

Cholic Acid-based Delivery System for Vaccine Candidates against Group A Streptococcus

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Supporting Information

MATERIALS

9-Fluorenylmethyl carbamate (Fmoc) protected L-amino acids were purchased from Novabiochem, Merck Chemicals (Darmstadt, Germany) and Mimotopes (Melbourne, Australia). 1-[6-Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxide hexafluorophosphate (HATU) and 1-hydroxybenzotriazole hydrate (HOBt) were also purchased from Mimotopes. Acetonitrile, dichloromethane (DCM), methanol, N,N-dimethylformamide (DMF), N,N-diisopropylethylamine (DIPEA), piperidine, rinkamide methylbenzhydrylamine (MBHA) resin, trifluoroacetic acid (TFA), triisopropylsilane (TIPS) were acquired from Merck (Hohenbrunn, Germany). Cholesterol, didodecyldimethylammonium bromide (DDAB), and dipalmitoylphosphatidylcholine (DPPC) were purchased from Avanti Polar Lipids, Inc. (Alabama, USA). Phosphate-buffered saline (PBS) was obtained from eBioscience (California, USA). C57BL/6 mice were purchased from The University of Queensland Biological Resources (UQBR) (Queensland, Australia). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Thermo Scientific (Victoria, Australia). Goat anti-mouse IgG (H+L)-HRP (IgG HRP) conjugate was purchased from Millipore, Temacula (California, USA). Goat anti-mouse IgA was purchased from Invivogen (San Diego, USA). Analytical-grade Tween 20 was purchased from VWR International (Queensland, Australia). GAS clinical isolate ACM-2002 (human abscess – lymph gland) was obtained from the Royal Brisbane Hospital (Brisbane, Australia). GAS clinical isolates ACM-5199 (ATCC 12344, NCIB 11841, scarlet fever); ACM-5203 (ATCC 19615, pharynx of child with episode of sore throat); GC2 203 (wound swab); D3840 (nasopharynx swabs); and D2612 (nasopharynx swabs) were provided by the Princess Alexandra Hospital (Brisbane, Australia). Todd–Hewitt broth (THB) and Roswell Park Memorial Institute (RPMI) medium 1640 were purchased from Oxoid, Thermo Fisher Scientific (South Australia, Australia). The human lung carcinoma (NCIH460) cell line was obtained from American Type Culture Collection (ATCC; Virginia, USA). Yeast extracts were purchased from Novabiochem, Merck Chemicals (Darmstadt, Germany). Chloroform, cholic acid, cholera toxin B subunit (CTB), L-glutamine, o-phenylenediamine dihydrochloride (OPD) substrate, foetal bovine serum (FBS), human embryonic kidney (HEK293), and human colon adenocarcinoma (SW620) cell lines, pilocarpine, penicillin, streptomycin, and all other reagents, were purchased from Sigma-Aldrich (Victoria, Australia).

Microwave-assisted Fmoc solid phase peptide synthesis (SPPS) was performed using an SPS CEM Discovery reactor from CME Corporation (North Carolina, USA). ESI-MS was performed on an LCMS-2020 Shimadzu (Kyoto, Japan) instrument (DGU-20A3, LC-20Ad x 2, SIL-20AHT, STO-20A). Analytical reverse-phase HPLC (RP-HPLC) was performed on a Shimadzu (Kyoto, Japan) instrument (DGU-20A5, LC-20AB, SIL-20AHT, SPD-M10AVP) using a Vydac analytical C18-column (218TP54; 5 μ m, 4.6 x 250 mm) or C4-column (214TP54; 5 μ m, 4.6 x 250 mm). Purification was performed on a Shimadzu preparative RP-HPLC (Kyoto, Japan) instrument (LC-20AP x 2, CBM-20A, SPD-20A, FRC-10A) with a 20.0 ml/min flow rate using a C18-column (218TP1022; 10 μ m, 22 x 250 mm) or C4-column (214TP1022; 10 μ m, 22 x 250 mm). Dynamic light scattering (DLS) measurements were taken using a Zetasizer Nano ZP instrument (Malvern Instrument, UK) with Dispersion Technology software. Particles were imaged using a JEM-1010 transmission electron microscope (TEM) (JEOL Ltd., Japan). Plate reading absorbance for antibody titres and cytotoxicity assays were measured using a Spectramax microplate reader (Molecular Devices, USA) and PowerWave XS microplate reader (Bio-Tek Instruments Inc., USA), respectively.

