Video Article

Analysis of the Solvent Accessibility of Cysteine Residues on *Maize rayado fino virus* Virus-like Particles Produced in *Nicotiana benthamiana* Plants and Cross-linking of Peptides to VLPs

Angela Natilla[1,2], Rosemarie W. Hammond[1,2]

[1]Plant Sciences Institute, Agricultural Research Service, United States Department of Agriculture

[2]Molecular Plant Pathology Laboratory, Agricultural Research Service, United States Department of Agriculture

Correspondence to: Angela Natilla at Angela.Natilla@ars.usda.gov

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Abstract

Mimicking and exploiting virus properties and physicochemical and physical characteristics holds promise to provide solutions to some of the world's most pressing challenges. The sheer range and types of viruses coupled with their intriguing properties potentially give endless opportunities for applications in virus-based technologies. Viruses have the ability to self- assemble into particles with discrete shape and size, specificity of symmetry, polyvalence, and stable properties under a wide range of temperature and pH conditions. Not surprisingly, with such a remarkable range of properties, viruses are proposed for use in biomaterials [9], vaccines [14, 15], electronic materials, chemical tools, and molecular electronic containers[4, 5, 10, 11, 16, 18, 12].

In order to utilize viruses in nanotechnology, they must be modified from their natural forms to impart new functions. This challenging process can be performed through several mechanisms including genetic modification of the viral genome and chemically attaching foreign or desired molecules to the virus particle reactive groups [8]. The ability to modify a virus primarily depends upon the physiochemical and physical properties of the virus. In addition, the genetic or physiochemical modifications need to be performed without adversely affecting the virus native structure and virus function. *Maize rayado fino virus* (MRFV) coat proteins self-assemble in *Escherichia coli* producing stable and empty VLPs that are stabilized by protein-protein interactions and that can be used in virus-based technologies applications [8]. VLPs produced in tobacco plants were examined as a scaffold on which a variety of peptides can be covalently displayed [13]. Here, we describe the steps to 1) determine which of the solvent-accessible cysteines in a virus capsid are available for modification, and 2) bioconjugate peptides to the modified capsids. By using native or mutationally-inserted amino acid residues and standard coupling technologies, a wide variety of materials have been displayed on the surface of plant viruses such as, *Brome mosaic virus* [3], *Carnation mottle virus* [12], *Cowpea chlorotic mottle virus* [6], *Tobacco mosaic virus* [17], *Turnip yellow mosaic virus* [1], and MRFV [13].

Video Link

The video component of this article can be found at http://www.jove.com/video/50084/

Protocol

1. Virus Inoculation and VLPs Purification from *Nicotiana benthamiana* Plants

1. Produce capped T7-RNA transcripts from *Potato virus* X (PVX)-based vector plasmids carrying MRFV wild-type (wt) and Cys-mutated coat protein (CP) genes [12], using Ambion's T7-mMessage mMachine Kit.
2. For each T7 transcript reaction, inoculate two fully expanded leaves of *N. benthamiana* with 10 µl reactions and incubate the plants for 10 days in greenhouse, at 60% humidity for 16 hr with light (25,000-30,000 lux) at 25 °C and 8 hr dark at 20 °C.
3. Harvest *N. benthamiana* virus-infected leaves 10 days post inoculation (dpi), weigh the plant tissue (50 grams) and place on ice.
4. Homogenize the plant material in a blender in presence of 100 ml of a chilled solution of 0.5 M Na-Citrate buffer, pH 5.5 (use 2 ml of buffer per gram of plant tissue).
5. Filter homogenate through 3 layers of cheesecloth and clarify by centrifugation at 27,000 x g for 30 min. Transfer the supernatant into a chilled graduated cylinder, measure the volume, and then transfer to a chilled sterile beaker. Discard the pellet.
6. Process the supernatant by adding 10% polyethylene glycol 8,000 (PEG 8,000), stir for 15 min at 4 °C, and incubate for an additional 45 min at 4 °C to precipitate the virus-like particles (VLPs)-PEG matrix.
7. Centrifuge the sample at 27,000 x g for 20 min at 4 °C and remove the supernatant.

