



Self-assembling small-molecule adjuvants as antigen nano-carriers†

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The effective co-delivery of antigens and immune potentiators (adjuvants) and the high degree of antigen presentation have been two major challenges in the development of subunit vaccines. Here, we address these issues by conjugating peptide antigens with cholicamide, a self-assembling small molecule adjuvant. Co-assemblies of the conjugates and cholicamide achieved high levels of both cytokine induction and MHC class II peptide presentation.

Traditional vaccines are derived from live-attenuated or inactivated pathogens, thereby potentially bringing along with them unexpected risks.¹ Subunit vaccines consisting of isolated protein antigens are a safer and cost-effective option. However, they often lack sufficient immunogenicity and require the inclusion of immune potentiators (termed adjuvants), which trigger early innate immune responses to ensure the generation of robust and long-lasting adaptive immune responses.²

The effective co-delivery of adjuvants and antigens to antigen-presenting cells (APCs) has been a major challenge in the development of subunit vaccines.^{3,4} To address this issue, conjugate vaccines, in which adjuvant and antigen are covalently attached, have been explored.⁵ Such self-adjuvanting vaccines benefit the cytokine induction to boost early innate immune responses of APCs and enhance the uptake of antigen through receptor-mediated endocytosis.⁶ The adjuvant components often used in conjugate vaccines are agonists of toll-like receptors (TLRs). For instance, HIV gag protein has been conjugated with

a TLR7/8 agonist, a thiazoloquinolone derivative CL-075,⁷ and tumor-associated carbohydrate antigen MUC1 has been combined with TLR1/2 agonists, Pam₃Cys and Pam₃CSK₄.⁸

Another important component of subunit vaccines is an antigen carrier that facilitates the delivery of antigens to immune cells. The immune system has developed the ability to recognize and respond to nano- and micron-sized particles such as viruses and bacteria.⁹ Nano- and micron-sized antigen carriers, including virus-like particles,¹⁰ viral protein cages,¹¹ and *de novo* self-assembling peptides,¹² hold great potential for delivering and presenting antigens in APCs.¹³

The TLR-agonist adjuvants that simultaneously serve as nano-carriers of antigens might be ideal constituents of minimalist conjugate vaccines. We previously discovered a small molecule vaccine adjuvant, cholicamide (molecule 1), which self-assembles to form a nanoparticle approximately 150 nm in size and stimulates APCs through the activation of TLR7, an endosomal Toll-like receptor.¹⁴ Activation of TLR7 has been demonstrated to induce highly potent humoral and cell-mediated immune responses. Taking advantage of the self-assembling property of cholicamide (1), we postulated that cholicamide (1), when covalently conjugated with peptides, serves simultaneously as a nano-carrier and an adjuvant for peptide antigens (Fig. 1A).

Structure-activity relationship study of cholicamide (1) has shown that the deoxycholate moiety is essential for both immunopotential and self-assembly, suggesting the linker as a potential conjugation site for peptide antigens. The original linker was replaced by lysine for the introduction of a branched carboxylic acid functional group (Fig. S1A, ESI†). The resulting molecule (molecule 2) was further functionalized by the introduction of an alkyne group (molecules 3 and 4), allowing subsequent copper-catalyzed azide-alkyne cycloaddition (CuAAC) with azide-peptides.

To examine the feasibility of our concept, we initially selected molecule 3 for conjugation with an azide-Flag peptide (N₃-GGSDYKDDDDK) through CuAAC (Fig. 2A). The resulting conjugate, 3-Flag, readily formed nanoparticles in water and

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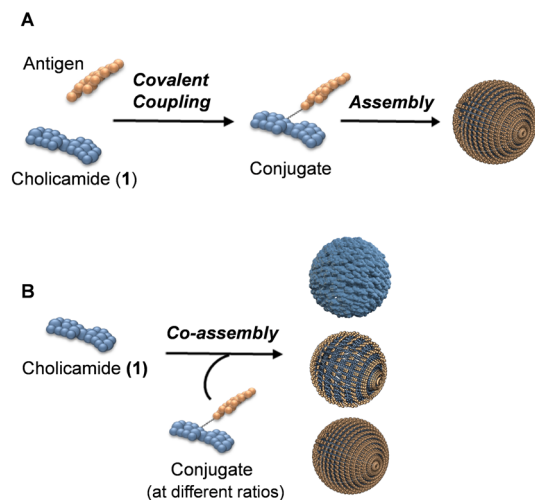


Fig. 1 Schematic representation of nano-carrier adjuvants for peptide antigens. (A) Nano-assemblies of a cholicamide-peptide conjugate. (B) Formation of co-assemblies of cholicamide and its peptide conjugate at different mixing ratios.