METHODS

Synthesis of Compounds 1-3

Compound **1** (Figure 1) was synthesised using microwave-assisted SPPS (70°C, 20 W) via Fmoc chemistry on MBHA resin at 0.2 mmol.¹ The coupling cycle included deprotection of Fmoc (2 and 5 min treatments with 20% piperidine in DMF), DMF wash, amino acid (0.84 mmol/g, 4.2 equivalent) activation using 0.5 M HATU (1.6 mL, 4 equivalent) and DIPEA (0.18 mL, 5.2 equivalent), and its coupling to the resin (5 and 10 min). This procedure was repeated for the addition of each amino acids, until the desired peptide was achieved. Special coupling condition was applied to arginine, with 10 mins (5 min at RT then 5 mins at 65°C) coupling, twice. Synthesised compound **1** was cleaved from the resin using a TFA:TIPS:water (9.5:2.5:2.5) solution for 4 h. TFA was then removed under reduced pressure. Compound **1** was precipitated and washed with cold diethyl ether. It was then dissolved in aqueous acetonitrile (50% acetonitrile, 50% milli-Q water, 0.1% TFA), and filtered. The purification of compound **1** was performed by preparative RP-HPLC on a C18 column using solvent B (90% acetonitrile, 10% milli-Q water, 0.1% TFA) at a gradient of 0-50% over 25 min; compound detection at 214 nm. The purified compound **1** was analysed using ESI-MS and analytical RP-HPLC.

Compound **1** purity: 98%. Chemical Formula: C₂₂₉H₃₉₄N₆₈O₆₄. Molecular weight: 5124.08. ESI-MS: [M+3H]³⁺ *m/z* 1708.85 (calculated: 1709.03), [M+4H]⁴⁺ *m/z* 1282.00 (calculated: 1282.02), [M+5H]⁵⁺ *m/z* 1025.80 (calculated: 1025.82), [M+6H]⁶⁺ *m/z* 855.05 (calculated: 855.01), [M+7H]⁷⁺ *m/z* 733.00 (calculated: 733.01), [M+8H]⁸⁺ *m/z* 641.55 (calculated: 641.51), [M+9H]⁹⁺ *m/z* 570.25 (calculated: 570.34). R_t: 19.0 min (0-100% solvent B, 40 min, C18 column).

Compound **2** was synthesised in the same manner as compound **1**, except that cholic acid was conjugated following coupling of the last amino acids in the compound **1** sequence. Cholic acid (0.84 mmol/g, 4.2 equivalent) was activated using 0.5 M HATU (1.6 mL, 4 equivalent) and DIPEA (0.18 mL, 5.2 equivalent) before being coupled to the resin twice for 45 min, each, at room temperature (RT). Compound **2** was cleaved

from the resin for 15 min (instead of 4 h) and purified using preparative RP-HPLC on a C4 column using solvent B at a gradient of 30-45% over 25 min; compound detection at 214 nm. Pure compound **2** was analysed using ESI-MS and analytical RP-HPLC.

Compound **2** purity: 98%. Chemical Formula: C₂₅₃H₄₃₂N₆₈O₆₈. Molecular weight: 5514.65. ESI-MS: [M+3H]³⁺ *m/z* 1839.50 (calculated: 1839.217), [M+4H]⁴⁺ *m/z* 1379.90 (calculated: 1379.66), [M+5H]⁵⁺ *m/z* 1104.15 (calculated: 1103.93), [M+6H]⁶⁺ *m/z* 920.30 (calculated: 920.11), [M+7H]⁷⁺ *m/z* 788.90 (calculated: 788.81), [M+8H]⁸⁺ *m/z* 690.50 (calculated: 690.33). R_t: 17.0 min (0-100% solvent B, 40 min, C4 column).