8. Process the pellet by adding 8 ml of 0.05 M Na-Citrate buffer, pH 5.5 containing 2% Triton X-100. Swirl the centrifuge tubes well to release the pellet. Transfer the pellet and buffer to a chilled sterile beaker. Rinse the tubes immediately with 2 ml of the same buffer and add to the beaker. Stir the suspension at 4 °C until the pellets are dissolved (generally 1 hr).
9. Clarify by centrifugation for 5 min at 27,000 x g, discard the pellet, and process the supernatant by centrifugation at 140,000 x g for 2 hr at 4 °C.
10. Resuspend the pellet in 1 ml 0.05 M Na-Citrate buffer, pH 5.5 and place the suspension on top of a 10-40% sucrose gradient made in 0.05 M Na-Citrate buffer, pH 5.5.
11. Centrifuge the gradient for 3 hr at 140,000 x g at 4 °C.
12. Shine a light over the top of the centrifuge tube and collect the light-scattering upper band at 4 cm from the top of the tube, dilute with 0.05 M Na-Citrate buffer, pH 5.5, and centrifuge for 3 hr at 140,000 x g at 4 °C.
13. Resuspend the pellet in 0.5 ml of sterile water and store at 4 °C for up to six months.
14. Quantitate the VLPs concentration by performing a Bradford protein assay. The yield of VLPs is between 1 and 3 µg/g of plant tissue.

2. Fluorescein-5-maleimide-labeling Reactions of VLPs

1. Prepare a 1 mM solution of fluorescein-5-maleimide (427.37 g/mole) in 50 mM sodium phosphate buffer, pH 7.0; 1 mM EDTA. Mix using the vortex and verify complete dissolution. Protect the tube from light using aluminum foil.
2. Add fluorescein-5-maleimide to VLPs produced in step 1 and mix. Use a 25-fold molar excess of fluorescein-5-maleimide for molar amount of sulfhydryls. Generally, using 30 µl of VLPs at the concentration of 1 µg/µl and 60 µl of the fluorescein-5-maleimide solution produces acceptable results.
3. Incubate the reaction for 2 hr at room temperature or overnight at 4 °C.
4. Terminate the reaction by adding dithiothreitol (DTT) to a final concentration of 50 mM.
5. Remove non-reacted fluorescein using a desalting spin column (Thermo Scientific Inc, Rockford IL) and centrifugation for 2 min at 1,500 x g at room temperature.
6. For SDS-PAGE analysis, add 10 µl of 2 x SDS-PAGE Laemmli sample buffer to 10 µl of each reaction. Store the labeled protein protected from light at 4 °C for up to one month or in single-use aliquots at -20 °C up to 3 months.

3. Labeling Reaction and Determination of Biotin Incorporation

1. Use desalting spin columns (Thermo Scientific Inc., Rockford IL) to perform a buffer exchange from water to phosphate-buffered saline (PBS), pH 7.0 of the VLPs produced in step 1.
2. Prepare a 20 mM stock solution of maleimide-PEG2-biotin by adding 190 µl of PBS in No-Weigh maleimide-PEG2-biotin and mix by pipetting up and down.
3. Add maleimide-PEG2-biotin to the VLPs and mix. Use a 10-fold molar excess of reagent for VLPs solutions ≤ 2 mg/ml.
4. Incubate the reaction at room temperature for 4 hr.
5. Remove non-reacted maleimide-PEG2-biotin using a desalting spin column (Thermo Scientific Inc, Rockford IL) and centrifugation for 2 min at 1,500 x g at room temperature.
6. Determine the moles of biotin per moles of VLPs using the Pierce Biotin Quantitation Kit (Thermo Scientific) and following manufacturer's instructions.

