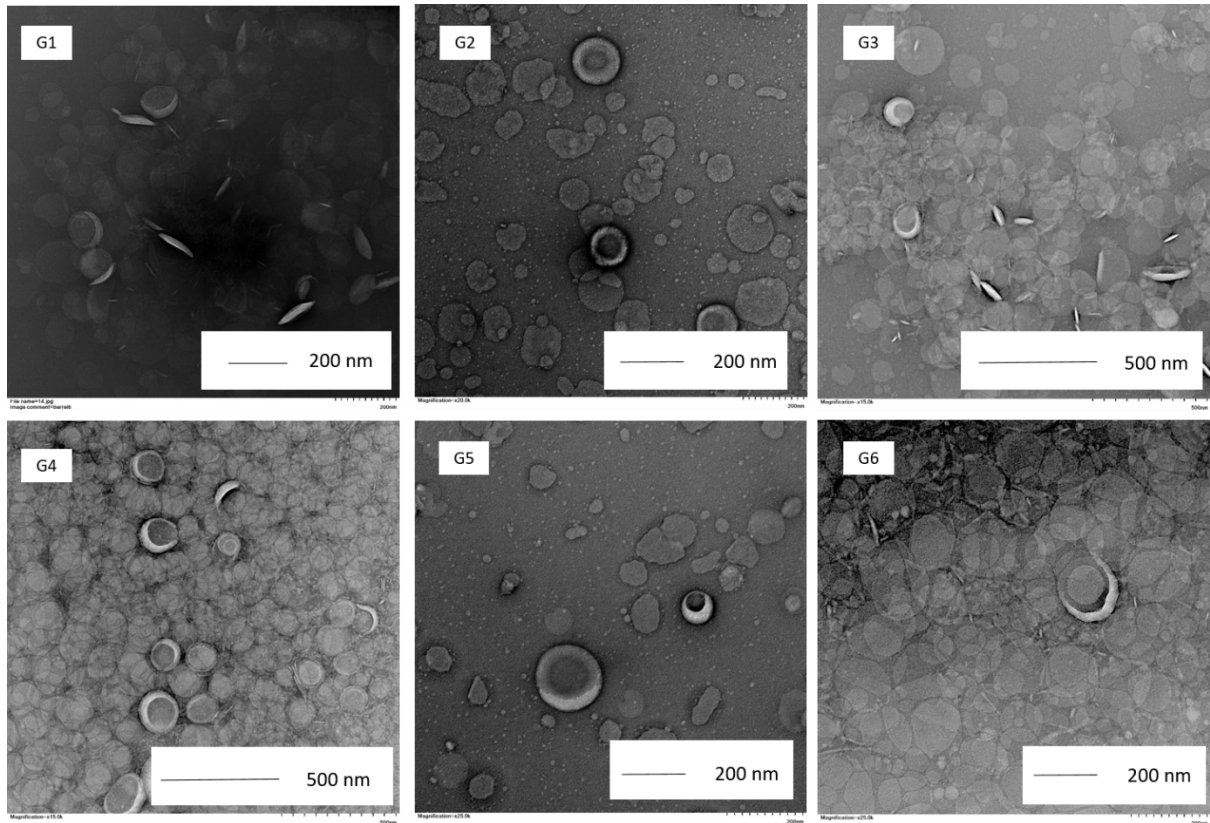
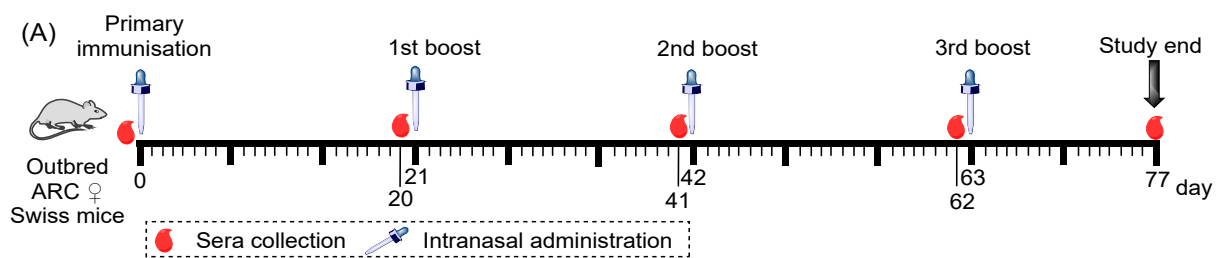
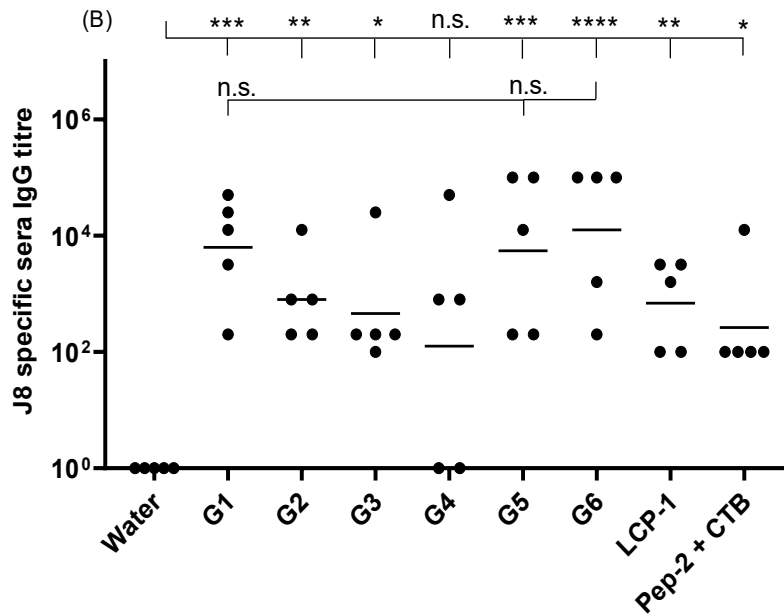


