

Comparison of Methods for Chemical Conjugation of an Influenza Peptide to Wild-Type and Cysteine-Mutant Virus-Like Particles Expressed in *Pseudomonas fluorescens*

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Abstract Chemical conjugation of the influenza peptide antigen M2E to different variants of virus-like particles (VLPs) was investigated. Wild-type cowpea chlorotic mottle virus (CCMV) and two novel cysteine mutants of CCMV, all expressed in *Pseudomonas fluorescens*, were utilized in this study. Two different conjugation schemes, primary amine-directed and cysteine-directed, were tested and compared. Both strategies were successfully used to attach M2E peptides to the surface of these VLPs. Ultimately, the cysteine-directed conjugation strategy using the CCMV cysteine mutant particles displayed key advantages over the primary amine-directed strategy.

Keywords Virus-like particle (VLP) · Cowpea chlorotic mottle virus (CCMV) · Influenza peptide conjugation · Conjugate vaccine · *Pseudomonas fluorescens* · Recombinant VLP Production

Introduction

Virus-like particles (VLPs) are being utilized in vaccine development for their ability to display antigens on the particle surface in an ordered fashion and, most importantly, increase the immunogenicity of antigens (Buonaguro et al. 2010). Antigens are typically displayed on VLPs either by genetic engineering or chemical conjugation.

With genetic engineering, a protein fusion is produced consisting of a viral surface protein and an antigenic amino acid sequence of interest. Thus, after expression, a VLP can be produced containing the antigen of interest displayed on the surface of the particle. With chemical conjugation, a similar result is achieved by covalently attaching a peptide or protein to a VLP, using a variety of chemistries (Gillitzer et al. 2002). Additionally, a unique chemical conjugation strategy can be created by using genetically engineered mutant VLPs with particular surface exposed amino acids (e.g. cysteine and lysine) for covalent attachment of peptides or proteins (Wang et al. 2002; Chatterji et al. 2004; Gillitzer et al. 2002).

In this report, we have used chemical conjugation to attach peptides to wild-type and mutant VLPs. Wild-type cowpea chlorotic mottle virus (CCMV), expressed in *Pseudomonas fluorescens* (Phelps et al. 2007), was used in one peptide-conjugation strategy. In another strategy, cysteine-mutant VLPs were used. In this second strategy, two different unique CCMV cysteine-substitution mutants were created, and ultimately expressed in *P. fluorescens*. The cysteine substitutions were made at surface exposed locations of the coat protein based on the previously published three-dimensional structure (Speir et al. 1995; Fox et al. 1997).

We carried out two different chemical conjugation schemes that utilized different cross-linking agents to covalently attach a peptide to one of the VLPs described above. The influenza antigen peptide M2E was used in all conjugations. In one scheme, we conjugated M2E peptide to the surface-exposed lysines and N-termini of wild-type CCMV coat protein (Fig. 1a). In another scheme, we conjugated M2E peptide to surface exposed cysteines of two different CCMV-cysteine mutants (Fig. 1b). Preparations from both conjugation schemes were analyzed and