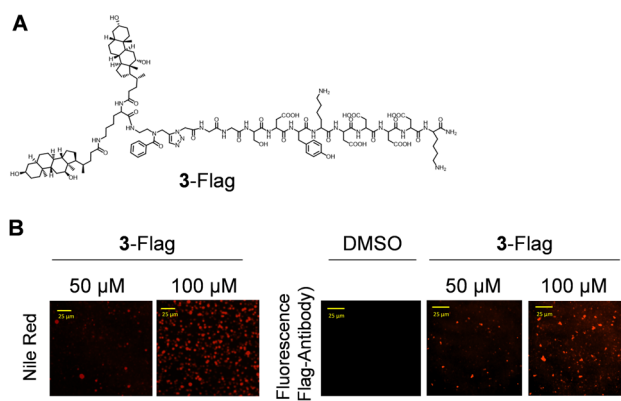


Fig. 2 Characterization of **3-Flag**. (A) Chemical structure of **3-Flag**. (B) Self-assemblies of **3-Flag** in H₂O (left) stained with Nile Red (15 μM) and PBS (right) recognized by Flag-antibody under a confocal microscope. Scale bar: 25 μm.

PBS, as observed under a confocal microscope when stained with Nile Red, an environment-sensitive fluorescent probe that was previously used for detecting cholicamide assemblies¹⁴ (Fig. 2B). To assess the surface exposure of the hydrophilic Flag peptide, we examined whether the particles of **3-Flag** can be recognized by a fluorescent Flag antibody, whose large size often limits its access to the inside of the nanoparticles. Confocal imaging revealed fluorescent dots whose sizes and numbers are consistent with those of Nile Red-stained particles of **3-Flag** (Fig. 2B). The antibody's recognition of the particles suggests that **3-Flag** forms nanoparticles in which the Flag peptide segments are, at least in part, accessible to a large macromolecule.

The surface fabrication of the adjuvant nanoparticles with antigens can be a double-edged sword. While the surface

fabrication endorses the ability to present antigens to APCs, it would also mask the adjuvant nanoparticles from TLR7, limiting its immunopotential activity. One potential solution is the preparation of co-assemblies by mixing cholicamide (**1**) and its peptide conjugate in an appropriate ratio (Fig. 1B). To analyze such co-assembly formation, we prepared a cholicamide-rhodamine conjugate (**4-PEG-rhodamine**) (Fig. 3A). This fluorescent conjugate permits the detection of the co-assembly under a confocal microscope. While cholicamide (**1**) alone displayed non-fluorescent particles, the mixture samples at a fixed total concentration (50 μM) but varied molar ratios (10–30% of **4-PEG-rhodamine**) showed fluorescent nanoparticles (Fig. 3B). Comparison with the bright-field images indicates that all the observed nanoparticles exhibit fluorescence, suggesting co-assembly formation.

As a model peptide antigen, we initially used OVA_{257–264} (SIINFEKL), an ovalbumin epitope peptide presented by MHC I molecule H-2K^b.¹⁵ Molecule **4** was coupled with OVA_{257–264} through two different linkers to yield **4-OVA**_{257–264} and **4-PEG-OVA**_{257–264} (Fig. S2A, ESI[†]). Dynamic light scattering (DLS) measurement confirmed the formation of ~130 nm particles at 30 μM irrespective of mixing ratios with cholicamide (**1**) (Fig. S2B, ESI[†]), suggesting the ability of these peptide conjugates to form co-assemblies with cholicamide (**1**). Each mixture was also added to the culture of RAW264.7 macrophage cells for examining its ability to elicit IL-6 and TNF-α production. As anticipated, peptide conjugates alone exerted little, if any, cytokine induction perhaps due to the masked adjuvant surface. In contrast, the cytokine-inducing ability of cholicamide (**1**) was maintained at mixing ratios up to 30% (Fig. S2C, ESI[†]). Encouraged by the result, we evaluated the antigen-presenting ability of the 20% and 30% co-assemblies in bone marrow-derived dendritic cells (BMDCs) using an antibody against H-2K^b-bound SIINFEKL (Fig. S2D, ESI[†]). To our