4. Cross-linking of Cys 2-VLPs and F Peptide

1. VLPs which resulted in available cysteine residues for cross-linking in step 2 (Cys 2-VLPs) are used in the following step. Prepare a fresh cross-linking stock solution (28.63 mM) by dissolving 10 mg of NHS-PEG4-maleimide cross-linker in 680 µl of dimethyl sulfoxide.
2. Dissolve a 17 (F)-amino acid long peptide (LLPNMOKDKEACAKAPL) in 50 µl of conjugation buffer (1 x PBS, pH 7.2; 1 mM EDTA) at a concentration of 0.1 mM.
3. Add 4 µl of cross-linker to 50 µl of the dissolved peptide (protein-NH2) at 1 mM final concentration (which is a 10-fold molar excess for 0.1 mM of peptide solution).
4. Incubate the reaction for 2 hr at 4 °C.
5. Purify cross-linked peptide using a desalting column (Thermo Scientific Inc., Rockford IL) equilibrated with conjugation buffer (1 x PBS, pH 7.2; 1 mM EDTA) by centrifugation at 1,500 x g for 1 min at room temperature.
6. Set up a reaction mix combining VLPs (protein-SH) and cross-linked peptide (protein-NH2) in a molar ratio determined by the number of thiols and activated amines involved in the reaction and the difference in molecular weight between VLPs and peptide.
7. Incubate the reaction mixture at room temperature for 2 hr at 4 °C.
8. For Western blot analysis, mix the resulting conjugated product 1:1 with Laemmli buffer, boil for 10 min, and proceed with SDS-PAGE on a pre-cast 10-20% Tris-Glycine gel (Invitrogen), followed by electroblotting onto a nitrocellulose membrane (Invitrogen). Block the membranes with 1 x PBS, pH 7.2 containing 0.05% Tween-20 (PBS-Tween) and 5% skim milk powder and probe with peptide-specific antibodies followed by the appropriate phosphatase-labeled secondary antibody.

Representative Results

Transient expression of mutant MRFV coat protein (CP) genes in *N. benthamiana* plants in a PVX-based vector producing VLPs is described in **Figure 1**. The modified MRFV coat protein gene is amplified by PCR and then placed under the transcriptional control of the duplicated subgenomic CP promoter in a PVX-based vector, pP2C2S [2], (a gift of D. Baulcombe, Sainsbury Laboratories, Norwich, England). *In vitro* RNA transcription produces RNA transcripts that are then used to inoculate *N. benthamiana* plants. In the infected plants (**Figure 2A**) MRFV-CP subunits form VLPs (**Figure 2B**) which are easily purified. Subsequently, the VLPs are cross-linked to peptides.

An example of reactive amino acids of the MRFV coat protein is shown in **Figure 3**. Each amino acid selected can be used to attach moieties if it is surface exposed. The chemical techniques include traditional bioconjugation strategies such as, the acylation of the amino group of lysine, alkylation of the sulfhydrilic group of cysteine, and activation of carboxylic acid residues and coupling with added amines. In addition, the aromatic groups of tyrosine and tryptophan can be attractive targets for bioconjugation through diazotization and alkylation.

The solvent accessibility of Cys residues of VLPs to thiol-specific reagents is illustrated in **Figure 4**. Purified wt-VLPs (lane 4) and Cys-VLPs mutants carrying cysteine residues in position 107-111 (lane 1) and 192-194 (lane 3) of the CP gene [12] are unreactive in native conditions with thiol-specific reagent (absence of fluorescence). Fluorescein-5-maleimide imparts orange fluorescence only for the Cys-VLPs mutant carrying cysteine residues in position 125-129 of the CP gene (lane 2). The result shows that VLPs in lane 2 have a geometric arrangement of cysteines residues resulting in solvent-exposed free thiol groups, whereas the cysteine residues in the samples in lanes 1 and 3 are perhaps involved in disulphide bridges in the folding of the coat protein.