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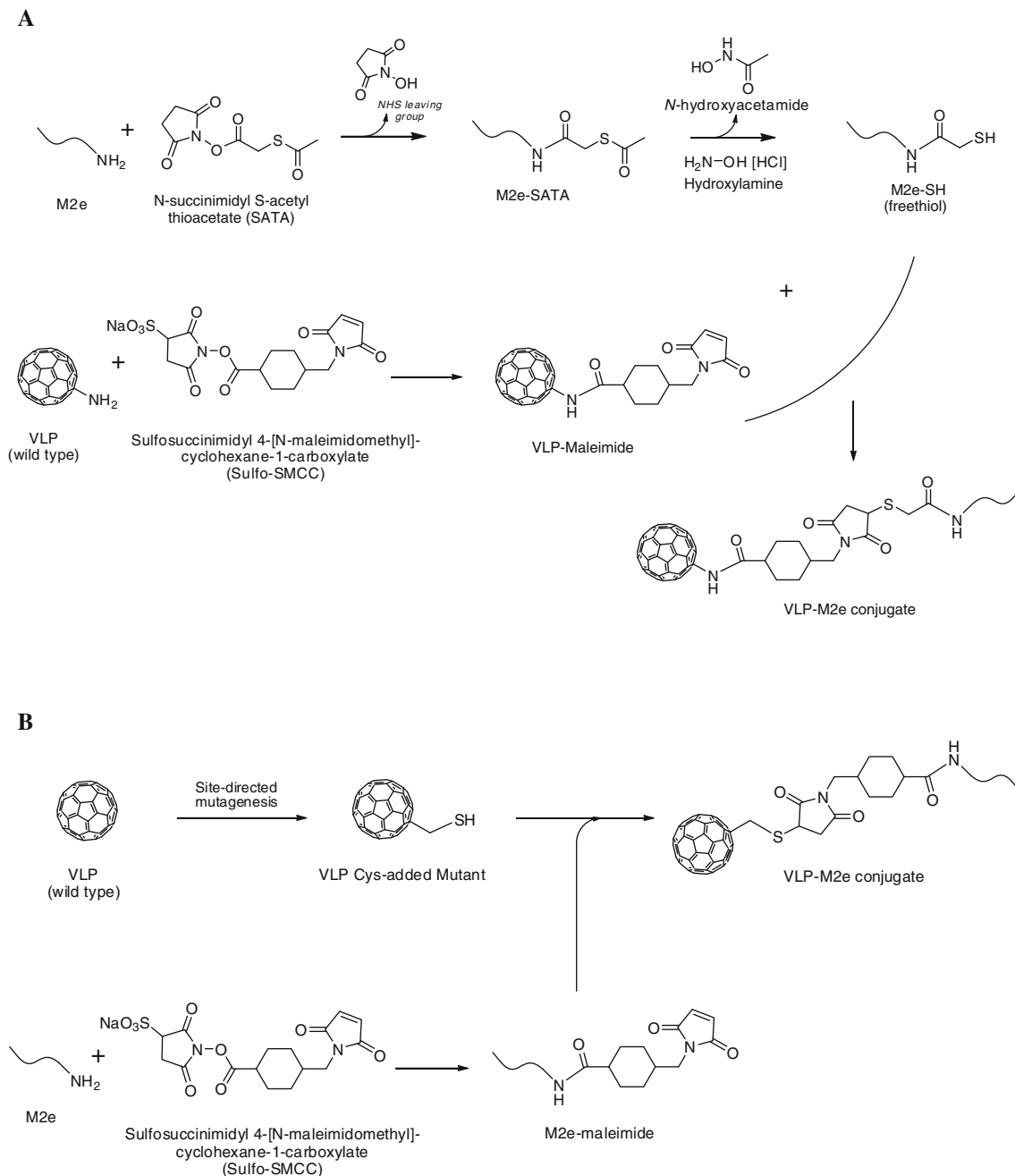


Fig. 1 Two different conjugation schemes used in this study are presented. One is a primary amine-directed conjugation strategy (**a**). In this strategy, a native VLP was modified at lysines and the N-termini of CCMV subunits, prior to direct cross-linking to a

modified M2E peptide (via a maleimide-thiol reaction). The other is a cysteine-directed conjugation strategy (**b**). In this strategy, a modified M2E peptide is directly cross-linked to surface exposed cysteines of cysteine-added VLP mutants (via a maleimide-thiol reaction)

compared by multiple analytical methods. Altogether, both conjugation schemes were successful in covalently attaching M2E peptide to the appropriate VLP. For the newly created CCMV cysteine-mutants, the experimental results demonstrate that the added cysteines are solvent-exposed and are thus good candidates for future vaccine studies involving VLP-peptide conjugations.

Materials and Methods

Molecular Biology, Growth, and Purification of CCMV and CCMVcys

CCMV coat protein was expressed in *Pseudomonas fluorescens* as previously reported (Phelps et al. 2007), using

strain DC916, consisting of host strain DC454 and plasmid pDOW2091. CCMV particles were purified by size exclusion chromatography and anion exchange chromatography, followed by buffer exchange into PBS pH 7.4.

For the CCMV-cysteine mutants (CCMVcys), two amino acids of the CCMV coat protein were individually targeted for replacement with cysteine. The two amino acids targeted were R82 and S130. Corresponding mutant plasmids were generated by SOE-PCR using plasmid pDOW2091 as the template. The two CCMVcys mutants (R82C and S130C) were expressed from plasmids pDOW2076 and pDOW2077, respectively. Both were expressed in the *P. fluorescens* host DC454. Purification of each cysteine-mutant VLP were carried out as the wild type, but in the presence of reducing agent (200 mM DTT).