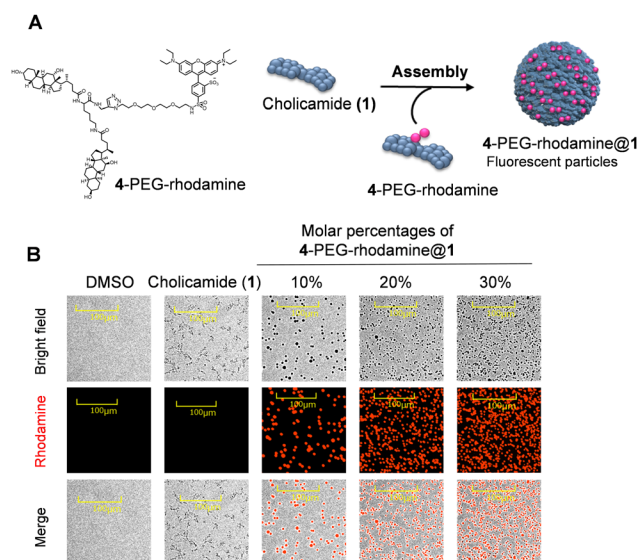


Fig. 3 Co-assembly approach confirmed by **4-PEG-rhodamine**. (A) Schematic illustration of **4-PEG-rhodamine** co-assemblies with cholicamide (**1**). (B) Co-assemblies of **4-PEG-rhodamine** with cholicamide (**1**) at various mixing ratios under a confocal microscope. Scale bar: 100 μm.

disappointment, flow cytometric analysis of the cells detected no significant enhancement of antigen presentation on the cell surface by the co-assemblies. In fact, their antigen presentation activities were even lower than that of OVA_{257–264} *per se*.

The antigen presentation in APCs is governed by two classes of major histocompatibility complex (MHC): classes I and II. Although both classes share the task of presenting antigen peptides on the cell surface for recognition by T cells, they capture the peptides from distinct sources. While MHC class I molecules bind usually to cytosolic peptides, MHC class II molecules bind selectively to peptide antigens generated by proteolysis of proteins in endosomes and lysosomes for subsequent presentation on the cell surface.¹⁶ OVA_{257–264} is an antigenic peptide that is known to be recognized by MHC class I molecules, but not by class II. We reasoned that OVA_{257–264} may not be suited for the cholicamide-based delivery for antigen presentation, since cholicamide particles have been proposed to enter cells through endocytosis.¹⁴ To confirm the post-uptake subcellular localization of cholicamide assemblies, we monitored confocal images of 20% 4-PEG-rhodamine@1 in HEK293 cells expressing GFP fusions of Rab5a and Rab7a, early and late endosome markers, respectively. As anticipated, confocal imaging analysis indicated co-localization of the nanoparticles of 20% 4-PEG-rhodamine@1 with either early or late endosomes (Fig. S1C, ESI†). These considerations led us to examine an MHC class II peptide for cholicamide conjugation.

