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A Potent Vaccine Delivery System

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[Abstract] Most vaccines require co-delivery of an adjuvant in order to generate the desired immune responses. However, many currently available adjuvants are non-biodegradable, have limited efficacy, and/or poor safety profile. Thus, new adjuvants, or self-adjuvanting vaccine delivery systems, are required. Here, we proposed a self-adjuvanting delivery system that is fully defined, biodegradable, and non-toxic. The system is produced by conjugation of polyleucine to peptide antigen, followed by self-assembly of the conjugate into nanoparticles. The protocol includes solid-phase peptide synthesis of the vaccine conjugate, purification, self-assembly and physicochemical characterization of the product. Overall, this protocol describes, in detail, the production of a well-defined and effective self-adjuvanting delivery system for peptide antigens, along with tips for troubleshooting.

Keywords: Poly (hydrophobic amino acid), Delivery system, Subunit vaccine, Peptide synthesis, Particle self-assembly, Self-adjuvanting nanoparticles, Polyleucine, Secondary structure

[Background] Peptide subunit vaccines, which use the small antigen fragments (epitopes) to trigger protective immune responses against infectious diseases, are one of the most promising vaccine technologies to have emerged in recent decades (Skwarczynski and Toth, 2016; Malonis *et al.*, 2020). However, as peptides, themselves, are always poorly immunogenic, they need to be co-administered with an adjuvant (immune stimulator) and/or delivery system (Azmi *et al.*, 2014; Nevagi *et al.*, 2018). Currently, only a few options exist when it comes to adjuvants that are safe enough to be administered to humans. While more numerous in options, experimental adjuvants are often poorly defined, toxic, or have limited efficacy (Shi *et al.*, 2019). One of the most recent strategies developed to deliver vaccines utilizes nanostructures with self-adjuvanting properties (Skwarczynski and Toth, 2014). Self-assembling polymers, in particular, have been widely investigated (Zhao *et al.*, 2017; Nevagi *et al.*, 2019). However, the structures of these polymers are not fully defined (number of units, stereochemistry) and, therefore, batch variability may affect vaccine activity and safety profile.

We have conceptualized, designed, and developed a new vaccine adjuvant/delivery system to overcome the disadvantages outlined above. This system is based on fully-defined and biodegradable polymers built from our own natural hydrophobic amino acids. The lead vaccine candidate produced based on this system was able to stimulate the production of highly opsonic antibodies against six clinical isolate strains of group A streptococcus in mice (Skwarczynski *et al.*, 2020). The compound was more efficient than the powerful, but toxic, “gold standard” Complete Freund’s Adjuvant and did not

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induce undesired inflammatory responses. The strategy to deliver antigenic epitopes attached to self-adjuvanting amino acid-based polymer described here offers an attractive, safe alternative to conventional vaccine adjuvants. Importantly, this approach can be fully customized to match the properties of the antigen of choice. The procedure on how to produce this vaccine candidate (Figure 1) is presented here, with reference to the published vaccine study (Skwarczynski *et al.*, 2020). Notes provide additional helpful information.