Primary Amine-Directed Conjugation of M2E Peptide to CCMV

M2E-serine-substituted peptide for N-terminal conjugation (referred to as M2E in the text) with the sequence MSLLETVETPIRNEWGSRNSNDSSD (2724 Da) was used for all conjugation reactions; the synthesized peptide had greater than 95% purity (New England Peptide). M2E peptide was dissolved in PBS pH 7.4 at 2 mg/ml. To 1 mg of peptide (0.5 ml), SATA (*N*-succinimidyl *S*-acetylthioacetate, Pierce) was added at a 20 mol excess (21 μ l at 80 mg/ml in DMSO) and incubated at room temperature (RT) for 1 h with mild shaking (400 rpm); the total volume of the reaction was 0.58 ml. This reaction was then desalted to remove free SATA and buffer exchanged into PBS pH 7.4 using a 5 ml D-Salt column (Pierce, 43426) with an exclusion range up to 1.8 kDa. Approximately 0.2 ml fractions were then collected and 10–20 μ l of each fraction were combined with 0.1 ml of Bradford reagent (Protein Assay, BioRad) for protein detection. The peak fractions were pooled (~0.8 ml), resulting in ~1.2 mg/ml M2E-SATA, assuming 100% recovery of peptide.

M2E-SATA was then deacetylated in order to expose a free thiol for subsequent conjugation. For deacetylation, one volume of deacetylation buffer (0.5 M hydroxylamine and 25 mM EDTA in PBS pH 7.4) was added to ten volumes of M2E-SATA, and incubated for 2 h. at RT with mild shaking (400 rpm, in an Eppendorf Thermomixer, 022670107).

CCMV at 5 mg/ml in PBS pH 7.4 was derivatized with SSMCC (sulfo-SMCC, sulfosuccinimidyl 4-*N*-maleimidomethyl]cyclohexane-1-carboxylate, Pierce) at 32 mg/ml in DMSO. SSMCC was added at either a 10 or 25 mol excess to CCMV (moles of coat protein) and incubated for 1 h. at RT with mild shaking (400 rpm); or at a 10 or 25 mol excess for 30 min., with another 10 or 25 mol excess added for an additional 30 min. CCMV-SSMCC

was subsequently desalted in either one of two ways. If the sample was under 0.12 ml, then it was desalted three times using spin columns (0.7 ml, Pierce, 89849), with an exclusion range up to 7 kDa, equilibrated in PBS, 20 mM EDTA, pH 7.4. For samples >0.12 ml, a 10 ml column (Econo-Pac 10DG, BioRad, 732-2010), with an exclusion range up to 6 kDa, was used to desalt into the above buffer; ~0.25 ml fractions were collected, and the peak fractions were pooled.

For the conjugation reaction, 0.2 mg of desalted CCMV-SSMCC was added to 10 mol excess of deacetylated M2E-SATA (moles of coat protein-SSMCC: moles of M2E-SATA), resulting in final concentrations of ~0.9 mg/ml CCMV-SSMCC and ~0.9 mg/ml M2E-SATA. This reaction was carried out for 1 h. at RT with mild shaking (400 rpm). Unreacted free maleimides of CCMV-SSMCC were covalently blocked by the addition of a 10 mol excess of L-cysteine. Reactions were then desalted one time in a spin column as described above.

Cysteine-Directed Conjugation of M2E Peptide to CCMVcys

M2E peptide was derivatized with SSMCC as follows. The peptide was dissolved in PBS pH 7.4 at 2 mg/ml, and 1 mg (0.5 ml) of peptide was derivatized with SSMCC at a 20 mol excess (0.1 ml of SSMCC 32 mg/ml in DMSO was added) and incubated at RT for 1 h with mild shaking (400 rpm); the total volume of the reaction was 0.6 ml. This reaction was then desalted to remove free SSMCC from M2E-SSMCC using a 5 ml D-Salt column as above.

Each CCMV cysteine-mutant and wild type (as a negative control) were used in conjugation reactions at a concentration of approximately 3 mg/ml. Immediately prior to the conjugation reactions, DTT in the CCMVcys preparations was removed by desalting as described above. For the conjugation reaction, 0.3 mg of desalted CCMVcys was added to 10 mol excess of M2E-SSMCC (moles of coat protein: moles of M2E-SSMCC), resulting in final concentrations of approximately 0.9 mg/ml CCMVcys and approximately 0.9 mg/ml M2E-SSMCC. This reaction was carried out for 1 h. at room temperature (RT) with mild shaking. Reactions were then desalted one time in a spin column as described above.

