

Synthetic Virus-Like Particles from Self-Assembling Coiled-Coil Lipopeptides and Their Use in Antigen Display to the Immune System

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Virus-like particles (VLPs) have attracted great interest in pharmaceutical and biotechnological research.^[1] VLPs are typically composed of recombinant proteins, for example, the capsid of the hepatitis B virus,^[2] sometimes in combination with nucleic acids, such as bacteriophage,^[3] or they may contain a viral envelope in addition to viral coat proteins, such as virosomes.^[4] The natural self-assembling core structures of many natural VLPs can be exploited to display multiple copies of an antigen across the surface of the particle, for example, by inserting oligonucleotides encoding peptide loops into the appropriate structural genes, or by chemically conjugating haptens to recombinant VLPs. The importance of displaying repeated antigenic structures across a viral or bacterial surface to generate robust immune responses is now well established.^[5,6] VLPs may also contain natural T-helper-cell epitopes and pathogen-associated molecular patterns that are recognized by the innate immune system, which further enhance immune responses. All the previously described VLPs, however, require the use of cell-culture or recombinant-DNA methods for production and/or engineering. We have explored the possibility of producing VLPs using artificial synthetically derived building blocks, which can self-assemble into particles having a size and composition similar to that of natural viruses, in this case containing protein and lipid, but not nucleic acids. Such synthetic virus-like particles (SVLPs) might then retain the advantages of naturally derived VLPs as antigen delivery vehicles, but in addition could be optimized and produced using a myriad of synthetic chemical methods.

The SVLPs described herein harness the principles of protein–protein as well as lipid–lipid recognition to drive self-assembly. The basic building block comprises a lipopeptide containing a coiled-coil sequence.^[7] The coiled-coil motif drives self-assembly of the peptide chains into parallel helical bundles. In addition, a lipid component, typically a phospholipid having two long fatty acid chains, is coupled at the N terminus of each peptide chain. After self-association of the coiled coil into a parallel helical bundle, the resulting cluster of lipid chains drives self assembly of multiple coiled-coil

bundles into stable nanosize SVLPs (Figure 1). The lipopeptide building block may also harbor T-helper-cell epitopes and conceivably also Toll-like receptor (TLR) ligands. Finally, synthetic antigens can be coupled to the C terminus of the lipopeptide building block. As a result, after self-assembly multiple copies of the attached antigen are displayed over the surface of the SVLP, for recognition by B cells.

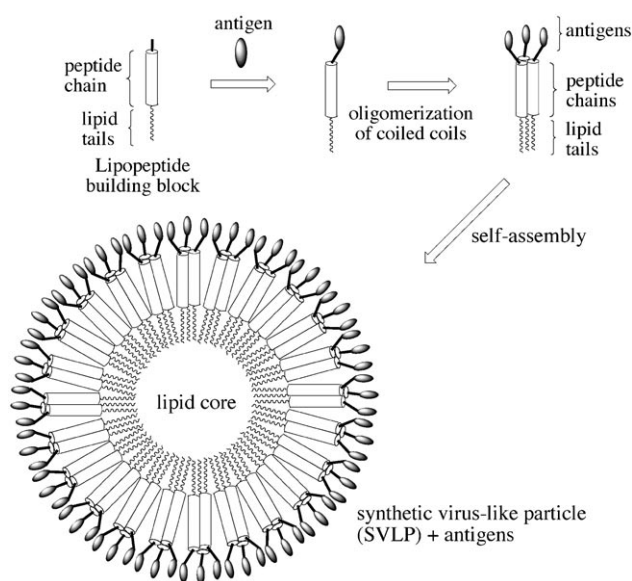


Figure 1. Representation of the components and assembly of a synthetic virus-like particle (SVLP).

Peptides modified with one or more alkyl chains have been shown to form a variety of supramolecular architectures, including parallel^[8] and antiparallel^[9] β sheets, β hairpins,^[10,11] ribbons,^[12–14] and fibres.^[15,16] In addition, the self-assembly of a collagen-derived lipopeptide into spherical micelles has been described.^[17] From the immunological standpoint, the use of lipopeptides in immunizations is also well documented, although the mechanisms by which lipopeptides induce B- and T-cell responses *in vivo* are not yet fully understood.^[18–21] Synthetic lipopeptides have also been used as antigen carriers, for example, in the study of synthetic carbohydrate vaccines.^[22] The novel aspects of the work herein, however, include the discovery of a family of self-assembling lipopeptides that form discrete, nanometer-scale, virus-sized particles, and their use for the multivalent display of synthetic antigens, features, which are important for the efficient adjuvant-free induction of antigen-specific antibody responses *in vivo*.