Figure 3. TEM of **G1 – G6** stained with 2% ammonium molybdate.

In vivo immunological evaluation was performed in outbred female ARC Swiss mice. All mice received four intranasal immunizations (a single primary immunization and three boosts; Figure 4A). Mice (5 mice/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**, **G1 – G6**; Figure 1). The positive control group was 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.





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197 **Figure 4.** (A) Immunization schedule. (B) J8-antigen specific sera IgG responses obtained in
198 mice sera on day 77. Geometric mean of antigen-specific IgG titers is represented as a
199 horizontal bar for each group of five mice. Statistical analysis was performed by one-way
200 ANOVA followed by Tukey post hoc test where, probability value of $p < 0.05$ was considered
201 statistically significant (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).
202). **G1:** PEI 600 (1 eq) liposome + **LCP-1**; **G2:** PEI 600 (0.5 eq) liposome + **LCP -1**; **G3:** PEI
203 600 (2 eq) liposome + **LCP -1**; **G4:** PEI 1800 (1 eq) liposome + **LCP-1**; **G5:** PEI 10k (1 eq)
204 liposome + **LCP-1**; **G6:** PEI 25k (1 eq) liposome + **LCP-1**.

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206 Following the third boost, sera collected from **G1 – G3**, **G5**, **G6** and **LCP-1** groups showed
207 significant J8-specific IgG antibody titers when compared with mice who received Milli-Q
208 water only (Figure 4B). **G1**, **G5** and **G6** mice also demonstrated the highest IgG titers with no
209 significant difference amongst the three vaccines. However, **G4** mice showed the lowest IgG
210 titers with two non-responding mice, and there was no significant difference between the **G4**
211 mice and the negative control group. Mice that received the **G3** vaccine also produced low IgG
212 titers. The weaker responses of **G3** and **G4** vaccines is related to the liposomes which had lower
213 stability over the course of the study, despite each vaccine being prepared fresh for each
214 immunization (Figure 2). All vaccinated mice displayed variations in IgG titers. These

variations might be due to the use of outbred mice, which is a heterogeneous population and this is consistent with previous immunological assays.^{16, 43-44}

To evaluate the opsonic activities of sera IgG antibodies from these liposome vaccines, we performed an opsonization study against two GAS clinical isolates (D3840 and GC2 203) obtained from Australian hospitals (Figure 5). Pooled sera collected from mice that received the **G1** (PEI 600 1 eq liposome + **LCP-1**) vaccine showed significantly strong opsonic activities against both GAS clinical isolates when compare to the negative control (water) group. Pooled sera obtained from mice administrated with the **G5** (PEI 10 k 1 eq liposome + **LCP-1**) and **G6** (PEI 25k 1 eq liposome+ **LCP-1**) vaccines also showed significantly strong opsonic activities against only the GC2 203 GAS isolate. There was no significant difference in opsonic activities within these groups (**G1**, **G5** and **G6**).