SDS-Capillary Gel Electrophoresis (CGE)

Protein samples were analyzed by HTP microchip SDS capillary gel electrophoresis using a LabChip GXII instrument (Caliper Life Sciences) with a HT Protein Express v2 chip and corresponding reagents (part numbers 760499 and 760328, respectively, Caliper Life Sciences). Samples were prepared following the manufacturer's

protocol (Protein User Guide Document No. 450589, Rev. 3). Briefly, in a 96-well polypropylene conical well PCR plate, 4 μ l of sample was mixed with 14 μ l of sample buffer (with or without 68 mM DTT), heated at 95°C for 5 min and diluted by the addition of 70 μ l water. Gel-like images were produced from resulting electropherograms using Caliper Life Sciences software.

SDS-PAGE and Western Blot Analysis

Samples were diluted in Laemmli sample buffer (Bio-Rad, 161-0737) with and without XT reducing agent (Bio-Rad, 161-0792) and heated at 95°C for 5 min prior to loading on 12% Bis-Tris PAGE gels (Bio-Rad, 345-0118). Gels were run in 1 \times MES running buffer (Bio-Rad, 161-0789). For Coomassie staining, gels were stained with Gel Code Blue (Thermo Scientific, 24592). For Western analyses, gels were transferred onto a 0.2 μ m nitrocellulose membrane (Bio-Rad, 162-0232) using 1 \times NuPAGE transfer buffer (Invitrogen, NP0006-1) with 20% methanol. Membranes were blocked and subsequently probed with anti-M2E antibody (LifeSpan Biosciences, LS-C34745) or anti-CCMV antibody (DSMZ, AS-0011). Peroxidase-conjugated anti-rabbit IgG secondary antibody (Sigma) in conjunction with Immunopure Metal Enhanced DAB substrate (Pierce, 34065) was used for detection. Imaging was performed with an Alpha Innotech FluorImager.

Analytical-Scale Size-Exclusion Chromatography (SEC)

Samples were analyzed on an Agilent 1100 HPLC in-line with a light-scattering detector (Wyatt). The following analytical-scale size-exclusion column was used: G5000 PWxl (Tosoh), 7.8 mm \times 30 cm, 10 μ m particles, attached to a guard column consisting of TSKgel Guard PWxl (Tosoh), 6 mm \times 4 cm, 12 μ m particles. The buffer was 0.1 M NaCl, 0.1 M Tris, pH 7.5 (filtered using 0.2 μ m sterile filter units). The flow rate was 0.5 ml/min, 30–50 μ l of each sample was injected, and a 32 min run time was used. Absorbance at 214, 254 and 280 nm was collected by Chemstation software (Agilent) and light-scattering data was collected by Astra software (Wyatt).

Results and Discussion

Initially, a method was tested for conjugating M2E peptide to recombinantly-produced wild type CCMV virus-like particles (Phelps et al. 2007). This method involves the conjugation of a derivatized peptide to exposed lysine residues and N-termini of a substrate protein via primary amines (Fig. 1a). In carrying out this conjugation, first the

peptide intermediate was made by derivatizing M2E peptide with SATA, a heterobifunctional crosslinker which reacts with primary amines via an NHS ester group. This occurs at the N-terminus of the M2E peptide (see Materials and Methods for peptide details). Subsequently, the derivatized peptide was deacetylated, exposing a free thiol (as described in the Materials and Methods section). The deacetylated peptide intermediate was subjected to intact mass analysis by LC-MS, and was shown to be the major species produced from these reactions (\sim 70% deacetylated, \sim 30% acetylated, and unmodified peptide was virtually undetectable, data not shown). Secondly, the CCMV intermediate was made by derivatizing CCMV with SSMCC, a heterobifunctional crosslinker which reacts with the primary amines of CCMV via an NHS ester group. For the purpose of optimization, different amounts of SSMCC were tested for derivatizing CCMV. Lastly, the deacetylated M2E-SATA was combined with the CCMV-SSMCC samples for the final conjugation reaction between the free thiol on deacetylated M2E-SATA and the maleimide of CCMV-SSMCC. Shown in Fig. 2 is the SDS-based capillary gel electrophoresis (CGE) analysis of four conjugation reactions where four different amounts of SSMCC were used for producing the CCMV-SSMCC intermediate. Multiple CCMV-M2E conjugation products were produced in these reactions, ranging from 1 to 5 copies of M2E per CCMV subunit. It is apparent that increasing the amount of SSMCC used for generating the CCMV-SSMCC intermediate resulted in a larger number of M2E peptides being conjugated to CCMV (Fig. 2).