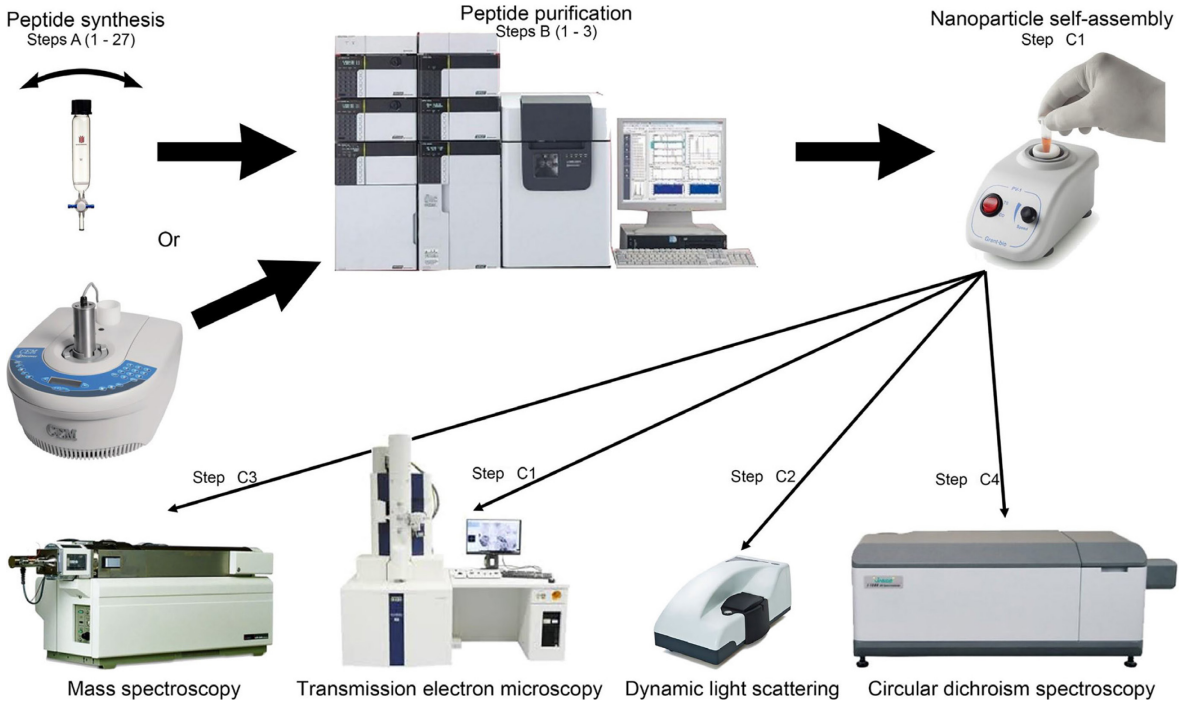

Figure 1. Flowchart of vaccine candidate synthesis, purification and characterization steps

Materials and Reagents

Note: All chemicals should be analytical grade, unless stated otherwise.

A. Vaccine candidate synthesis

1. Chemical resistance gloves (Ansell, catalog number: 02-100)
2. Rink amide p-methyl-benzhydrylamine hydrochloride (pMBHA·HCl) resin (substitution: 0.59 mmol/g; 100-200 mesh; Peptides International, catalog number: RMB-1045-PI)
3. N,N-dimethylformamide (DMF; Merck, catalog number: 227056) (see Note 1)
4. N,N-diisopropylethylamine (DIPEA; 6.2 equivalent; Merck, catalog number: 387649)
5. Trifluoroacetic acid (TFA; Merck, catalog number: 302031)
6. Butyloxycarbonyl (Boc)–protected L-amino acids (0.84 mmol/g; 4.2 equivalent; Novabiochem Merck Chemicals and Mimotopes)
7. 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU; 0.5 M; 4 equivalent; Mimotopes, catalog number: 148893-10-1) solution: 9.5 g HATU in 50 ml DMF (store solution at 0 °C for no longer than 1 week after preparation) (see Note 2)

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8. Capping solution: 5% acetic anhydride (Sigma-Aldrich, catalog number: 320102), 5% DIPEA, and 90% DMF (v/v/v)
9. Dichloromethane (DCM; Merck, catalog number: 270997)
10. Piperidine deprotection solution: 20% piperidine (Sigma-Aldrich, catalog number: 8.22299) and 80% DMF (v/v)
11. Methanol (Merck, catalog number: 34860)
12. p-Cresol (Sigma-Aldrich, catalog number: C85751)
13. Hydrofluoric acid (HF; Ghc Gerling, Holz & Co. Handels gmbh, catalog number: 3100, Hydrogen Fluoride [99.95%])
14. Diethyl ether (Sigma-Aldrich, catalog number: 91238)
15. n-hexane (Merck, catalog number: 1.04367)
16. Acetonitrile (Merck, catalog number: 271004)
17. Endotoxin-free Milli-Q water (sensitivity of 18.2 MΩ.cm at 25 °C and total organic content below 5 parts per billion)
18. Solvent A: 100% Milli-Q water and 0.1% TFA (v/v; solution can be stored at room temperature for up to 3 months)
19. Solvent B: 90% acetonitrile, 10% Milli-Q water, and 0.1% TFA (v/v/v; solution can be stored at room temperature for up to 3 months)

B. Vaccine candidate purification
   1. Phenex syringe filter (0.45 µm; Phenomenex, catalog number: AF3-3107-52)
   2. Reagents listed previously (Solvent A and B)

C. Vaccine candidate characterization
   1. Disposable capillary cuvettes (Malvern Analytical, model: DTS1070)
   2. Whatman filter paper (Merck, catalog number: WHA1005090)
   3. Phosphate-buffered saline (PBS; ThermoFisher Scientific, catalog number: 10010031)
   4. Phosphotungstic acid stain (2%): 2 mg phosphotungstic acid hydrate (Sigma-Aldrich, catalog number: P4006-25G) in 100 ml Milli-Q water (stir for 1 h, then filter; solution can be stored at 2-8 °C for up to 3 months)

Equipment

A. Vaccine candidate synthesis
   1. Laboratory glassware
   2. CEM Discover Solid Phase Synthesis (SPS) reactor (CEM Corporation, model: Discover SPS; see Software 1) (see Note 3)
   3. Peptide synthesis vessel (CEM Corporation, catalog number: 170897) (see Note 4)
   4. Glass peptide synthesis vessel (Sigma-Aldrich, catalog number: Z41,850-1)