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From the many known naturally occurring and artificially designed coiled coils, we began with a peptide sequence derived from heptad repeat region 1 (HR1) from the F₁ glycoprotein of respiratory syncytial virus (RSV), which has been shown to form a parallel three-helix bundle.^[23] This three-helix bundle can also associate with the HR2 region of the F₁ glycoprotein to form a six-helix bundle of known 3D structure.^[24] A peptide from the HR1 region (residues Ala153–Gln202) with two additional Gly residues at the N terminus (see Figure 2a) was assembled by solid-phase peptide chemistry (see Supporting Information). A phospholipid comprising 1,3-(dipalmitoyl)glycero-2-phosphoethanolamine (Bachem) was coupled by a succinate linker to the free N terminus of the peptide on the resin. The final product of the synthesis (FB-6) was released from the resin by treatment with trifluoroacetic acid, and was purified and characterized.

Initial hydrodynamic studies using analytical ultracentrifugation (Figure 3a) indicated that FB-6 self-associates in aqueous buffered solution. The average apparent mass obtained by sedimentation equilibrium was 431 ± 14 kDa, corresponding to an oligomer composed of approximately 72 lipopeptide monomers, or 24 three-helix lipopeptide bundles. The apparent mass was independent of lipopeptide concentration in the range studied (10–240 μM), although

there was evidence of slight aggregation at the highest lipopeptide concentration. Sedimentation velocity centrifugation yielded a sedimentation coefficient distribution in the range 9.5–10 S, independent of the analytical method used, although there was slight tailing to the low-value side of the distribution at low lipopeptide concentration (Supporting Information, Figure S1). No significant statistical improvement was found when models involving discrete non-interacting species were tested and inspection of the individual S values showed no apparent concentration dependence. Dynamic light scattering measurements (Supporting Information, Figure S2) and a number-weighted distribution analysis based on a spherical-particle model yielded a mean diameter of about 17–20 nm (see Supporting Information).

The far-UV circular dichroism (CD) spectrum of the 52-residue peptide moiety (**1**) of FB-6, lacking the phospholipid and linker, is characteristic of a random-coil/ α -helix equilibrium, which is both temperature and concentration dependent (Figure 3b). The spectrum indicates a high α -helical content at 1 °C and the thermal denaturation transition exhibited a mid-point of about 40 °C at a peptide concentration of 35 μM . A closely related peptide forms a trimeric coiled coil in equilibrium with the monomer,^[23] with an estimated association constant of circa $5 \times 10^8 \text{ M}^{-2}$. The lipopeptide FB-6 has a

markedly more intense CD spectrum, and the estimated helix content rises to about 100% (Figure 3b). Direct contributions of the phospholipid and linker to the CD spectrum are likely to be negligible. The introduction of a lipid tail should have little influence on the oligomerization state (trimer) of the helical bundle, given that the lipid tails are likely to be sequestered in the interior away from polar interactions with both the peptide and solvent. This situation suggests that the apparent increase in helical content, detected by CD, is due to the association of the lipid chains. These data support a structural model in which it is the subunits composed of three-helix lipopeptide bundles that associate to form the much larger particles.

A computer model, based on these experimental considerations, is shown in Figure 3c in which the lipid chains form a micelle-like core from which the coiled coils extend outwards into solution. Electron microscopy (EM) supports this model (Figure 3d). Thus star-like particles having a remarkably uniform appearance and aggregation state are apparent by transmission EM. It is probable that the particles are spherical (although they could also be disk-shaped) with an average diameter of 20 ± 5 nm. Pure phosphatidylethanolamine forms lamellar or hexagonal phases rather than vesicles in neutral aqueous solution.^[25] Packing considerations within the particles suggest that the lipid tails adopt a micelle-like organization, possibly owing to restrictions imposed by the presence of the peptide headgroups. An estimate of the overall stability of the particle can be made by measurement of the apparent critical micelle concentration (CMC; Supporting Information, Figure S2). How-