Pooled sera from mice that received the **G2** (PEI 600 0.5 eq liposome + **LCP-1**), **G3** (PEI 600 2 eq liposome + **LCP-1**) or **G4** (PEI 1800 1 eq liposome + **LCP-1**) formulations failed to show any opsonic activity against both GAS clinical isolates, which was consistent with the low IgG antibody titers obtained (Figure 4B). However, pooled sera from **G2** and **G4** groups showed similar opsonic activities with the positive control (**Pep-2** + CTB) group in the GC2 203 isolate. The average opsonic activities of the sera collected from the mice that received **G1**, **G5** and **G6** vaccines were around 35 – 45%, which was higher than the opsonic rate of the positive control group. However, the average opsonic rate from the **G1** vaccinated group was lower than that of the previous study due to the low average IgG antibody in this study.¹⁶ This opsonization study suggested that all the liposomal vaccine candidates adjuvanted with PEI 600 (**G1**), 10k (**G5**) and 25k (**G6**) elicit protective immunity against GAS where the opsonic activities are not significantly related to the molecular weight of PEI used. However, the quantity of PEI used (e.g. **G2**) and liposome stability (e.g. **G3** and **G4**) are critical for intranasal liposomal vaccine formulation moving forward.

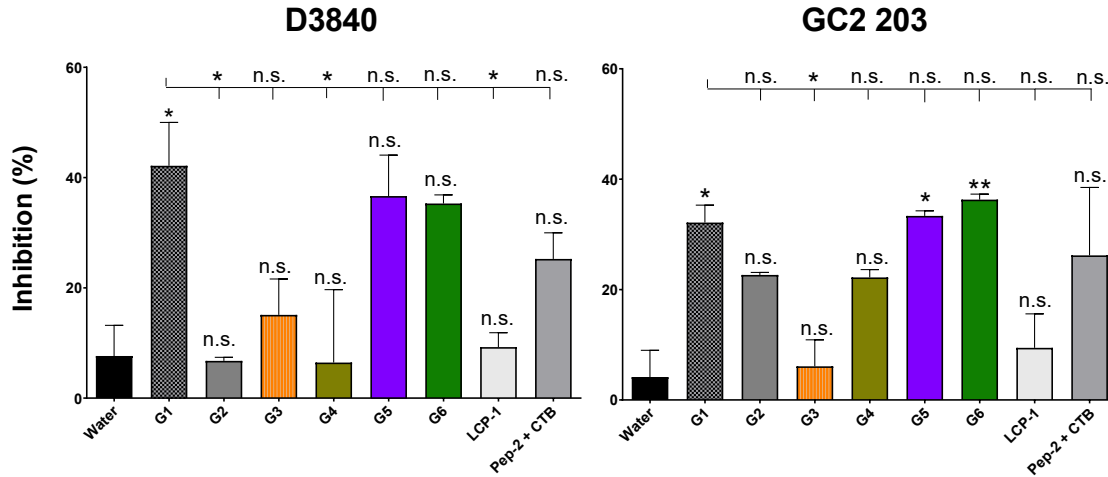


Figure 5: Average opsonization (duplicate from two independent cultures) of sera collected from mice on day 77 against two clinical isolates (D3840 and GC2 203) of GAS. **G1:** PEI 600 (1 eq) liposome + **LCP-1**; **G2:** PEI 600 (0.5 eq) liposome + **LCP -1**; **G3:** PEI 600 (2 eq) liposome + **LCP -1**; **G4:** PEI 1800 (1 eq) liposome + **LCP-1**; **G5:** PEI 10k (1 eq) liposome + **LCP-1**; **G6:** PEI 25k (1 eq) liposome + **LCP-1**. Negative control pooled sera from five mice received Milli-Q water only. Positive control pooled sera from five mice received **Pep-2** co-administered with CTB adjuvant. Results are represented as an opsonization percentage that statistically 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.