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5. CEM Discovery SPS vacuum manifold filtration apparatus (CME Corporation, catalog number: 167993) (see Note 4)
6. Scintillation vials (Merck, catalog number: DWK986568)
7. Vortex mixer (Phoenix Instruments, model: RS-VA 1) or sonicator (Baranson Ultrasonicator Corporation, catalog number: 2510E-MTH)
8. Rotary mixer (Ratek Instruments, catalog number: RSM7DC)
9. Desiccator
10. Hydrofluoric acid (HF)-reaction apparatus (including HF reaction vessel) for peptide cleavage from the resin (refer to Jadhav *et al.*, 2020)
11. Alpha 2-4 LD freeze dryer (John Morris Scientific, catalog number: 101521)

B. Vaccine candidate purification
1. Shimadzu preparative reverse-phase HPLC (RP-HPLC) instrument (Shimadzu, models: LC-20AP × 2, CBM-20A, SPD-20A, FRC-10A) with a 20.0 ml/min flow rate (see Software 2)
2. Vydac C4 (Hichrom, catalog number: 214TP54, 5 µm, 4.6 × 250 mm; and 214TP1022, 10 µm, 22 × 250 mm) or C8 columns (Hichrom, catalog number: 208TP54; 5 µm, 4.6 × 250 mm)
3. Perkin-Elmer-Sciex API3000 electrospray ionization mass spectrometry (ESI-MS) instrument (Applied Biosystems/MDS Sciex, model: Sciex API3000; see Software 3)
4. Shimadzu analytical RP-HPLC instrument (Shimadzu, models: DGU-20A5, LC-20AB, SIL-20ACHT, SPD-M10AVP) with a 1.0 ml/min flow rate (see Software 2)

C. Vaccine candidate characterization
1. Malvern Zetasizer dynamic light scattering (DLS; Malvern Instruments, model: Nano ZS; see Software 4)
2. JEM-1010 transmission electron microscope (TEM; JEOL, see Software 5)
3. Carbon-coated copper grids (Pure Carbon Film 200 mesh, Ted Pella, catalog number: 01840-F)
4. Jasco J710 circular dichroism (CD) spectrometer (JASCO Corporation, model: J710; see Software 6)
5. CD 1.0 mm cell (Starna, catalog number: 21/Q/1/CD)

Software

1. Synergy™ (CME Corporation, North Carolina, USA, www.cemsynthesis.com)
2. LabSolutions (Shimadzu, Kyoto, Japan, www.shimadzu.com)
3. Analyst® 1.6 (Applied Biosystems/MDS Sciex, Toronto, Canada, www.sciex.com)
4. Malvern Zetasizer Analyzer 6.2 (Malvern Instruments, Worcestershire, UK, www.malvernpanalytical.com)
5. Olympus Soft Imaging Solutions (Olympus Corporation, Tokyo, Japan, www.olympus-global.com)

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6. Spectra Manager™ (JASCO Corporation, Tokyo, Japan, www.jascoinc.com)

Procedure

A. Vaccine candidate synthesis (see Note 5)

1. **Resin swelling:** Weight out 339 mg pMBHA•HCl resin (0.2 mmol equivalent) into a peptide synthesis vessel and add 10 ml of DMF and 0.216 ml of DIPEA (see Note 6). Let the resin swell for at least 2.5 h (see Note 7).
2. **Resin washing:** Drain the solvent using vacuum filtration and wash the resin three times using DMF (~5 ml per wash; see Notes 8-9).
3. **Boc deprotection** (see Note 9)**:** Drain all of the solvent before adding ~5 ml of neat TFA. Stir the resin gently for 2 min with a stirring rod (see Note 10).
4. Drain the TFA, and repeat Step A3.
5. **Resin washing:** Drain the solvent using vacuum filtration and wash the resin five times with DMF (~5 ml per wash; see Notes 8-9).
6. **Amino acid activation:** Weigh out each amino acid in scintillation vials. Add 1.6 ml of HATU and 0.181 ml of DIPEA (see Note 11).
7. **Amino acid coupling:** Drain all of the solvent off before adding the preactivated amino acid to the washed resin and stir gently (see Note 12). Insert the vessel, with temperature probe, into the microwave and heat for 5 min at 70 °C (see Note 13).
8. **Resin washing:** Drain the solvent using vacuum filtration and wash the resin three times with DMF (~5 ml per wash; see Note 8).
9. Repeat Steps A6-A7 for the second coupling (10 min at 70 °C).
10. **Resin washing:** Drain the solvent using vacuum filtration and wash the resin five times with DMF (~5 ml per wash; see Note 8).
11. **Acetylation:** Drain the solvent completely, then add 5 ml of capping solution to the resin. Stir gently, then insert the vessel, with temperature probe, into the microwave and heat the mixture for 10 min at 70 °C (see Note 14).
12. **Resin washing:** Drain the solvent using vacuum filtration and wash the resin three times with DMF (~5 ml per wash; see Note 8).
13. Repeat Step A11 for the second acetylation.
14. **Resin washing:** Drain the solvent using vacuum filtration and wash the resin five times with DMF (~5 ml per wash; see Note 8).
15. Repeat Steps A2-A10 for the following amino acid sequence, until the **Peptide 1** (Figure 2) sequence is finished (Table 1, entry 1-42).