Compound **3** was synthesised in the same manner as compound **2**. However, instead of coupling cholic acid, two additional Fmoc-Ser(tBu)-OH (0.84 mmol/g, 4.2 equivalent) were conjugated to compound **1**. Dde-protected C16 (0.84 mmol/g, 4.2 equivalent; synthesised following the previously reported procedure¹) was activated using 0.5 M HATU (1.6 mL, 4 equivalent) and DIPEA (0.18 mL, 5.2 equivalent) for 10 min and was coupled to the resin for 1 h twice at RT. The Dde group was removed using 2% hydrazine in DMF for 1 h before coupling another Dde-C16. N-terminal amine was acetylated with 5% acetic anhydride, 5% DIPEA, in DMF following the deprotection of Dde of second C16. Compound **3** was then cleaved from the resin for 4 h, before it was purified using preparative RP-HPLC on a C4 column using solvent B at a gradient of 40-60% over 25 min; compound detection at 214 nm. Pure compound **3** was analysed using ESI-MS and analytical RP-HPLC.

Compound **3** purity: 96%. Chemical Formula: C₂₆₉H₄₆₈N₇₂O₇₁. Molecular weight: 5847.14. ESI-MS: [M+3H]³⁺ *m/z* 1949.95 (calculated: 1950.05), [M+4H]⁴⁺ *m/z* 1462.70 (calculated: 1462.79), [M+5H]⁵⁺ *m/z* 1170.50 (calculated: 1170.43), [M+6H]⁶⁺ *m/z* 975.75 (calculated: 975.52), [M+7H]⁷⁺ *m/z* 836.45 (calculated: 836.31), [M+8H]⁸⁺ *m/z* 732.00 (calculated: 731.89), [M+9H]⁹⁺ *m/z* 650.50 (calculated: 650.68), [M+11H]¹¹⁺ *m/z* 532.45 (calculated: 532.56). R_t: 21.0 min (0-100% solvent B, 40 min, C4 column).

Nanoparticle Formation

Compound **1-3** were individually dissolved in endotoxin-free water at concentration 1 mg/mL. Each mixture was sonicated for 30 min.

Preparation of Liposomes L1-L3

Compounds **1-3** were encapsulated into liposome delivery systems to produce **L1-L3**, respectively. The liposomes were firstly formulated using the film hydration method.² Compound **1** (1 mg) was dissolved in 1 mL methanol and mixed with 0.5 mL chloroform solution of DPPC (10 mg/mL), 0.2 mL of DDAB (10 mg/mL), and 0.2 mL of cholesterol (5 mg/mL). An additional 2 mL of chloroform was added to the formulation. The organic solvent in the mixture was slowly removed under reduced pressure on a rotary evaporator, then dried under vacuum for 24 h. The dried thin lipid film was rehydrated using 1 mL endotoxin-free water and extruded through a 100 nm polycarbonate membrane at 55°C to produce uniform-sized liposomes. **L2** and **L3** were formulated in the same manner as **L1**, using compounds **2** and **3**, respectively.

Dynamic Light Scattering (DLS)

Compounds **1-3** and liposomes **L1-L3** in endotoxin-free water were analysed using DLS²⁻⁴ to measure particle size (intensity), polydispersity index (PDI), and charge (zeta

potential) of the vaccine constructs. The samples were transferred into disposable cuvettes before measurements were taken at 25°C and 173° light scattering.

Transmission Electron Microscopy (TEM)

The same samples of compounds **1-3** and liposomes **L1-L3** (as for DLS) were applied to glow-discharged carbon-coated copper 200 mesh grids and negative-stained with 1% uranyl acetate.^{3, 4} The images of the samples were taken using a JEM-1010 TEM, operated at 80kV.