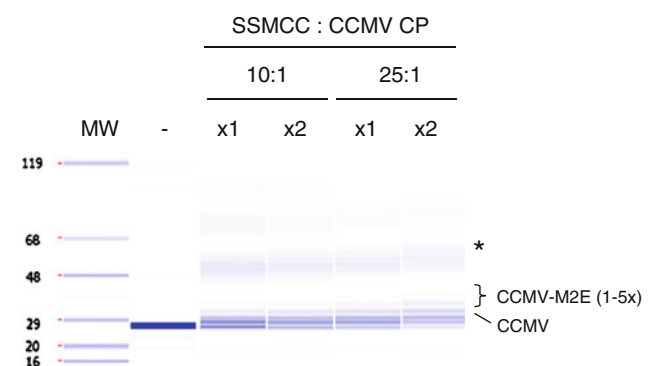


Fig. 2 SDS-CGE analysis of CCMV-M2E conjugation reactions. SSMCC was added to CCMV at different molar ratios (10:1 or 25:1, SSMCC: CCMV coat protein (CP)), and added either once (1 \times) or twice (2 \times) over a 1 h. period. Deacetylated M2E-SATA was conjugated with each of the different preparations of CCMV-SSMCC. CCMV conjugated to multiple copies (1–5) of M2E was observed as increases in apparent MW by \sim 3 kDa per M2E. It appeared that increased amounts of SSMCC resulted in a higher number (on average) of M2E peptides being conjugated to CCMV. Dimers of CCMV-M2E conjugation products were likely formed as well, denoted by *asterisk* symbol. Lane-to-lane visualization was not set to the same scale

We next wanted to confirm that the M2E peptides were in fact being conjugated to CCMV, and this was done by Western blot analysis using anti-M2E and anti-CCMV antibodies. Figure 3 shows the results of this analysis for a CCMV-M2E conjugation reaction, where a ladder of bands (approximately 3 kDa apart) was observed starting slightly above the expected position of CCMV (approximately 20 kDa). This pattern is similar to what was observed in the SDS-CGE analysis (Fig. 2), confirming that 1–5 copies of M2E were being conjugated to CCMV subunits. Additionally, multiple M2E conjugated CCMV dimers were observed, as was seen by CGE analysis. Lastly, a mixture of high MW species was also observed in the CCMV-M2E conjugation sample analyzed by Western blot (Fig. 3). This suggests that crosslinked multimers of CCMV-M2E subunits were also formed during the reaction. A similar high MW mixture was also observed in a CCMV-M2E conjugation reaction when analyzed by SDS-PAGE followed by Coomassie staining (data not shown). These covalent linkages may be between the maleimide of CCMV-SSMCC and a primary amine of an adjacent CCMV subunit; though unfavored compared to the reaction between the maleimide and free thiol of M2E-SATA, this reaction is possible, and may have occurred at a low frequency. Conversely, these covalent linkages could be due to the maleimide of CCMV-SSMCC reacting with native cysteines of a neighboring CCMV subunit.

The CCMV-M2E conjugation reactions analyzed by SDS-CGE (Fig. 2) were analyzed by analytical-scale size-exclusion chromatography (SEC) (Fig. 4). The results showed that the majority of the final product of each reaction

was still in the form of a particle. Additionally, the retention times of the CCMV-M2E conjugates shifted slightly earlier, and this shift was proportional to the amount of SSMCC in each reaction. This suggests that the increased peptide conjugation per subunit could be observed by analysis of the intact VLP using SEC. The hydrodynamic radius (Rh) of CCMV and the CCMV-M2E conjugates were determined by light scattering data collected during SEC. The hydrodynamic radius for all of the CCMV-M2E conjugates were ~24 nm compared to ~18 nm for CCMV alone, confirming the increased particle size of CCMV-M2E conjugates compared to unmodified CCMV. The amount of particle recovered (yield) for these reactions were in the range of 40–51% (determined by SEC). However, when calculated using SDS-CGE data, the yield was much lower (data not shown). This discrepancy is most likely due to the complex pattern of the high MW inter-crosslinked subunits of CCMV-M2E that were visible by CGE (Fig. 2) and Western analysis (Fig. 3). Together, these data suggest that these high MW inter-crosslinked subunits of CCMV-M2E were probably the result of intraparticle subunit crosslinking, rather than interparticle crosslinking, since inter-crosslinked particles would be expected to have a significantly earlier SEC retention time than what was observed.