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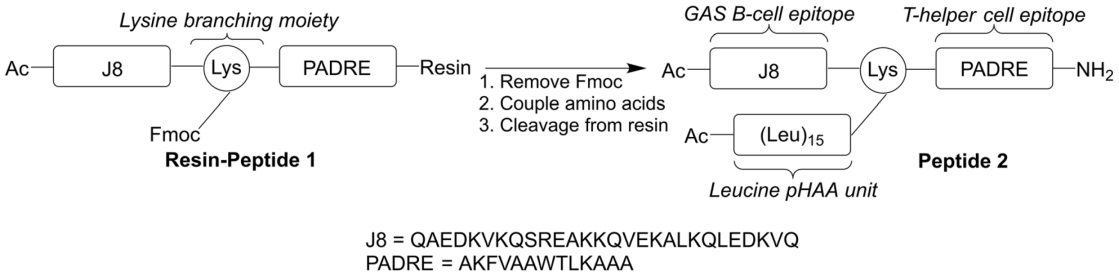

Figure 2. Synthesis of the vaccine candidate Peptide 2, which is comprised of (i) GAS J8 B-cell epitope, and (ii) PADRE T-helper cell epitope, branched with (iii) a leucine poly(hydrophobic amino acid) (pHAA) unit via lysine linker

Table 1. List of amino acids used for the synthesis of vaccine candidate Peptide 2

PADRE	1	Boc-Ala-OH	A	J8 (continue)	30	Boc-Lys(2Cl-Z)-OH	K
	2	Boc-Ala-OH	A		31	Boc-Ala-OH	A
	3	Boc-Ala-OH	A		32	Boc-Glu(OcHx)-OH	E
	4	Boc-Lys(2Cl-Z)-OH	K		33	Boc-Arg(Tos)-OH	R
	5	Boc-Leu-OH H_2O	L		34	Boc-Ser(Bzl)-OH	S
	6	Boc-Thr(Bzl)-OH	T		35	Boc-Gln-OH	Q
	7	Boc-Trp(For)-OH	W		36	Boc-Lys(2Cl-Z)-OH	K
	8	Boc-Ala-OH	A		37	Boc-Val-OH	V
	9	Boc-Ala-OH	A		38	Boc-Lys(2Cl-Z)-OH	K
	10	Boc-Val-OH	V		39	Boc-Asp(OcHx)-OH	D
	11	Boc-Phe-OH	F		40	Boc-Glu(OcHx)-OH	E
	12	Boc-Lys(2Cl-Z)-OH	K		41	Boc-Ala-OH	A
	13	Boc-Ala-OH	A		42	Boc-Gln-OH	Q
Branching moiety	14	Boc-Lys(Fmoc)-OH	K	Leucine pHAA unit	43	Boc-Leu-OH H_2O	L
J8	15	Boc-Gln-OH	Q		44	Boc-Leu-OH H_2O	L
	16	Boc-Val-OH	V		45	Boc-Leu-OH H_2O	L
	17	Boc-Lys(2Cl-Z)-OH	K		46	Boc-Leu-OH H_2O	L
	18	Boc-Asp(OcHx)-OH	D		47	Boc-Leu-OH H_2O	L
	19	Boc-Glu(OcHx)-OH	E		48	Boc-Leu-OH H_2O	L
	20	Boc-Leu-OH H_2O	L		49	Boc-Leu-OH H_2O	L
	21	Boc-Gln-OH	Q		50	Boc-Leu-OH H_2O	L
	22	Boc-Lys(2Cl-Z)-OH	K		51	Boc-Leu-OH H_2O	L
	23	Boc-Leu-OH H_2O	L		52	Boc-Leu-OH H_2O	L
	24	Boc-Ala-OH	A		53	Boc-Leu-OH H_2O	L
	25	Boc-Lys(2Cl-Z)-OH	K		54	Boc-Leu-OH H_2O	L
	26	Boc-Glu(OcHx)-OH	E		55	Boc-Leu-OH H_2O	L
	27	Boc-Val-OH	V		56	Boc-Leu-OH H_2O	L
	28	Boc-Gln-OH	Q		57	Boc-Leu-OH H_2O	L
	29	Boc-Lys(2Cl-Z)-OH	K				

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After coupling the final amino acid of **Peptide 1**, repeat Steps A2-A5 (washing and Boc deprotection), then proceed with Steps A11-A14 (**Peptide 1** acetylation at the N-terminus; see Note 15).