In the development of efficient and safe GAS vaccines, PEI has the potency to act as an adjuvant for liposome-based GAS intranasal vaccines. In this study, we successfully formulated a library of liposome-based GAS intranasal vaccines containing different quantities of PEI (e.g. 0.5 eq, 1 eq and 2 eq) and molecular weights of PEI (e.g. 600, 1800, 10k and 25k Da), where PEI was externally and internally associated with liposomes leading to an immediate and sustained-released immune response in each formulation. Following a single primary immunization and three boosts of the vaccine liposomes intranasally to Swiss outbred mice, the mice developed J8-specific IgG immune responses. Mice that received the **G1** (PEI 600 1 eq liposome + **LCP-1**), **G5** (PEI 10k 1 eq liposome + **LCP-1**) and **G6** (PEI 25k 1 eq

liposome + **LCP-1**) formulations showed the highest IgG antibody titers and opsonic activities out of all groups assessed. However, the mice that received the **G2** (PEI 600 0.5 eq liposome + **LCP-1**) formulation showed weaker immune responses when compared to the **G1**, **G3** (PEI 600 2 eq liposome + **LCP-1**) and **G4** (PEI 1800 1eq liposome + **LCP-1**) vaccines which failed to form stable liposomes leading to a reduced immune response *in vivo*. This study highlights that ratio of PEI present in the liposome vaccines impacting the adjuvanting activity, however, PEI molecular weight did not enhance its adjuvanting properties. The stability of PEI liposomes is also critical for vaccines to elicit the desired immune response.

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References

1. Yadav, D. K.; Yadav, N.; Khurana, S. M. P., Vaccines. In *Animal Biotechnology*, 2014; pp 491-508. DOI: 10.1016/b978-0-12-416002-6.00026-2.
2. Rappuoli, R.; Pizza, M.; Del Giudice, G.; De Gregorio, E., Vaccines, new opportunities for a new society. *Proc Natl Acad Sci U S A* **2014**, *111* (34), 12288-93. DOI: 10.1073/pnas.1402981111.
3. Azmi, F.; Ahmad Fuaad, A. A.; Skwarczynski, M.; Toth, I., Recent progress in adjuvant discovery for peptide-based subunit vaccines. *Hum Vaccin Immunother* **2014**, *10* (3), 778-96. DOI: 10.4161/hv.27332.
4. Plotkin, S. A., Vaccines: past, present and future. *Nat Med* **2005**, *11* (S4), S5-S11.
5. Azuar, A.; Zhao, L.; Hei, T. T.; Nevagi, R. J.; Bartlett, S.; Hussein, W. M.; Khalil, Z. G.; Capon, R. J.; Toth, I.; Skwarczynski, M., Cholic Acid-based Delivery System for Vaccine

295 Candidates against Group A Streptococcus. *ACS Med Chem Lett* **2019**, *10* (9), 1253-1259. DOI:
 296 10.1021/acsmedchemlett.9b00239.

297 6. Skwarczynski, M.; Toth, I., Peptide-based synthetic vaccines. *Chem Sci* **2016**, *7* (2),
 298 842-854. DOI: 10.1039/c5sc03892h.

299 7. Mbow, M. L.; De Gregorio, E.; Valiante, N. M.; Rappuoli, R., New adjuvants for
 300 human vaccines. *Curr Opin Immunol* **2010**, *22* (3), 411-6. DOI: 10.1016/j.coi.2010.04.004.

301 8. Andrianov, A., Polymeric carriers for oral uptake of microparticulates. *Adv Drug Deliv*
 302 *Rev* **1998**, *34* (2-3), 155-170. DOI: 10.1016/s0169-409x(98)00038-6.

303 9. Payne, L. G.; Jenkins, S. A.; Woods, A. L.; Grund, E. M.; Geribo, W. E.; Loebelenz, J.
 304 R.; Andrianov, A. K.; Roberts, B. E., Poly[di(carboxylatophenoxy)phosphazene] (PCPP) is a
 305 potent immunoadjuvant for an influenza vaccine. *Vaccine* **1998**, *16* (1), 92-98. DOI:
 306 10.1016/s0264-410x(97)00149-7.