We selected a peptide segment encompassing amino acids 323–339 of ovalbumin (OVAp: ISQAVHAAHAEINEAGR), a model antigen peptide presented by MHC II molecule I-A^d.^{17–19} This peptide was conjugated with molecules 2 or 4 through CuAAC or amide coupling. The resulting four conjugates were designated as 2-OVAp, 2-PEG-OVAp, 4-OVAp and 4-PEG-OVAp, respectively (Fig. S3, ESI†). Their ability to elicit TNF- α production was first examined at varied mixing ratios in RAW264.7 macrophage cells (Fig. S4, ESI†). Among the four conjugates, 2-PEG-OVAp, 4-OVAp and 4-PEG-OVAp tend to lose the TNF- α -inducing activity at the mixing ratios above 50%. Another conjugate, 2-OVAp, maintained its activity at the mixing ratios up to 70% both for TNF- α and IL-6 (Fig. S4, ESI†). When compared at the mixing ratio of 50%, 2-OVAp displayed the highest ability to induce TNF- α production (Fig. 4B). Both DLS measurements and confocal images of 2-OVAp@1 confirmed that the co-assembly had limited effects on the cholicamide assemblies (Fig. S5, ESI†). We decided to focus on 2-OVAp for in-depth studies.

To assess the antigen-presenting ability of 2-OVAp, we prepared a biotinylated version of OVAp (OVAp-B) for a detection purpose and coupled it with molecule 2 (2-OVAp-B) (Fig. S6A, ESI†). Confocal imaging analysis confirmed that this biotinylated version of 2-OVAp formed co-assemblies with cholicamide (1) and exposed the biotinylated OVAp on the surface of the assemblies, as detected by FITC-labeled streptavidin (Fig. S6B, ESI†). To assess its antigen-presenting ability, BMDCs were treated with 2-OVAp-B@1 at varied mixing ratios, and the amounts of the cell surface-exposed biotinylated peptide were quantified by flow cytometry using FITC-labeled streptavidin.

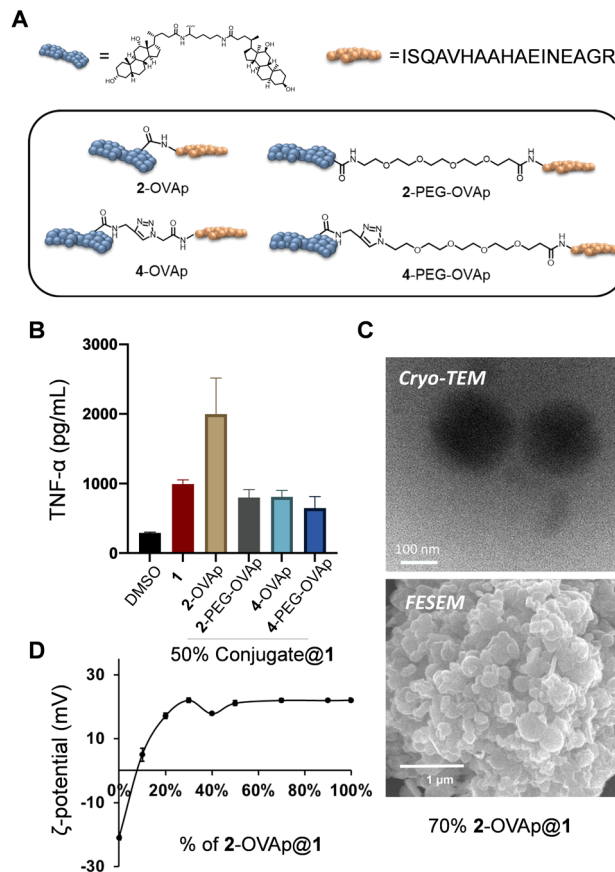


Fig. 4 Evaluation of conjugates and characterization of 2-OVAp@1. (A) Structure illustration of the conjugates. (B) TNF- α production in RAW264.7 cells stimulated with conjugates@1 (30 μ M). (C) Cryo-TEM (50 μ M) and FESEM (powder) observation of 70% 2-OVAp@1 particles. (D) Surface ζ -potential of 2-OVAp@1 (30 μ M) in H₂O.

The cells treated with 2-OVAp-B@1 exhibited higher mean fluorescence intensities than those with DMSO control, OVAp-B alone, or OVAp-B + 1, indicating the ability of 2-OVAp-B@1 to present the biotinylated peptide on the cell surface (Fig. 5). In particular, 2-OVAp-B@1 at the mixing ratio of 70% exhibited the highest level of peptide presentation. It remains unclear why 2-OVAp-B alone displayed antigen presentation less than the 70% sample. Given that cytokines have been reported to promote antigen presentation,²⁰ the inability of 2-OVAp to induce cytokines may be a reason for its moderate antigen presentation. In contrast, OVAp-B + 1, where OVAp-B was simply mixed with cholicamide (1) at 30%, 50% or 70% mixing ratio, had limited levels of peptide presentation on the cell (Fig. 5), indicating that the covalent conjugation is required for the peptide presentation.