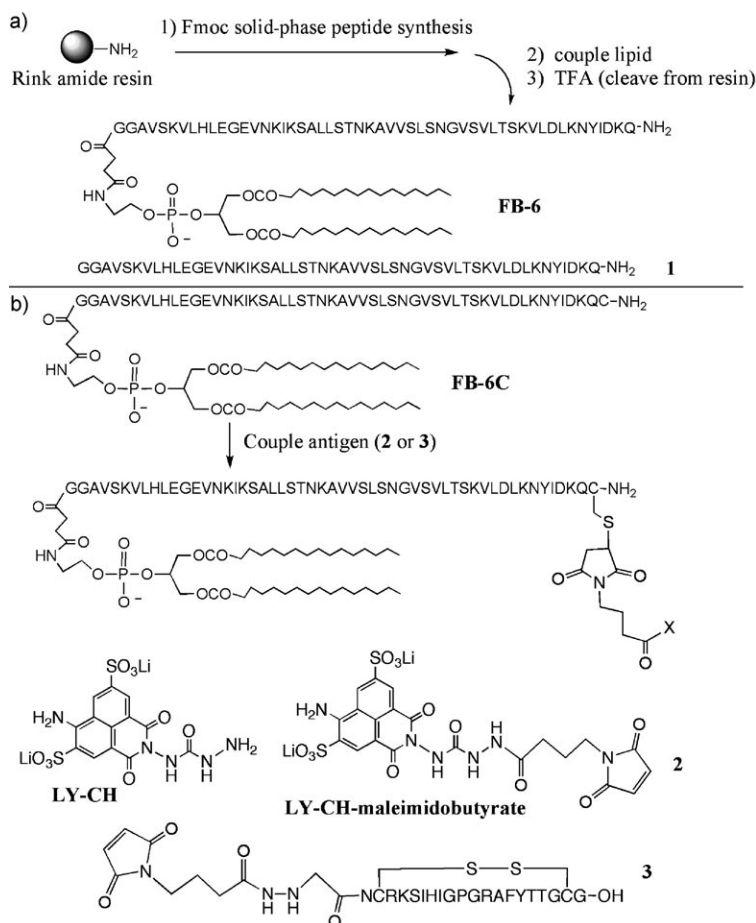


Figure 2. a) Synthesis of the lipopeptide building block FB-6. See text for details. TFA = trifluoroacetic acid, Fmoc = [(9H-fluorenyl)methoxy]carbonyl. b) Coupling of antigens **2** or **3** at the additional Cys residue of FB-6C. X denotes non-maleimidobutyrate portion of **2** or **3**.