307 10. Hussein, W. M.; Liu, T. Y.; Jia, Z. F.; McMillan, N. A. J.; Monteiro, M. J.; Toth, I.;
 308 Skwarczynski, M., Multiantigenic peptide-polymer conjugates as therapeutic vaccines against
 309 cervical cancer. *Bioorgan Med Chem* **2016**, *24* (18), 4372-4380. DOI:
 310 10.1016/j.bmc.2016.07.036.

311 11. Liu, T. Y.; Hussein, W. M.; Giddam, A. K.; Jia, Z.; Reiman, J. M.; Zaman, M.;
 312 McMillan, N. A.; Good, M. F.; Monteiro, M. J.; Toth, I.; Skwarczynski, M., Polyacrylate-based
 313 delivery system for self-adjuvanting anticancer peptide vaccine. *J Med Chem* **2015**, *58* (2),
 314 888-896. DOI: 10.1021/jm501514h.

315 12. Liu, T. Y.; Hussein, W. M.; Jia, Z. F.; Ziora, Z. M.; McMillan, N. A. J.; Monteiro, M.
 316 J.; Toth, I.; Skwarczynski, M., Self-adjuvanting polymer-peptide conjugates as therapeutic
 317 vaccine candidates against cervical cancer. *Biomacromolecules* **2013**, *14* (8), 2798-2806. DOI:
 318 10.1021/bm400626w.

319 13. Marasini, N.; Giddam, A. K.; Khalil, Z. G.; Hussein, W. M.; Capon, R. J.; Batzloff, M.
 320 R.; Good, M. F.; Toth, I.; Skwarczynski, M., Double adjuvanting strategy for peptide-based
 321 vaccines: trimethyl chitosan nanoparticles for lipopeptide delivery. *Nanomedicine (Lond)* **2016**,
 322 *11* (24), 3223-3235. DOI: 10.2217/nnm-2016-0291.

323 14. Nevagi, R. J.; Dai, W.; Khalil, Z. G.; Hussein, W. M.; Capon, R. J.; Skwarczynski, M.;
 324 Toth, I., Structure-activity relationship of group A Streptococcus lipopeptide vaccine
 325 candidates in trimethyl chitosan-based self-adjuvanting delivery system. *Eur J Med Chem* **2019**,
 326 *179*, 100-108. DOI: 10.1016/j.ejmech.2019.06.047.

327 15. Nevagi, R. J.; Khalil, Z. G.; Hussein, W. M.; Powell, J.; Batzloff, M. R.; Capon, R. J.;
 328 Good, M. F.; Skwarczynski, M.; Toth, I., Polyglutamic acid-trimethyl chitosan-based

329 intranasal peptide nano-vaccine induces potent immune responses against group A
 330 Streptococcus. *Acta Biomater* **2018**, *80*, 278-287. DOI: 10.1016/j.actbio.2018.09.037.

331 16. Dai, C. C.; Yang, J.; Hussein, W. M.; Zhao, L.; Wang, X.; Khalil, Z. G.; Capon, R. J.;
 332 Toth, I.; Stephenson, R. J., Polyethylenimine: An Intranasal Adjuvant for Liposomal Peptide-
 333 Based Subunit Vaccine against Group A Streptococcus. *ACS Infect Dis* **2020**, *6* (9), 2502-2512.
 334 DOI: 10.1021/acsinfecdis.0c00452.

335 17. Wegmann, F.; Gartlan, K. H.; Harandi, A. M.; Brinckmann, S. A.; Coccia, M.; Hillson,
 336 W. R.; Kok, W. L.; Cole, S.; Ho, L. P.; Lambe, T.; Puthia, M.; Svanborg, C.; Scherer, E. M.;
 337 Krashias, G.; Williams, A.; Blattman, J. N.; Greenberg, P. D.; Flavell, R. A.; Moghaddam, A.
 338 E.; Sheppard, N. C.; Sattentau, Q. J., Polyethyleneimine is a potent mucosal adjuvant for viral
 339 glycoprotein antigens. *Nat Biotechnol* **2012**, *30* (9), 883-U116. DOI: 10.1038/nbt.2344.