MTT Cytotoxicity Assay

Cytotoxicity evaluation of compound **2** and liposome **L2** was performed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on HEK293, SW620 and NCIH460 adherent cell lines. These cell lines were cultured in culture medium (RPMI-1640 medium for SW620 and NCIH460 cells and DMEM medium for HEK293 cells) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, and incubated at 37°C with 5% CO₂. All cell lines were plated in an individual 96-well round bottom plate with a density of 2,000 cells/well for SW620 and 5,000 cells/well for NCIH460 and HEK293 in 180 µL of culture medium. The cells were incubated at 37°C with 5% CO₂ for 18 h to allow cell attachment. The cells were treated with 20 µL/well of compound **2** and liposome **L2** (at concentrations of 0.5, 1, and 2 mg/mL, each) and the plates were incubated at 37°C with 5% CO₂ for 68 h. Verapamil was used as a positive control, while untreated cells were used as negative controls. All experiments were performed in duplicate. After incubation, 100 µL of fresh culture medium and 20 µL of MTT reagent (5 mg/mL in PBS) were added and mixed well. The plates were further incubated at 37°C with 5% CO₂ for 4 h. The culture medium was then aspirated to form formazan crystals. The crystals were solubilised with 100 µL/well of DMSO and incubated for 30 min at 37°C with 5% CO₂ before a Powerwave XS microplate reader was used to read the absorbance at 580 nm. The IC₅₀ data were analysed as the concentration of analyte required for 50% inhibition of cancer cell growth (compared to negative controls) using GraphPad Prism 7 software (GraphPad Software, Inc., USA).

Intranasal Immunisation in an *in vivo* Model

Six-week-old female C57BL/6 mice were housed in the Australian Institute for Bioengineering and Nanotechnology (AIBN) Animal Facility at The University of Queensland and allowed to acclimatise for 7 d before experimentation. Mice were intranasally immunised with 30 µL (15 µL per nare) of compound **2**, compound **3** or liposome **L1**, **L2** or **L3** (each containing 30 µg of antigen) on day 0, followed by two boosts of the same dose on days 21 and 42. Positive control mice were given 30 µg of peptide **1** mixed with 10 µg CTB dissolved in water, whereas negative control mice received 30 µL of PBS.

Collection of Serum and Saliva

Serum samples were collected on day 60 after primary immunisation to measure J8-specific IgG antibody titres. Blood (1 mL) was collected via heart puncture on day 60 and centrifuged at 3,600 rpm for 10 min. Serum was collected from the supernatant and kept at -80°C until further analysis.

Saliva samples were collected on day 49 after primary immunisation to measure J8-specific IgA antibody titres. Salivation was induced by administering 50 µL of 0.1% pilocarpine intraperitoneally to the mice. 100 µL samples of saliva were collected into 2 µL of protease inhibitor in 100 mM PMSF solution. Saliva samples were kept at -80°C until further analysis.

Antibody Titre Detection by ELISA

J8-specific antibody (IgG and IgA) titres were detected using an enzyme-linked immunosorbent assay (ELISA), as detailed elsewhere.^{2, 5} Two-fold serial dilution was performed starting at a 1:100 concentration of serum and 1:4 concentration of saliva. Naïve mice sera and saliva were used as controls. Secondary antibody was added to the plate before adding OPD substrate. A SpectraMax microplate reader was used to read the absorbance at 450 nm. Antibody titres (IgG and IgA) were expressed as the lowest possible dilution with absorbance above the mean plus three times the standard deviation of control wells.

The data were analysed using GraphPad Prism 7 software. Multiple comparisons were performed using one-way ANOVA with Tukey's multiple comparison test. Data were considered significantly different at (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$.