To better control the number of peptides attached per subunit, we created cysteine mutants to allow for site-specific conjugation using a slightly different cross-linking strategy. Two CCMV mutants containing cysteine-substituted residues (CCMVcys) were generated. These mutants were designed to introduce a cysteine on the CCMV coat protein that could be utilized in site-directed conjugation of

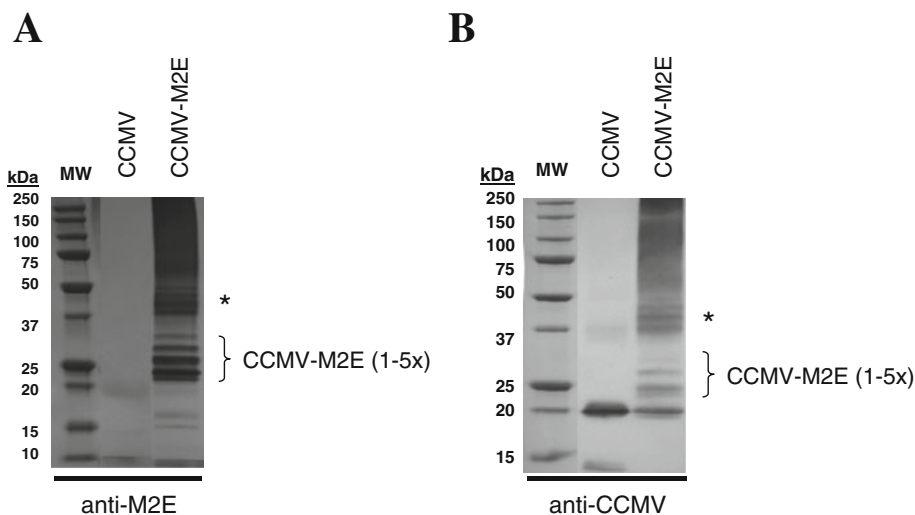
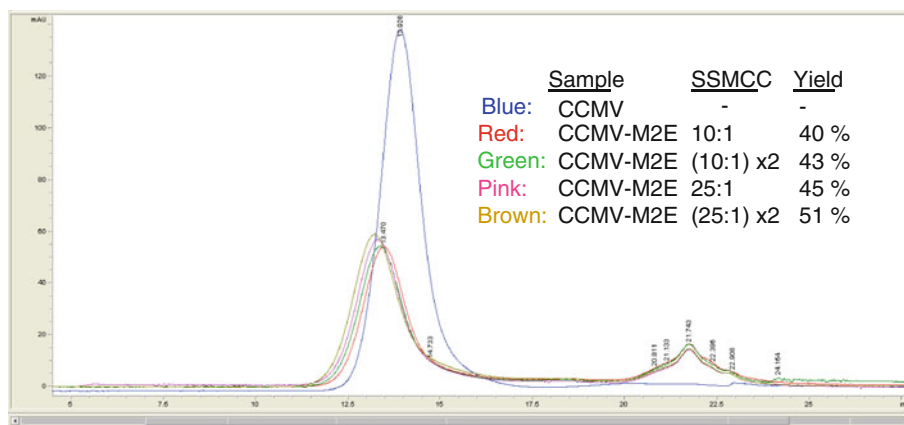


Fig. 3 Western blot analysis of M2E conjugated to CCMV coat protein using anti-M2E antibody (a) and anti-CCMV antibody (b). SSMCC was added to CCMV at a molar ratio of 10:1. Subsequently, deacetylated M2E-SATA was conjugated to CCMV-SSMCC. CCMV conjugated to multiple copies (1–5) of M2E were observed. Dimers of

CCMV-M2E conjugation products were likely formed as well, denoted by *asterisk* symbol. Additionally, it appeared that even higher molecular weight species of CCMV-M2E were produced as well, probably due to intermolecular crosslinks between multiple (greater than two) CCMV subunits (coat proteins)

Fig. 4 Analytical-scale size-exclusion chromatography of CCMV-M2E conjugation reactions. Reactions described in Fig. 2 were subjected to SEC. The amount of CCMV in the conjugation reactions loaded should equal the amount loaded of the unconjugated CCMV material, if no sample loss occurred



peptides to an intact VLP (Fig. 1b). These locations were chosen based on the known structure of the wild-type VLP, and are at putative surface exposed locations (Speir et al. 1995; Fox et al. 1997). These two CCMVcys mutants contained individual cysteine substitutions at amino acids R82 and S130. Both VLPs were subsequently expressed in *P. fluorescens* and purified to test the feasibility of each in peptide-conjugation reactions.