16. **Fmoc group deprotection:** Drain all of the solvent, then add 5 ml of piperidine deprotection solution to the resin and stir gently. Insert the vessel and temperature probe into the microwave. Heat to 70 °C for 2 min.
17. **Resin washing:** Drain the solvent using vacuum filtration, then wash the resin three times with DMF (~5 ml per wash; see Note 8).
18. Repeat Step A16 for the second Fmoc group deprotection (5 min at 70 °C; see Note 16).
19. **Resin washing:** Drain the solvent using vacuum filtration, then wash the resin five times with DMF (~5 ml per wash; see Note 8).
20. Repeat Steps A6-A10 for the first leucine in the pHAAs.
21. Repeat Steps A2-A10 for the remaining leucine's, until the **Peptide 2** (Figure 2) sequence is complete (Table 1, entry 43-57).
22. After the final amino acid coupling of **Peptide 2**, repeat Steps A2-A5 (washing and Boc deprotection), then proceed with Steps A11-A14 (**Peptide 2** acetylation at the N-terminus; see Note 15).
23. **Resin drying:** Drain the solvent using vacuum filtration, then wash the resin using ~5 ml of DMF (three times), DCM (three times), then methanol (once). Remove all solvent and leave the resin under vacuum filtration for 5 min.
24. Leave the vessel with the resin in a desiccator under reduced pressure overnight to fully remove the solvents.
25. Measure the finished resin-**Peptide 2**, then weigh out 500 mg of the resin into a 15 ml Falcon tube (see Note 17).
26. **Peptide cleavage:** Transfer the 500 mg of resin into a HF reaction vessel, then add 0.25 ml of p-cresol scavenger (0.5 ml/g of resin) and 5 ml of HF (10 ml/g of resin). Cleave the peptide from the resin following the HF-cleavage protocol (see Note 18 and Jadhav *et al.*, 2020).
27. **Reaction work up:** Wash the resin with 30 ml of cold diethyl ether:n-hexane (4:1; v/v) twice, then remove the solution through filtration. Dissolve the white precipitate using 50% Solvent A and 50% Solvent B (v/v), then filter the solution (from the resin) into a round-bottom flask. Wash the resin with Solvent B and collect the filtrate into a round-bottom flask. Freeze-dry the filtrate to obtain a yellowish-white powder (crude **Peptide 2**).

B. Vaccine candidate purification

1. **Purification** (see Note 19)**:** Dissolve 30 mg crude **Peptide 2** in 2 ml of 50% Solvent A and 50% Solvent B (v/v). Load the solution into a 5 ml syringe and filter the solution through syringe-filter into a scintillation vial. Wash the filter with 2 ml of 50% Solvent A and 50% Solvent B (v/v). Run the filtered crude **Peptide 2** using preparative RP-HPLC on a C4 column with solvent B gradients (65-85%) for 25 min, with compound detection at 214 nm.

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2. Analyze the fractions of the purified **Peptide 2** using ESI-MS and analytical RP-HPLC on a C4 column with a 0-100% gradient of solvent B for 40 min and compound detection at 214 nm (see Note 20).
3. Combine and freeze-dry the fraction based on its purity (as analyzed by ESI-MS and analytical RP-HPLC; Figure 3; Note 21). The final product should be a white solid. Molecular weight: 6521.03. ESI-MS $[M + 4H]^{4+}$ m/z 1631.8 (calc. 1631.3), $[M + 5H]^{5+}$ m/z 1305.6 (calc. 1305.2), $[M + 6H]^{6+}$ m/z 1088.0 (calc. 1087.8), $[M + 7H]^{7+}$ m/z 932.9 (calc. 932.6), $[M + 8H]^{8+}$ m/z 816.3 (calc. 816.1), $[M + 9H]^{9+}$ m/z 725.6 (calc. 725.6). t_R = 30.9 min (0 to 100% solvent B; C4 column); purity ≥ 99%.