Antibody Opsonisation Assay

Opsonisation assays were performed as previously published,⁶ on ACM-5199, ACM-5203, GC2 203, D3840 and D2612 GAS clinical isolates. Bacteria were prepared by streaking on THB agar plates (supplemented with 5% yeast extract), then incubated (37°C, 24 h). A single colony from each bacterium was transferred to 5 mL of THB (supplemented with 5% yeast extract) and incubated (37°C, 24 h) to replicate the bacteria to approximately 4.6×10^6 colony forming units (CFU)/mL. The cultures were serially diluted to 10^{-2} in PBS with a 10 µL aliquot that was mixed with 10 µL heat-inactivated sera collected on day 60, together with 80 µL horse blood. Sera were inactivated by heating in a 50°C water bath for 30 min. The assay was performed in triplicate from three independent cultures. Bacteria were incubated in a 96-well plate (37°C for 3 h) in the presence of sera, before 10 µL of the aliquot was plated on THB agar plates (supplemented with 5% yeast extract and 5% horse blood) and incubated (37°C for 24 h). The bacterial survival rate was analysed based on CFU enumerated from the plates. The opsonic activity (% reduction in mean CFU) of the antibody sera was calculated as:

$$\frac{(1 - CFU_{ab})}{CFU_{PBS}} \times 100\%$$

CFU_{ab} : CFU in presence of antibodies sera mean

CFU_{PBS} : CFU in presence of PBS

Ethic Statement

This study was performed according to the regulations set by the National Health and Medical Research Council (NHMRC) of Australia (Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th edition 2013). All animal

procedures and protocols were approved by The University of Queensland Animal Ethics Committee (AEC), AEC Approval Number: SCMB/AIBN/069/17.

References:

- (1) Ahmad Fuaad, A. A. H.; Skwarczynski, M.; Toth, I., The use of microwave-assisted solid-phase peptide synthesis and click chemistry for the synthesis of vaccine candidates against hookworm infection. *Methods in Molecular Biology* **2016**, *1403*; pp. 639-653.
- (2) 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 candidates against Group A Streptococcus. *Acta Biomaterialia* **2016**, *41*; pp. 161-168.
- (3) Azmi, F.; Fuaad, A. A. A.; Giddam, A.; Batzloff, M.; Good, M.; Skwarczynski, M.; Toth, I., Self-adjuvanting vaccine against Group A Streptococcus: Application of fibrillized peptide and immunostimulatory lipid as adjuvant. *Bioorganic and Medicinal Chemistry* **2014**, *22*; pp. 6401-6408.
- (4) Chan, A.; Hussein, W.; Ghaffar, K.; Marasini, N.; Mostafa, A.; Eskandari, S.; Batzloff, M.; Good, M.; Skwarczynski, M.; Toth, I., Structure-activity relationship of lipid core peptide-based Group A Streptococcus vaccine candidates. *Bioorganic and Medicinal Chemistry* **2016**, *24*; pp. 3095-3101.
- (5) Zaman, M.; Abdel-Aal, A.; Phillipps, K.; Fujita, Y.; Good, M.; Toth, I., Structure-activity relationship of lipopeptide Group A streptococcus (GAS) vaccine candidates on toll-like receptor 2 *Vaccine* **2010**, *28*; pp. 2243-2248.
- (6) Marasini, N.; Giddam, A. K.; Khalil, Z. G.; Hussein, W. M.; Capon, R. J.; Batzloff, M. R.; Good, M. F.; Toth, I.; Skwarczynski, M., Double adjuvanting strategy for peptide-based vaccines: trimethyl chitosan nanoparticles for lipopeptide delivery. *Nanomedicine (London, U. K.)* **2016**, *11*; pp. 3223-3235.