In this conjugation procedure (Fig. 1b), a maleimide-derivatized M2E peptide was first made using the hetero-bifunctional crosslinker SSMCC which reacts with primary amines via an NHS ester group, and occurs at the N-terminus of the M2E peptide. M2E peptide derivatized with SSMCC was subjected to intact mass analysis by LC-MS, and the M2E-SSMCC modified peptide was found to be the major product (~74% M2E-SSMCC, and ~26% consisted of hydrolyzed M2E-SSMCC or other unknown byproducts, and unmodified peptide was virtually undetectable, data not shown).

Both cysteine mutants and wild-type CCMV were subjected to conjugation using SSMCC-derivatized M2E peptide (as described in the Materials and Methods section). Figure 5 shows an SDS-PAGE Coomassie stain analysis of

the aforementioned conjugation experiment, where more than one conjugation product was observed for both cysteine mutant R82C (four major conjugation products/bands) and S130C (three major conjugation products). Additionally, two minor conjugation products were observed for the reaction using wild-type CCMV. Altogether, these results suggest that SSMCC-M2E was conjugated to the two native cysteines at a lower, yet detectable, rate compared to the genetically-introduced cysteines of the two mutants; and these observations were most likely due to the differences in solvent exposure of each cysteine within each assembled VLP. One unexplained event is the fact that there were four conjugation products in the reaction using mutant R82C, while there were only three available cysteines for both CCMVcys mutants. It is possible that SSMCC-M2E was conjugated to primary amines within this mutant VLP. Though this reaction is possible, and expected to occur at a low rate, there was no verification of this conjugation event. The same reactions were subjected to SDS-PAGE followed by Western blot analysis using antibodies against CCMV and M2E (Fig. 6). This analysis showed that each unique conjugation product in all three reactions contained M2E-labeled CCMV (wild-type or mutant). Additionally,

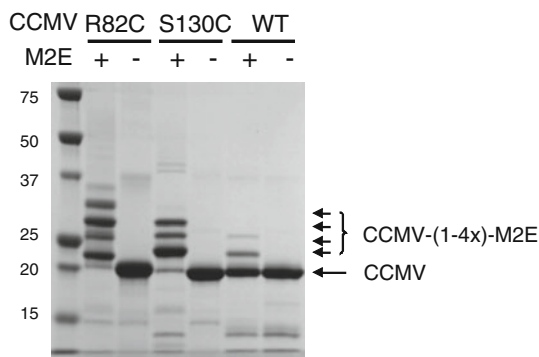


Fig. 5 SDS-PAGE followed by Coomassie stain analysis of CCMVcys and CCMV wild-type conjugation reactions. CCMVcys mutants (R82C and S130C) and wild-type were analyzed before and after conjugation to M2E