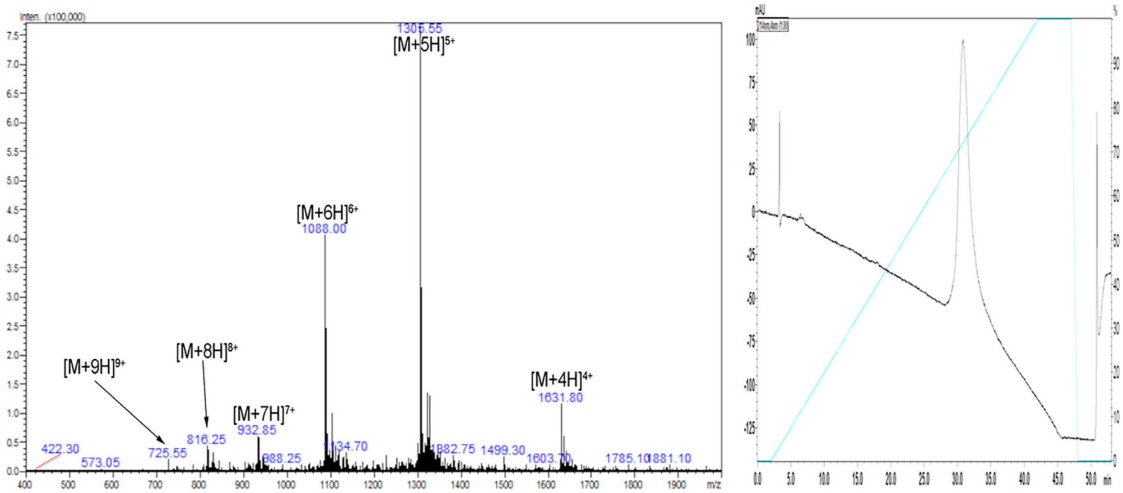

Figure 3. Analysis of the purified vaccine candidate Peptide 2 by ESI-MS (left) and analytical RP-HPLC (right)

C. Vaccine candidate characterization

1. Measure 1.5 mg of pure **Peptide 2** into a 2 ml microcentrifuge tube. Add 1 ml of PBS to prepare a 1.5 mg/ml solution (concentration of the vaccine candidate for the *in vivo* study). Vortex the mixture for 2-30 min (with 1-min intervals) until none of the solid peptide is visible (see Note 22).
2. Size and PDI:
   a. Transfer 0.5 ml of self-assembled vaccine candidate (from Step C1) into a new 2 ml microcentrifuge tube, then add 1 ml of PBS to prepare a 0.5 mg/ml solution (1:3 dilution) for DLS analysis.
   b. Turn on the Malvern Zetasizer DLS, open the Analyzer software, and connect with the instrument before starting measurements.
   c. Fill the disposable cell with diluted solution (~800 µl) and insert the cell into the instrument.
   d. Perform measurements (size, PDI) at 25 °C with non-invasive backscatter at a backscattering angle of 173°. Correlation times are based on 10 s per run; at least 10 consecutive runs should be made per measurement. Take at least five measurements per

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sample, and measure PBS as a blank (negative control).

e. Calculate the mean ± standard deviation for each sample using the five measurements.

f. In general, compounds should self-assemble into a mixture of small nanoparticles (10-30 nm) and larger aggregates with high polydispersity indexes (PDI > 0.3, according to DLS).

3. Morphology:

a. Pipette 5 µl of the 1:3 diluted solution (the same as what was used for DLS) onto a glow-discharged carbon-coated copper grid and leave for 2 min to dry (see Note 23).

b. Gently drain the excess liquid with a piece of filter paper and allow the grid to dry.

c. Add one drop (~5 µl) of 2% phosphotungstic acid to the dried grid for 20 s to negatively-stain the sample, then drain the excess stain solution off with a piece of filter paper.

d. Air-dry the grid for 5 min before observing it under TEM. Take images at an accelerating voltage of 100 kV (Figure 4).

e. Distinct nanoparticles and chain-like aggregates of nanoparticles (CLAN) should be visible in **Peptide 2** when viewed under TEM (Figure 4).

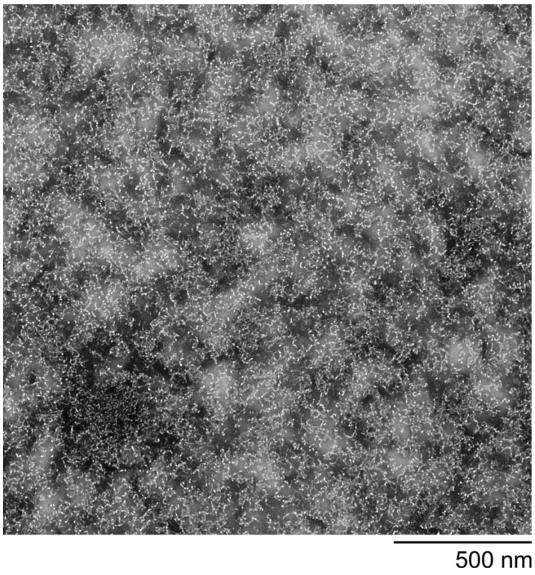

Figure 4. Particle image of vaccine candidate Peptide 2 captured by TEM [bar 500 nm; the dark areas are a result of the negative stain (2% phosphotungstic acid)]