Labeling reactions with maleimide-PEG2-biotin followed by a biotinylation assay represent another example of analysis of solvent accessibility of Cys residues. Because biotin is a relatively small molecule, it can be conjugated in several copies on the VLPs, each of which can bind one molecule of avidin as shown in **Figure 5**. The level of biotinylation, 84.74 mole of biotin per mole of protein [13], indicates the number of the biotin molecules attached to the VLPs and consequently the number of ligands that can be displayed on the exterior surface. Because of the availability of several thiol groups on the VLPs, peptides can be attached by cross-linking reactions with NHS-PEG4-maleimide. This reagent is a hetero-bifunctional cross-linker which contains reactive ends such as *N*-hydroxysuccinimide (NHS) ester and maleimide groups, allowing covalent conjugation of amine- and sulfhydryl-containing molecules. As shown in **Figure 6A**, the NHS ester reacts with primary amines forming amide bonds, whereas maleimide reacts with sulfhydryl groups forming stable thioether bonds. Cross-linking reactions produce VLPs-peptide complexes that are immunoreactive with the specific antibodies in Western blot as shown in **Figure 6B**.

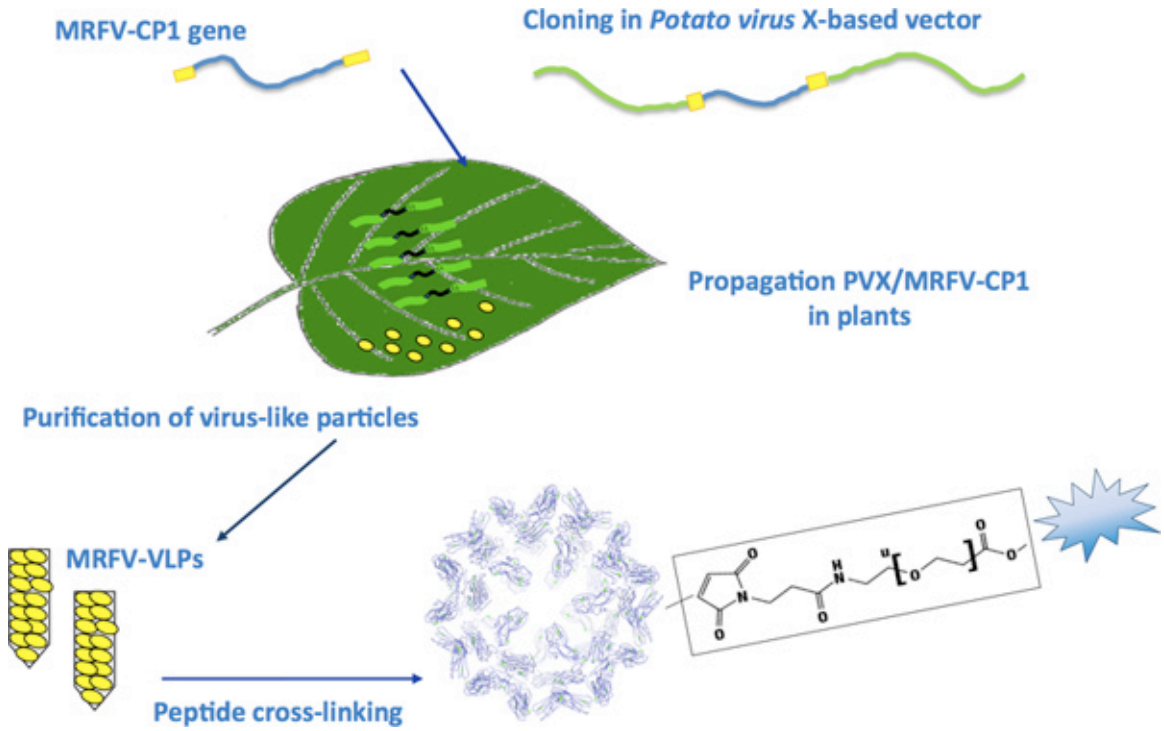

Figure 1. Schematic diagram of the production of VLPs in plants, followed by VLPs purification and subsequent cross-linking of peptides. Click here to view larger figure.