Our results collectively demonstrate that the 70% co-assemblies of 2-OVAp with cholicamide (1) induce high levels of both cytokine production and antigen presentation in APCs. The FESEM and Cryo-TEM observation revealed a spherical virus-like structure of 70% 2-OVAp@1 particles with an average size of $\sim$ 150 nm (Fig. 4C). To obtain insights into the surface charges, we also measured ζ -potential values of 2-OVAp@1 at varied mixing ratios. The ζ -potential value of cholicamide (1)

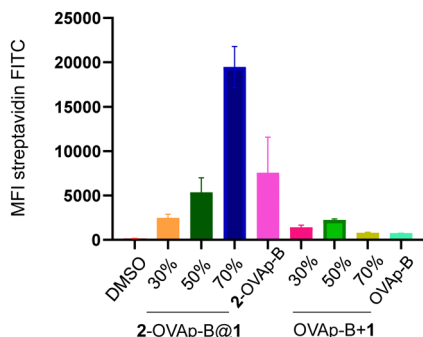


Fig. 5 Antigen presentation efficiency as measured by flow cytometry. BMDCs were treated with 2-OVAp-B@1 (30 μ M) or OVAp-B + 1 (30 μ M) at 30–70% mixing ratios and incubated for 24 h. The activities of 2-OVAp-B alone (30 μ M) and OVAp alone (30 μ M) are also shown. The presented antigens were detected with FITC-labeled streptavidin.

alone (0%) was estimated to be -21.3 mV. When the mixing ratio increases, the ζ -potential values became positive most likely due to the surface exposure of OVAp. A high value of ζ -potential, either positive or negative, can be translated as the high physical stability of nano-particles due to the electrostatic repulsion of individual particles. At the mixing ratio of 70%, the value was $+22.02$ mV, suggesting the formation of a stable co-assembly (Fig. 4D). Interestingly, the ζ -potential value did not change significantly from a mixing ratio of 50 to 100%, despite of the inability of 2-OVAp alone (100%) to induce cytokines. It remains unclear how 2-OVAp maximizes its cytokine induction activity when mixed with cholicamide (1) at 70%.

Given that the cellular uptake of the cholicamide assemblies leads to their localization in the early and late endosome, a highly acidic subcellular organelle, we next examined the stability of 70% 2-OVAp@1 in phosphate buffers of varied pHs. The average sizes of the particles decreased at pH 2 and 4 compared with those of pH 6 and 8. Additionally, the average size of 2-OVAp@1 gradually decreased under pH 2 over 24 h without significant change of derived count rates (Fig. S7, ESI[†]). Considering the fact that the pH values in late endosome and lysosome are 5.5 and 4.5, respectively, it is possible that the 70% 2-OVAp@1 particles disassemble to release the peptide conjugates for subsequent cell-surface presentation.²¹

The lack of efficacy of peptide-based subunit vaccines often results from extracellular degradation of peptides, rapid diffusion of the vaccine components, and poor antigen presentation in APCs.²² Cholicamide-based co-assemblies may permit co-delivery of antigen peptides and adjuvants to the target sites and a high-level presentation of MHC class II peptides in APCs. Unfortunately, the model MHC class II peptide used in this study has an insufficient level of antigenic activity for vaccination studies in animals. The cholicamide conjugation of highly antigenic MHC class II peptides derived from pathogens might lead to the development of minimalist adjuvant-built-in vaccines that simultaneously achieve co-delivery, cytokine induction, and antigen presentation.

S. J. performed all the experimentations. S. J., Y. T., S. Z., Y. L., and M. U. contributed to the analysis. S. J. and M. U. prepared the manuscript with edits from all co-authors.

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Conflicts of interest

There are no conflicts to declare.

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