340 18. Sheppard, N. C.; Brinckmann, S. A.; Gartlan, K. H.; Puthia, M.; Svanborg, C.; Krashias,
 341 G.; Eisenbarth, S. C.; Flavell, R. A.; Sattentau, Q. J.; Wegmann, F., Polyethyleneimine is a
 342 potent systemic adjuvant for glycoprotein antigens. *Int Immunol* **2014**, *26* (10), 531-538. DOI:
 343 10.1093/intimm/dxu055.

344 19. Bieber, T.; Elsässer, H. P., Preparation of a Low Molecular Weight Polyethylenimine
 345 for Efficient Cell Transfection. *BioTechniques* **2001**, *30* (1), 74-81. DOI: 10.2144/01301st03.

346 20. Shen, C.; Li, J.; Zhang, Y.; Li, Y.; Shen, G.; Zhu, J.; Tao, J., Polyethylenimine-based
 347 micro/nanoparticles as vaccine adjuvants. *Int J Nanomedicine* **2017**, *12*, 5443-5460. DOI:
 348 10.2147/IJN.S137980.

349 21. Schafer, J.; Hobel, S.; Bakowsky, U.; Aigner, A., Liposome-polyethylenimine
 350 complexes for enhanced DNA and siRNA delivery. *Biomaterials* **2010**, *31* (26), 6892-900.
 351 DOI: 10.1016/j.biomaterials.2010.05.043.

352 22. Henriksen-Lacey, M.; Korsholm, K. S.; Andersen, P.; Perrie, Y.; Christensen, D.,
 353 Liposomal vaccine delivery systems. *Expert Opin Drug Deliv* **2011**, *8* (4), 505-19. DOI:
 354 10.1517/17425247.2011.558081.

355 23. Gregoriadis, G., Engineering liposomes for drug delivery: progress and problems.
 356 *Trends Biotechnol* **1995**, *13* (12), 527-537. DOI: [https://doi.org/10.1016/S0167-](https://doi.org/10.1016/S0167-7799(00)89017-4)
 357 [7799\(00\)89017-4](https://doi.org/10.1016/S0167-7799(00)89017-4).

358 24. Allison, A. C.; Gregoriadis, G., Liposomes as Immunological Adjuvants. In
 359 *Lymphocytes, Macrophages, and Cancer*, Mathé, G.; Florentin, I.; Simmler, M.-C., Eds.
 360 Springer Berlin Heidelberg: Berlin, Heidelberg, 1976; pp 58-64. DOI: 10.1007/978-3-642-
 361 81049-7_8.

25. Delany, I.; Rappuoli, R.; De Gregorio, E., Vaccines for the 21st century. *EMBO Mol Med* **2014**, *6* (6), 708-720. DOI: 10.1002/emmm.201403876.
26. Tart, A. H.; Walker, M. J.; Musser, J. M., New understanding of the Group A *Streptococcus* pathogenesis cycle. *Trends Microbiol* **2007**, *15* (7), 318-25. DOI: 10.1016/j.tim.2007.05.001.
27. Watkins, D. A.; Johnson, C. O.; Colquhoun, S. M.; Karthikeyan, G.; Beaton, A.; Bukhman, G.; Forouzanfar, M. H.; Longenecker, C. T.; Mayosi, B. M.; Mensah, G. A.; Nascimento, B. R.; Ribeiro, A. L. P.; Sable, C. A.; Steer, A. C.; Naghavi, M.; Mokdad, A. H.; Murray, C. J. L.; Vos, T.; Carapetis, J. R.; Roth, G. A., Global, Regional, and National Burden of Rheumatic Heart Disease, 1990-2015. *N Engl J Med* **2017**, *377* (8), 713-722. DOI: 10.1056/NEJMoa1603693.
28. Carapetis, J. R.; Steer, A. C.; Mulholland, E. K.; Weber, M., The global burden of Group A *Streptococcal* diseases. *Lancet Infect Dis* **2005**, *5* (11), 685-694. DOI: 10.1016/s1473-3099(05)70267-x.
29. Wiseman, J. W.; Goddard, C. A.; McLelland, D.; Colledge, W. H., A comparison of linear and branched polyethylenimine (PEI) with DCChol/DOPE liposomes for gene delivery to epithelial cells in vitro and in vivo. *Gene Ther* **2003**, *10* (19), 1654-62. DOI: 10.1038/sj.gt.3302050.
30. Dai, C.; Khalil, Z. G.; Hussein, W. M.; Yang, J.; Wang, X.; Zhao, L.; Capon, R. J.; Toth, I.; Stephenson, R. J., Opsonic activity of conservative versus variable regions of the group A *Streptococcus* M protein. *Vaccines (Basel)* **2020**, *8* (2). DOI: 10.3390/vaccines8020210.
31. Madge, H. Y. R.; Sharma, H.; Hussein, W. M.; Khalil, Z. G.; Capon, R. J.; Toth, I.; Stephenson, R. J., Structure-Activity Analysis of Cyclic Multicomponent Lipopeptide Self-Adjuvanting Vaccine Candidates Presenting Group A *Streptococcus* Antigens. *J Med Chem* **2020**, *63* (10), 5387-5397. DOI: 10.1021/acs.jmedchem.0c00203.
32. Good, M. F.; Batzloff, M. R.; Pandey, M., Strategies in the development of vaccines to prevent infections with group A *Streptococcus*. *Hum Vaccin Immunother* **2013**, *9* (11), 2393-2397. DOI: 10.4161/hv.25506.
33. Albert, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P., *Molecular Biology of the Cell*. 4th Edition ed.; Garland Science: New York, 2002.
34. Ghaffar, K. A.; Marasini, N.; Giddam, A. K.; Batzloff, M. R.; Good, M. F.; Skwarczynski, M.; Toth, I., Liposome-based intranasal delivery of lipopeptide vaccine