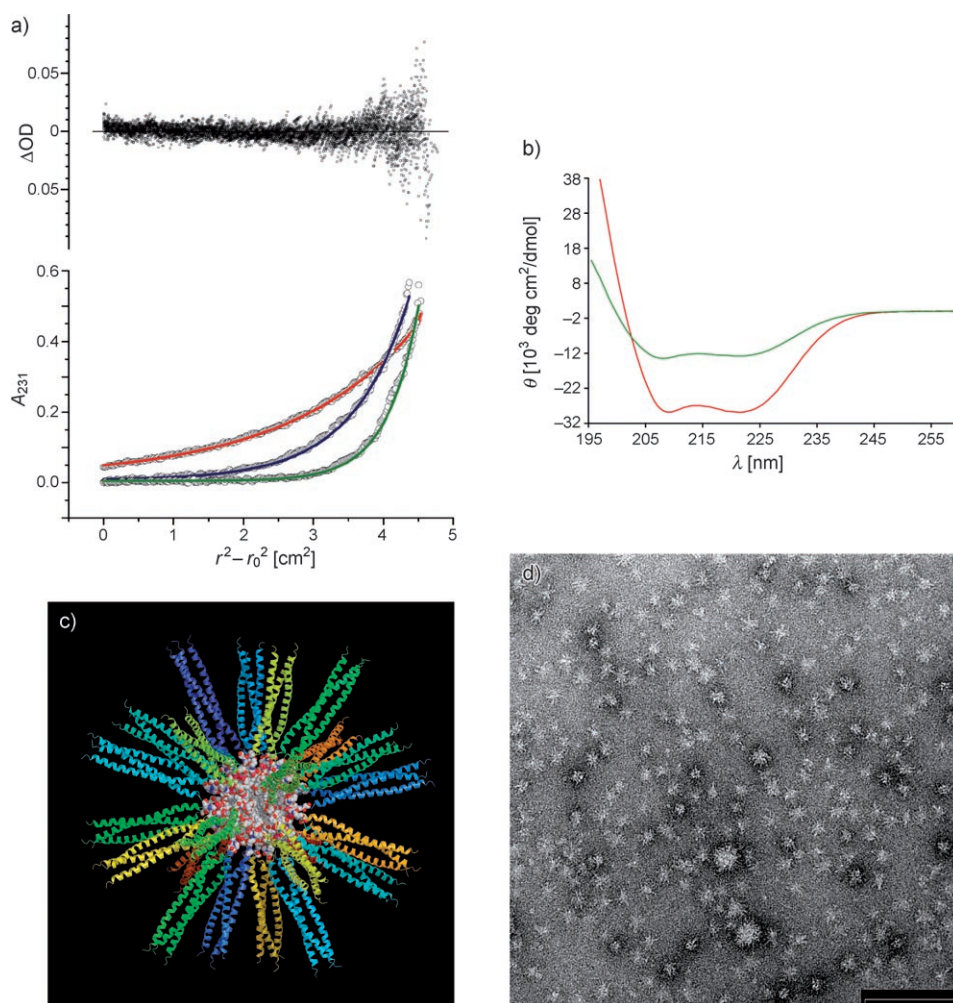


Figure 3. a) Representative sedimentation equilibrium profile for lipopeptide FB-6 aggregates. Lower panel: A_{231} denotes absorbance at 231 nm. Curves represent 5000 (red), 7500 (blue) and 10000 rpm (green). Lipopeptide concentration: 24 μ M in 0.01-M sodium phosphate, pH 7.4, containing 0.09-M sodium chloride (PBS). Upper panel: Optical density difference (Δ OD). Residuals to the global fit for 18 data sets at 24 and 48 μ M and 3 rotor speeds. b) CD spectra of lipopeptide FB-6 (red, 20 μ M) and peptide 1 aggregates (green, 50 μ M) in PBS at pH 7.4. θ is the mean residue ellipticity. c) Computer model of the SVLP formed from FB-6 and composed of 24 subunits (helical bundles). Each subunit is formed by association of three copies of the lipopeptide FB-6 to give a trimeric coiled coil. d) Negative-staining electron micrographs of SVLPs formed by the lipopeptide FB-6 in tris(hydroxymethyl)amino-methane (Tris) buffer, pH 7.4. Scale bar 100 nm.

ever, the sensitivity of methods available for this determination are such that it was only possible to obtain an upper limit of 20 nm for the CMC, indicating that the particle is stable well into the low nanomolar range.

To couple a synthetic antigen to SVLPs, the new lipopeptide FB-6C was produced, which contains an additional C terminal Cys residue (Figure 2b). Two synthetic antigens were then chosen: The dye Lucifer Yellow CH (LY-CH; Fluka; **2**) as a model organic hapten, and the disulfide-bridged peptide **3**, with a sequence derived from the V3 region of gp120 from HIV-1. Each of these antigens were linked to a maleimidobutyl linker. The C terminal Cys of FB-6C was coupled through the maleimide ring to **2** or **3** (see Figure 2b and Supporting Information).

The immunological properties of the conjugates were then tested by immunizing New Zealand white rabbits with each

conjugate alone, or in combination with Freund's adjuvant. The immune sera from the rabbits were analyzed by ELISA (enzyme-linked immunosorbent assay). The results (Table 1) showed that all rabbits receiving the conjugates had generated high titers of antigen-specific antibody after the second booster immunization. A response was already detectable after the initial immunization, but the steepest rise in titer was seen after the first boost, and a smaller increase was observed after the second boost. It was also apparent that antibody titers attained without the use of adjuvant were as high, or higher, than those achieved by co-injection with Freund's complete/incomplete adjuvant. The specificity of each immune response was analyzed by measuring the ability of antibodies raised against each immunogen to cross-react with the same antigens attached to a different lipopeptide carrier (see Supporting Information). The results in Table 2 show that a significant part of the immune response is directed towards the antigen (i.e. LY-CH or the V3 loop mimetic).