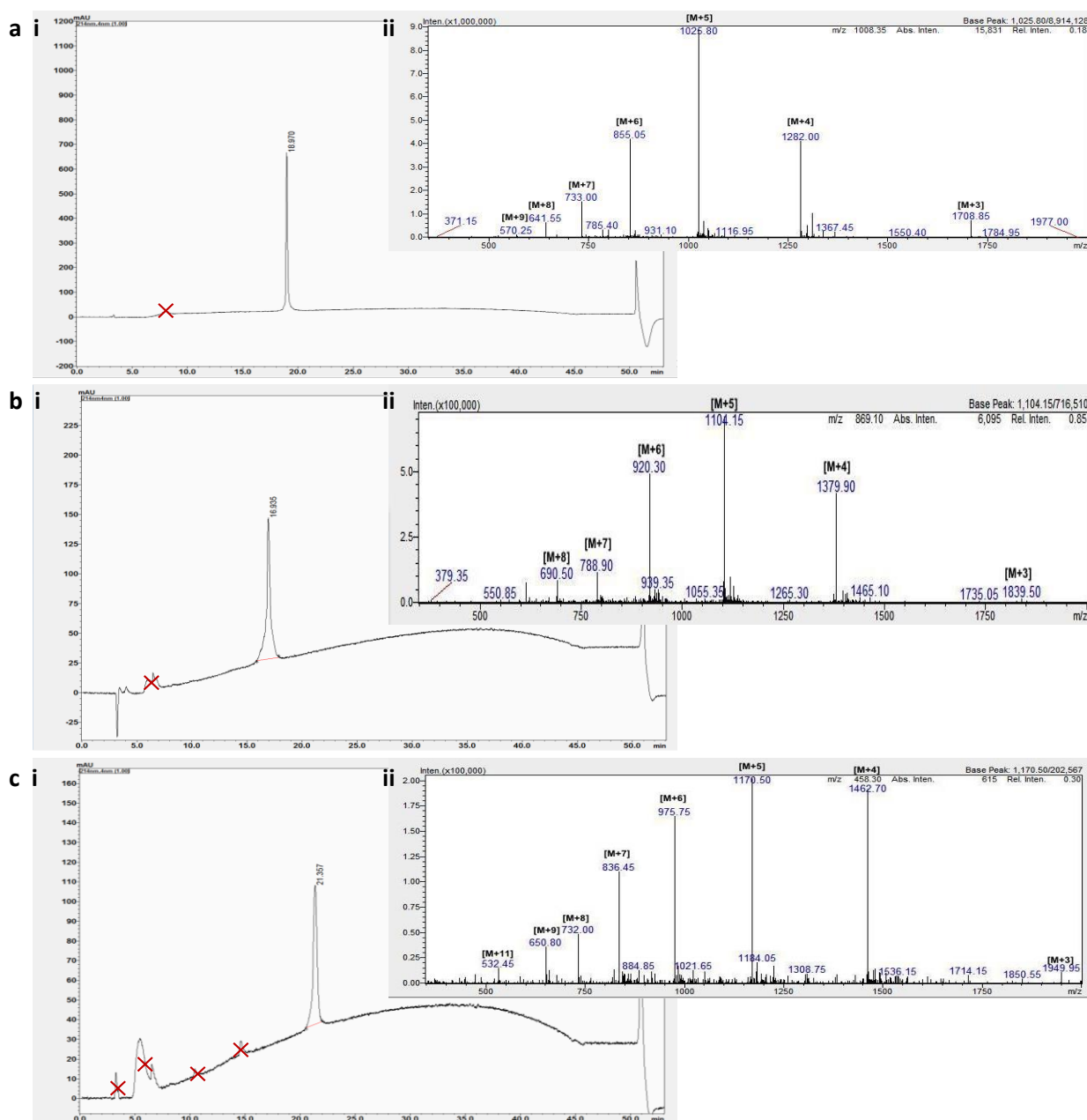


Figure S1. Analysis of purified compounds **1-3** by analytical RP-HPLC (**ai-ci**) and ESI-MS (**aii-cii**). The compounds were purified using preparative RP-HPLC with solvent B at concentration gradients of 0-50% (**1**) 30-45% (**2**) and 40-60% (**3**) from R_t 5 min to 30 min. “X” marks the background noise of the RP-HPLC column.