4. Secondary structure:

a. Transfer 0.1 ml of the self-assembled vaccine candidate (non-diluted sample from Step C1) into a new 2 ml microcentrifuge tube and add 1.4 ml PBS to prepare 0.1 mg/ml solution (1:15 dilution) for CD analysis (see Note 24).

b. Set up the Spectra Manager software with the following parameters: bandwidth, 5 nm; scan rate, 50 nm/min; response time, 2 s; interval, 1 nm over the wavelength range of 195 to 260 nm.

c. Insert the cell into the instrument and run a quick scan on an empty CD cell to make sure the cell is clean (the flat, horizontal line should be visible without substantial deviation from

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zero mdeg; see Note 25). Wash the cell if needed (see Note 26).

d. Fill the CD cell with diluted solution (~200 µl) and insert the cell into the instrument. Take at least six accumulations per measurement. Run PBS or water as a blank (negative control). After all measurements, clean the cell with water or methanol, only.

e. Using Spectra Manager, subtract the baseline spectra (PBS blank reading) from the vaccine candidate spectra before converting millidegrees (mdeg) to molar ellipticity ([θ]; $deg \cdot cm^2 \cdot dmol^{-1}$).

f. The conversion is done via the following formula:

$$[\theta] = mdeg/(l \times c \times n) \times 1000$$

where:

l = path length (0.1 cm),

c = peptide concentration (mM), and

n = number of amino acids in the peptide.

g. The vaccine candidate **Peptide 2** should adopt a helical conformation with a minimum at 222 nm and a lower-intensity minimum at 208 nm (Figure 5).

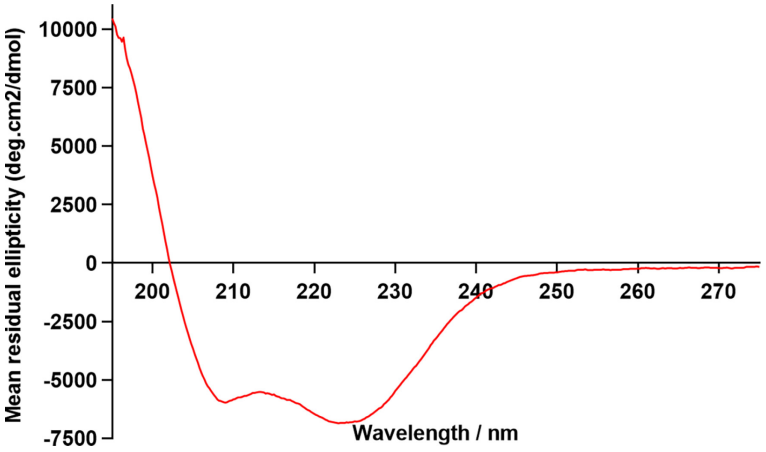

Figure 5. Circular dichroism (CD) spectra of vaccine candidate Peptide 2

Notes

1. Normal nitrile or latex gloves provide poor protection against DMF. Chemical resistance gloves should instead be worn when dealing with DMF.
2. HATU/HBTU/HCTU can induce an allergic response if it comes in contact with skin; caution should be taken when handling these chemicals.
3. The reactor is equipped with a vacuum manifold for liquid transfers and a fiber optic temperature probe. Each microwave system usually has its own complementary peptide synthesis vessels and vacuum filtration apparatus. Consult with your microwave provider for further information.

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4. This peptide synthesis vessel is a 20 ml, microwave-safe, open-vessel apparatus equipped with porous frit (to allow filtration of the peptide from the resin) and a cap at the bottom. Alternatively, synthesis can be done without the assistance of a microwave (heat); a glass peptide synthesis vessel may be used instead. Draining and washing of this glass vessel need to be done through filtration under vacuum. A rotary mixer is also required to provide continuous mixing.
5. Synthesis goes from the C- to N-terminus of the peptide amino acid sequence. Make sure the sequence is correct before starting. The information provided here is calculated for a 0.2 mmol synthesis scale.
6. During resin swelling and peptide synthesis, the resin should remain submerged in DMF solvent, with solvent level at least 2 cm higher than the resin. A rotary mixer can be used to improve swelling by continuous mixing.
7. The resin can be left to swell for up to 24 h. Alternatively, insert the resin-containing vessel into the microwave, together with the temperature probe, and heat to 70 °C for 10 min for fast swelling.
8. Perform each wash carefully to ensure complete removal of all reagents. Inadequate washing could result in the formation of side products during synthesis. If the microwave was used, remove the vessel from the microwave, then wash the temperature probe along with the resin and stirring rod (used in Boc deprotection).
9. Wash the resin five times with DCM (~5 ml for each wash; instead of DMF) before and after TFA deprotection of Boc from Boc-Gln(Xan)-OH. This prevents the cyclization of glutamine.
10. Alternatively, if the synthesis is done in a glass peptide synthesis vessel (for synthesis without microwave (heat) assistance), stirring can be done by placing the TFA/resin mixture on a rotary mixer for 2 min. Make sure the vessel is tightly closed on both ends after adding the TFA.
11. Each amino acid should be preactivated 2-5 min before reaction. Dissolve the amino acid fully before coupling to the resin. Use a sonicator or vortex to speed up the process.
12. Add additional DMF to the vessel to make sure the resin stays submerged.
13. Boc-amino acids are commonly heated at 70 °C for 10 min during peptide synthesis. The double-couplings are done for 5 min, then 10 min. However, cystine, histidine, and arginine must be coupled for 15 min, twice, at 50 °C. Alternatively, for coupling without microwave (heat) assistance, place the glass vessel onto a rotary mixer for 10 min (first coupling), then 20 min (second coupling), or 20 min each for cystine, histidine, and arginine. Make sure the vessel is tightly closed on both ends.
14. Double-acetylations are done for 10 min, each. The acetylation is performed after the first amino acid coupling to cap the unreacted site to the resin to avoid the formation of side products, which affect the purity of the final product. Do not remove Boc prior to this acetylation (non-N terminus acetylation), as it can impede any additional amino acid coupling. Alternatively, for acetylation without microwave (heat) assistance, place the glass vessel on a rotary mixer for 20 min. Make sure the vessel is tightly closed on both ends.
15. After all amino acids have been coupled, remove the Boc protective group of the final amino