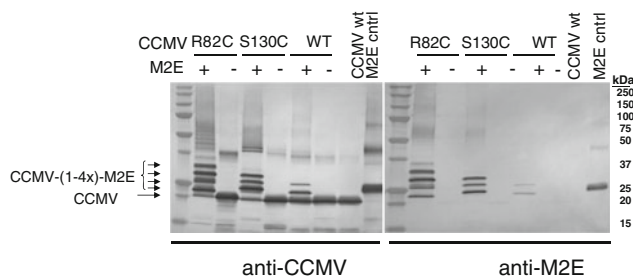
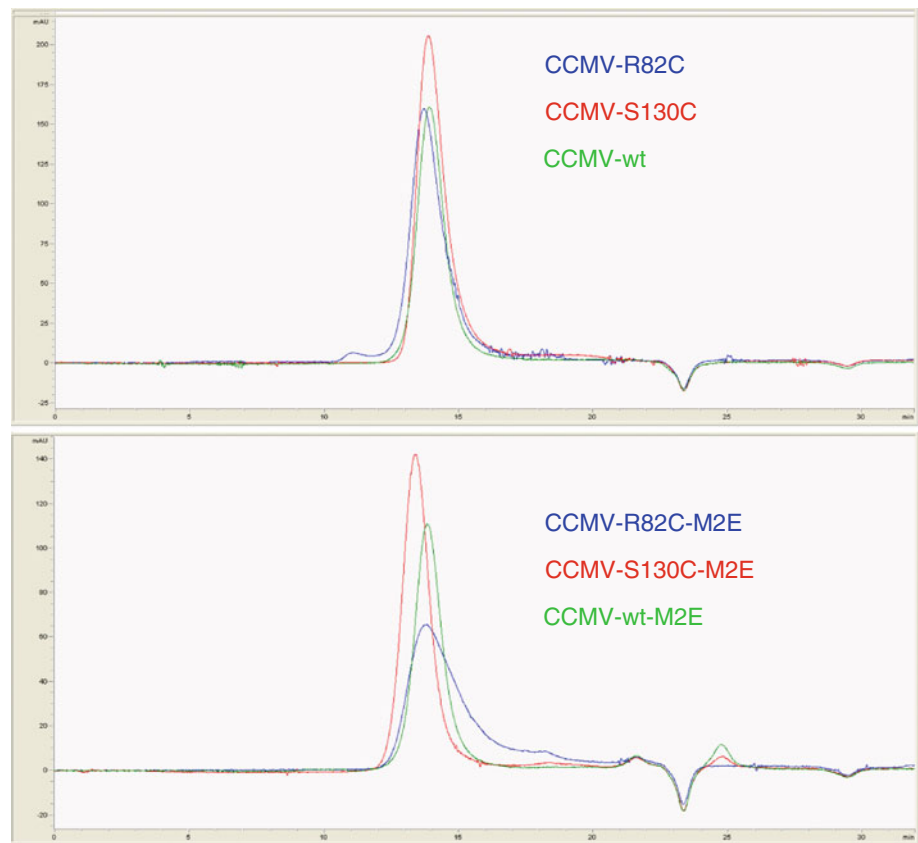


Fig. 6 Western blot analysis of M2E conjugation to CCMVcys mutants (R82C and S130C) and wild-type. Each VLP was analyzed before and after conjugation. A positive control for the antibody detection of M2E (M2E ctrl) was a CCMV-M2E genetic fusion protein. Anti-CCMV (left panel) and anti-M2E (right panel) antibodies were used on duplicate blots

Fig. 7 Analytical-scale size-exclusion chromatography of conjugation reactions. CCMVcys mutants and wild-type before (*upper panel*) and after (*lower panel*) M2E conjugation reactions are shown. Recovery for each conjugation product was calculated using peak areas of product-VLPs divided by peak areas of VLPs before conjugation. The recoveries for CCMV-R82C-M2E (*blue*), CCMV-S130C-M2E (*red*), and CCMV-wt-M2E (*green*) were 68, 67, and 69%, respectively



it appears that significantly less high molecular weight crosslinked multimers were formed in these cysteine-targeted conjugation reactions (Fig. 6) compared to the primary amine-targeted conjugation reactions (Fig. 3). Using SDS-CGE-analysis of the two mutant conjugations, it was determined that there was an average of 2.4 M2E peptides per CCMVcys R82C subunit (coat protein) and 1.5 M2E peptides per CCMVcys S130C subunit (data not shown).

The reaction products and starting VLP material was analyzed by size-exclusion HPLC to assess the structural integrity of the VLPs before and after the reaction, the results of which are shown in Fig. 7. Each VLP had a similar retention time before and after conjugation, suggesting that the structural integrity of the VLP was not significantly changed due to the conjugation. Using the SEC data, the yields for the CCMVcys conjugation products were calculated to be 67–68% (Fig. 7).

Conclusions

The chemical conjugation of the influenza antigen M2E peptide to two different virus-like particles was investigated. Wild-type cowpea chlorotic mottle virus (CCMV) and cysteine mutants of CCMV, all expressed in *Pseudomonas fluorescens*, were tested. Two different conjugation schemes

were successfully used to attach the M2E peptide to the surface of the aforementioned VLPs. The use of CCMVcys mutant VLPs allowed for the conjugation of M2E peptide at defined positions and reduced the formation of high molecular weight side products relative to the use of wild type CCMV (Figs. 3, 6). Additionally, the yields for the CCMVcys conjugation products (67–68%) using the cysteine-directed conjugation strategy were significantly higher than those for CCMV wild type conjugation products (40–51%) using the primary amine-directed conjugation scheme (Figs. 4, 7). Future work will focus on determining the immunogenicity of these different VLP-M2E conjugations in cell-based and animal studies. The ultimate goal of such work would be to develop new vaccines consisting of VLP-antigenic peptide conjugates.

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