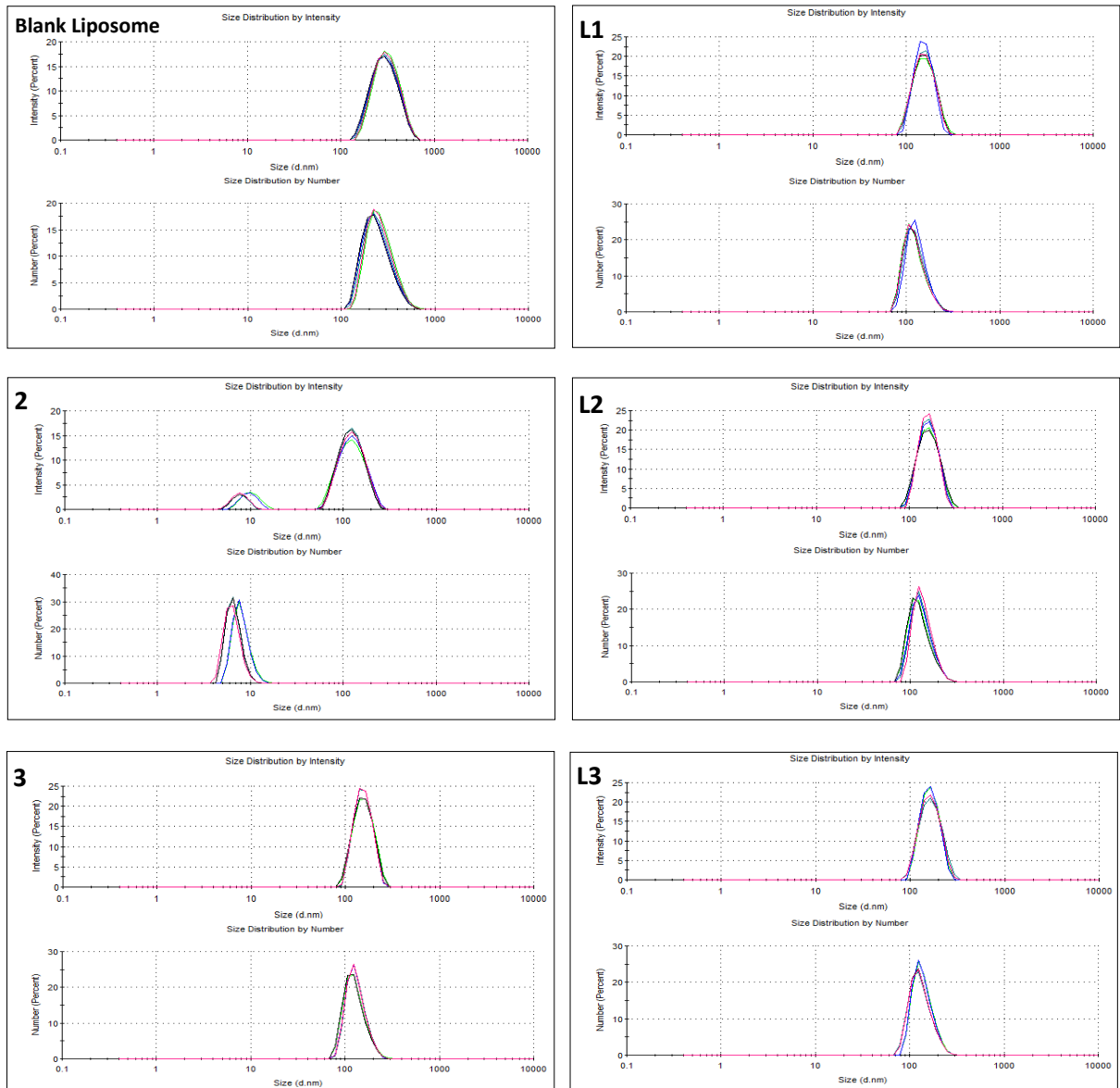


Figure S2. DLS spectra of particle size, by intensity (top) and number (bottom) for peptide 1, lipopeptides 2-3, blank liposome, and encapsulated liposomes L1-L3.