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acid before proceeding with acetylation at the N-terminus of the peptide.

16. Double-deprotections are done for 2 min, then 5 min. Make sure that these deprotection steps are done after the acetylation of the N-terminus. The Fmoc protective group on the lysine (branching moiety) is removed using 20% piperidine. Piperidine also removes the formyl group of tryptophan (Boc-Trp(For)-OH; PADRE sequence). According to our experience, this does not trigger the production of side products. Alternatively, if the synthesis is done in a glass peptide synthesis vessel (for synthesis without microwave (heat) assistance), stirring can be done by placing the piperidine/resin mixture on a rotary mixer for 5 min (first deprotection), then 10 min (second deprotection). Make sure the vessel is tightly closed on both ends.
17. 500 mg is the maximum amount of resin for efficient HF cleavage. A 15 ml Falcon tube allows for easy transfer of the resin into a HF reaction vessel.
18. HF is highly toxic and corrosive. Follow the protocol attached to the HF apparatus precisely. If there is cysteine or methionine in the peptide sequence, add additional p-thiocresol scavenger (0.25 ml; 0.5 ml/g of resin) to the p-cresol/resin mixture. Make sure the HF is completely evaporated before proceeding to workup.
19. Make sure the peptide is fully soluble before filtration. Add a few drops of Solvent B and use a sonicator or vortex to aid solubility. Always filter the solution before running it through ESI-MS and RP-HPLC machines. **Peptide 2** is hydrophobic and needs to be run through a C4 column. More hydrophilic peptides may be purified using a C8 column for better separation. The crude compound can degrade easily and is not stable; therefore, it must be purified for long-term storage.
20. Analytical RP-HPLC graphs show pure compounds in a single peak. The mass from this peak must match the mass of the desired peptide in ESI-MS. **Peptide 2** is hydrophobic and needs to be run through a C4 column. Run more hydrophilic peptides through C8 columns (as described above).
21. Analyze the combined fraction through analytical RP-HPLC and ESI-MS to confirm purity. Keep the pure compound in powder form at -20 °C to ease storage and prolong shelf-life.
22. The incorporation of a polyleucine tail increases the hydrophobicity of the compound and makes it difficult to dissolve. Using both sonication and vortex can help with the process. The end product should be a white, semi-cloudy or clear solution, which contains the self-assembled vaccine candidate. Vortex the solution before use.
23. The appropriate dilution is necessary for visualization; sample that contains too high concentration of nanoparticles will result in overlapping particles, which affects the visibility of particle morphology. Dilution ratio variability depends on the particular sample, but a concentration of 0.5 mg/ml is a good place to start.
24. Dilution is required to avoid measurement with high tension (HT) > 600 V.
25. A significant deviation from zero (> or < 1 mdeg) indicates that the CD cell is contaminated.
26. Clean the cell by filling the cell with 5M nitric acid (nitric acid doesn't damage the quartz) and leave it for a few hours or overnight. A sonicator can be used to speed up the process. Rinse

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the cell with water before a final wash with methanol. Remove the contents and dry the cell completely before use or storage. Clean the outside of the cell with Kimwipes, only.

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Competing interests

M.S., G.Z., and I.T. are co-inventors in a patent application entitled “Self assembling, self adjuvanting system for delivery of vaccines” filed by The University of Queensland (application number AU 2019900328). The remaining authors declare that they have no competing interests.

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