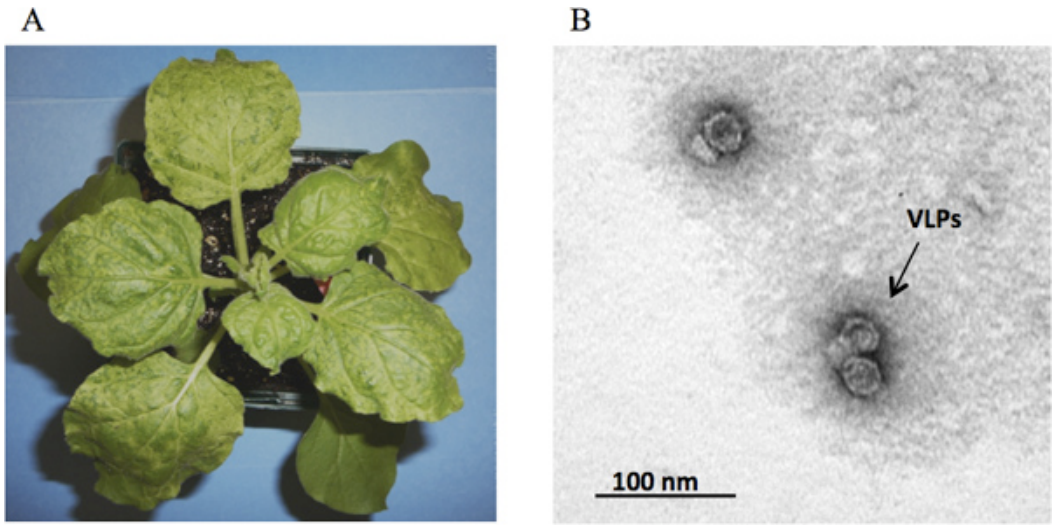

Figure 2. Symptoms produced by T7 transcripts inoculation on *N. benthamiana* plants **(A)** and transmission electron microscopy of the produced VLPs **(B)**.

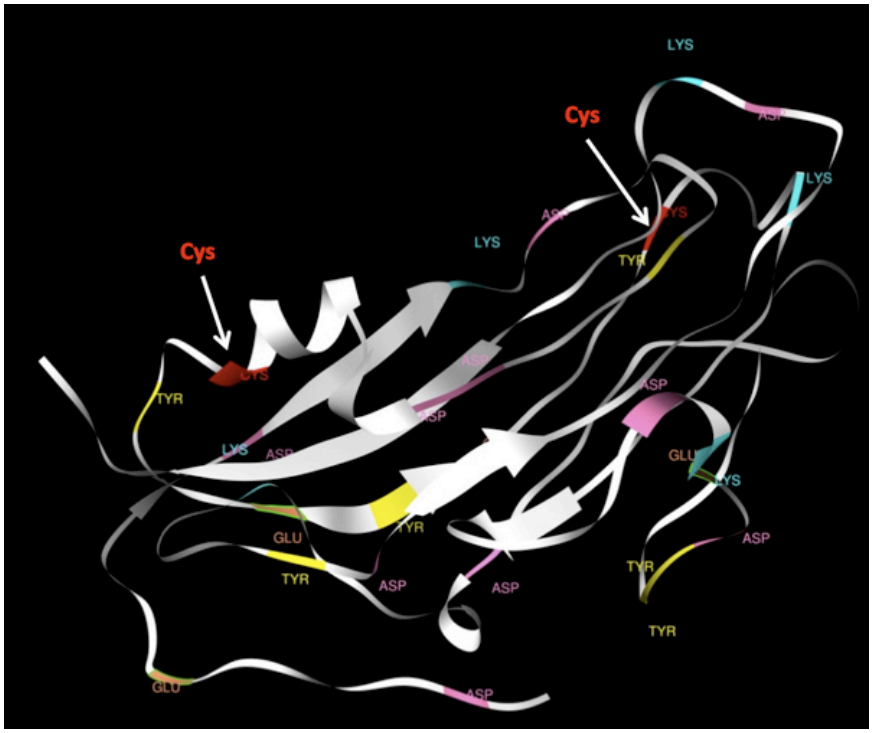

Figure 3. A protein structure prediction of wild-type (wt)-MRFV-CP showing reactive cysteine amino acids (indicated by arrows).