As these ELISAs detect antigen-specific IgG antibodies, the peptide sequence in FB-6C should include a CD4⁺ T-helper epitope. However, no systematic search has been per-

formed for rabbit T-helper epitopes in the human F1 glycoprotein. To assess the quality of the immune responses obtained, and whether antibody affinity maturation has occurred, mean avidity indices were also assessed (the avidity index (AI) is the concentration of NH_4SCN required to dissociate 50% of bound antibodies in ELISA). The results (see Supporting Information, Figure S5) revealed rather high avidities for the antigen-specific antibodies, and showed a significant avidity improvement when comparing the primary (AI = 0.5), secondary (AI = 1.5–2.5), and tertiary (AI = 2.0–3.0) sera, consistent with the induction of antibody affinity maturation. These results highlight the potential advantages of using this, and related, naturally occurring virally derived coiled-coil sequences in the design of the SVLP lipopeptide building blocks.

Table 1: Immunogenicity of conjugates FB-6C linked to **2** or **3**, each with two rabbits (a and b).^[a]

Conjugate used ^[c]	Rabbit	log ₁₀ (Endpoint titers) ^[b]		
		1°	2°	3°
FB-6C-3 ^[c]	1a	3.51	4.41	5.01
FB-6C-3 ^[c]	1b	<1.70	4.51	5.41
FB-6C-3	2a	3.51	5.01	5.01
FB-6C-3	2b	2.00	3.81	4.71
FB-6C-2 ^[c]	3a	4.11	5.11	5.71
FB-6C-2 ^[c]	3b	3.51	4.41	5.31
FB-6C-2	4a	3.90	5.11	5.71
FB-6C-2	4b	4.20	5.11	5.40

[a] Preimmune serum samples showed no significant reactivity with the corresponding immunogen. [b] Endpoint titers of the primary (1°), first booster (2°) and second booster (3°) immunizations were defined as the last reciprocal dilution for which the UV absorbance was higher than twice the value corresponding to the preimmune serum. A log₁₀(end point) titer of 5.00 corresponds to a titer of 100000. [c] Freund's complete adjuvant (FA, primary) and Freund's incomplete adjuvant (FIA, boosts) coadministered.

Table 2: Specificity of the hapten-specific immune responses following one (1°), two (2°), and three (3°) immunizations with each conjugate, each with two rabbits (a and b).^[a]

Immunogen	Rabbit	Antigen	Endpoint titers (log ₁₀) ^[b]		
			1°	2°	3°
FB-6C-3 ^[c]	1a	gp41-3	3.20	4.71	4.71
FB-6C-3 ^[c]	1b	gp41-3	<1.70	5.11	5.41
FB-6C-3	2a	gp41-3	3.60	5.11	5.41
FB-6C-3	2b	gp41-3	<1.70	3.81	4.11
FB-6C-2 ^[c]	3a	gp41-2	3.20	4.71	5.01
FB-6C-2 ^[c]	3b	gp41-2	2.90	3.81	4.41
FB-6C-2	4a	gp41-2	4.51	5.11	5.41
FB-6C-2	4b	gp41-2	4.20	5.71	5.71

[a] The antibodies generated against each immunogen were tested for cross reaction to the same antigens (**2** and **3**) linked to a different lipopeptide carrier (gp41) by ELISA. [b] Endpoint titers of the primary (1°), first booster (2°) and second booster (3°) immunizations were defined as the last reciprocal dilution for which absorbance value was higher than twice the value corresponding to the preimmune serum dilution. [c] with Freund's complete adjuvant (FA, primes) and Freund's incomplete adjuvant (FIA, boosts).

These results show that nanoparticles the size of some small virus capsids (ca. 20 nm) can be produced in a remarkably homogeneous form, starting from a designed self-assembling coiled-coil lipopeptide building block. We have also shown that multiple copies of an antigen can be displayed on these particles, and that they can be used for the adjuvant-free stimulation of antigen-specific humoral immune responses. The lipopeptide building blocks can, in principle, now be varied and optimized in many ways. For example, a wide range of naturally occurring and engineered sequences are known, ranging from those forming dimeric through to heptameric coiled coils.^[7,26,27] Conceivably, these peptides may be further engineered to include "universal", or artificial T-helper-cell epitopes, as well as Toll-like receptor ligands, such as lipid headgroups based on Pam3Cys and related lipids.^[28] The way is now open to explore systemati-

cally the scope and limitations of using these SVLPs in synthetic vaccine design.

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