395 candidates against group A streptococcus. *Acta Biomater* **2016**, *41*, 161-8. DOI:
396 10.1016/j.actbio.2016.04.012.

397 35. Batzloff, M. R.; Hartas, J.; Zeng, W.; Jackson, D. C.; Good, M. F., Intranasal
398 vaccination with a lipopeptide containing a conformationally constrained conserved minimal
399 peptide, a universal T cell epitope, and a self-adjuvanting lipid protects mice from group A
400 streptococcus challenge and reduces throat colonization. *J Infect Dis* **2006**, *194* (3), 325-30.
401 DOI: 10.1086/505146.

402 36. Ghosh, S.; Walker, J.; Jackson, D. C., Identification of canine helper T-cell epitopes
403 from the fusion protein of canine distemper virus. *Immunology* **2001**, *104* (1), 58-66. DOI:
404 10.1046/j.0019-2805.2001.01271.x.

405 37. Shabbits, J. A.; Mayer, L. D., Intracellular delivery of ceramide lipids via liposomes
406 enhances apoptosis in vitro. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2003**, *1612*
407 (1), 98-106. DOI: 10.1016/s0005-2736(03)00108-1.

408 38. Monteiro, N.; Martins, A.; Reis, R. L.; Neves, N. M., Liposomes in tissue engineering
409 and regenerative medicine. *J R Soc Interface* **2014**, *11* (101), 20140459. DOI:
410 10.1098/rsif.2014.0459.

411 39. Black, M.; Trent, A.; Tirrell, M.; Olive, C., Advances in the design and delivery of
412 peptide subunit vaccines with a focus on toll-like receptor agonists. *Expert Rev Vaccines* **2010**,
413 *9* (2), 157-73. DOI: 10.1586/erv.09.160.

414 40. Rattanapisit, K.; Bhoo, S. H.; Hahn, T. R.; Mason, H. S.; Phoolcharoen, W., Rapid
415 transient expression of cholera toxin B subunit (CTB) in *Nicotiana benthamiana*. *In Vitro Cell*
416 *Dev Biol Plant* **2012**, *49* (2), 107-113. DOI: 10.1007/s11627-012-9484-6.

417 41. Zhao, L.; Jin, W.; Cruz, J. G.; Marasini, N.; Khalil, Z. G.; Capon, R. J.; Hussein, W.
418 M.; Skwarczynski, M.; Toth, I., Development of Polyelectrolyte Complexes for the Delivery
419 of Peptide-Based Subunit Vaccines against Group A Streptococcus. *Nanomaterials (Basel)*
420 **2020**, *10* (5). DOI: 10.3390/nano10050823.