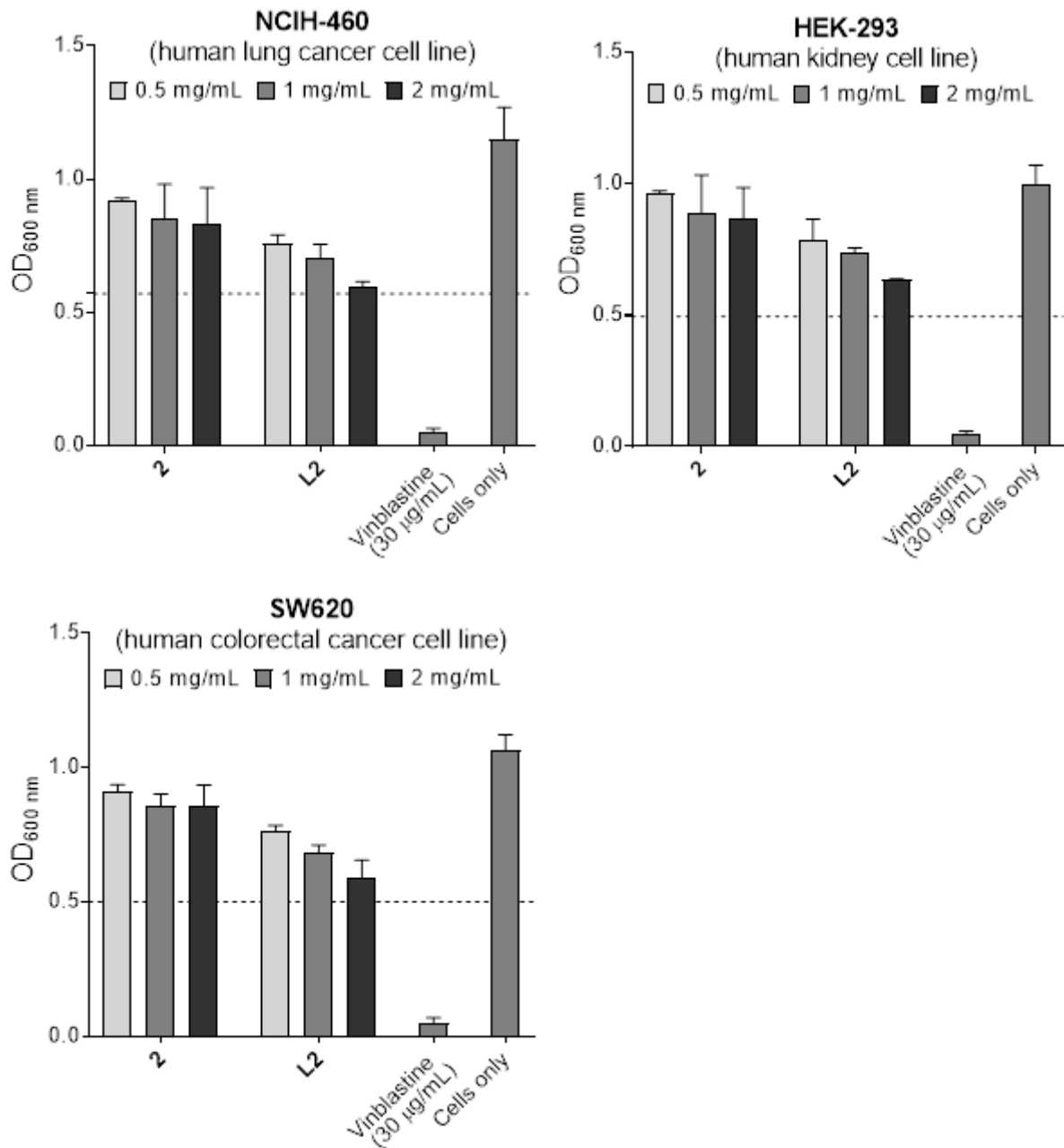


Figure S3. MTT cytotoxicity assay of lipopeptide 2 and encapsulated liposome L2.

Table S1. Liposome size over 11 weeks at room temperature.

Vaccine candidates	Particle size (nm)		Polydispersity index (PDI)	
	Day 0	Day 79	Day 0	Day 79
L1	159±2	160±3	0.04±0.02	0.04±0.01
L2	163±1	170±3	0.03±0.02	0.04±0.02
L3	165±2	169±4	0.03±0.02	0.04±0.01
Blank liposome	161±1	162±1	0.04±0.01	0.05±0.01