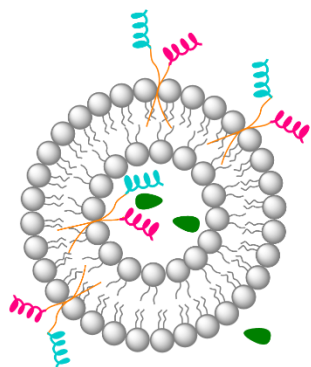
421 42. Skwarczynski, M.; Zhao, G.; Boer, J. C.; Ozberk, V.; Azuar, A.; Cruz, J. G.; Giddam,
422 A. K.; Khalil, Z. G.; Pandey, M.; Shibu, M. A.; Hussein, W. M.; Nevagi, R. J.; Batzloff, M. R.;
423 Wells, J. W.; Capon, R. J.; Plebanski, M.; Good, M. F.; Toth, I., Poly(amino acids) as a potent
424 self-adjuvanting delivery system for peptide-based nanovaccines. *Sci Adv* **2020**, *6* (5),
425 eaax2285. DOI: 10.1126/sciadv.aax2285.

426 43. Marasini, N.; Giddam, A. K.; Ghaffar, K. A.; Batzloff, M. R.; Good, M. F.;
427 Skwarczynski, M.; Toth, I., Multilayer engineered nanoliposomes as a novel tool for oral

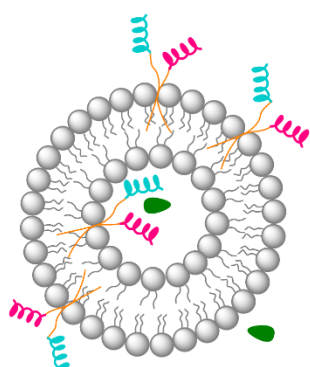
428 delivery of lipopeptide-based vaccines against group A Streptococcus. *Nanomedicine (Lond)*
429 **2016**, *11* (10), 1223-1236. DOI: 10.2217/nnm.16.36.
430 44. Nevagi, R. J.; Dai, W.; Khalil, Z. G.; Hussein, W. M.; Capon, R. J.; Skwarczynski, M.;
431 Toth, I., Self-assembly of trimethyl chitosan and poly(anionic amino acid)-peptide antigen
432 conjugate to produce a potent self-adjuvanting nanovaccine delivery system. *Bioorg Med Chem*
433 **2019**, *27* (14), 3082-3088. DOI: 10.1016/j.bmc.2019.05.033.

434

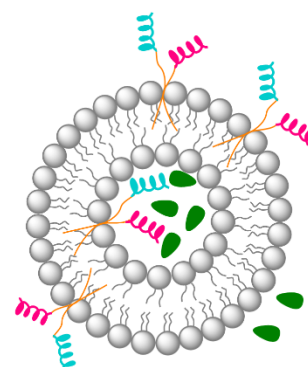
G1:
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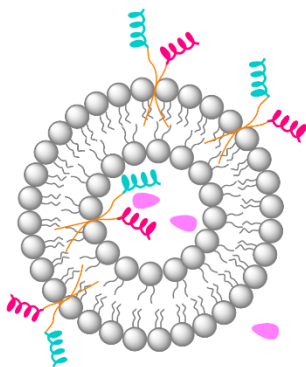
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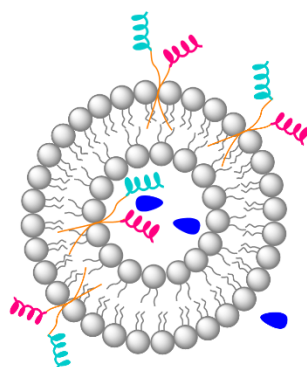
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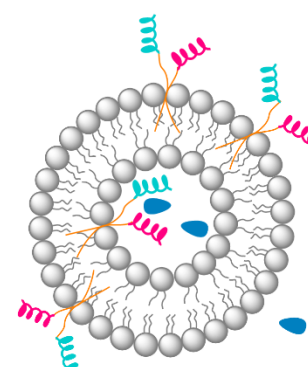
G4:
PEI 1800 (1 eq) liposome + LCP-1



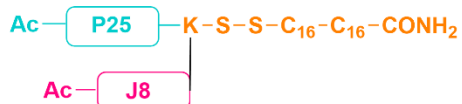
G5:
PEI 10k (1 eq) liposome + LCP-1



G6:
PEI 25k (1 eq) liposome + LCP-1



LCP-1



Pep-2