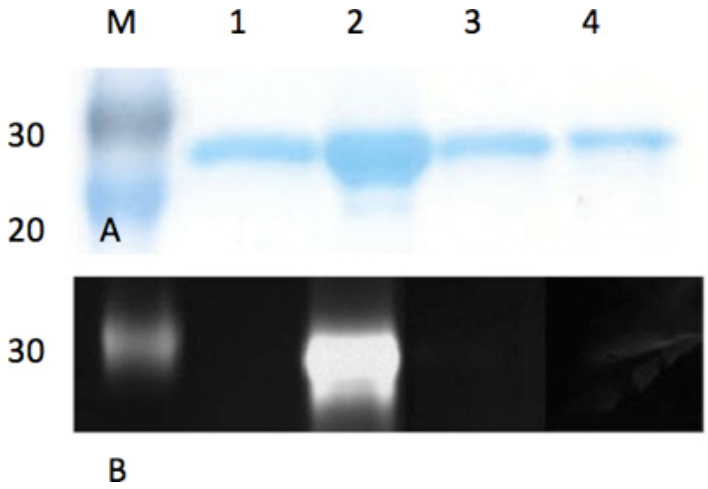

Figure 4. SDS-PAGE analysis of purified VLPs conjugated to fluoroscein-5-maleimide. **(A)** Simply blue safe stain was used to stain proteins. **(B)** Fluorescein-5-maleimide detection by photography under UV illumination. M: Prestained broad range protein marker (Bio-Rad, Hercules, CA), 1: purified Cys 1-VLPs, 2: purified Cys 2-VLPs, 3: purified Cys 3-VLPs, 4: purified wt VLPs. (Cys 1-VLPs, Cys 2-VLPs, and Cys 3-VLPs are Cys-MRFV-VLPs mutants [13]).

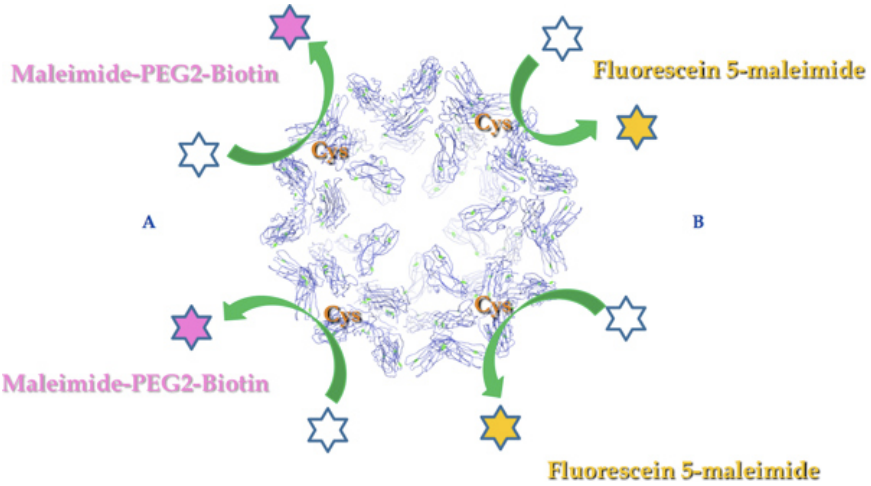

Figure 5. A T=3 ribbon model of isometric VLPs showing the sulfhydryl reactive chemistry for **(A)** biotin labeling and **(B)** fluorescein-5-maleimide labeling.

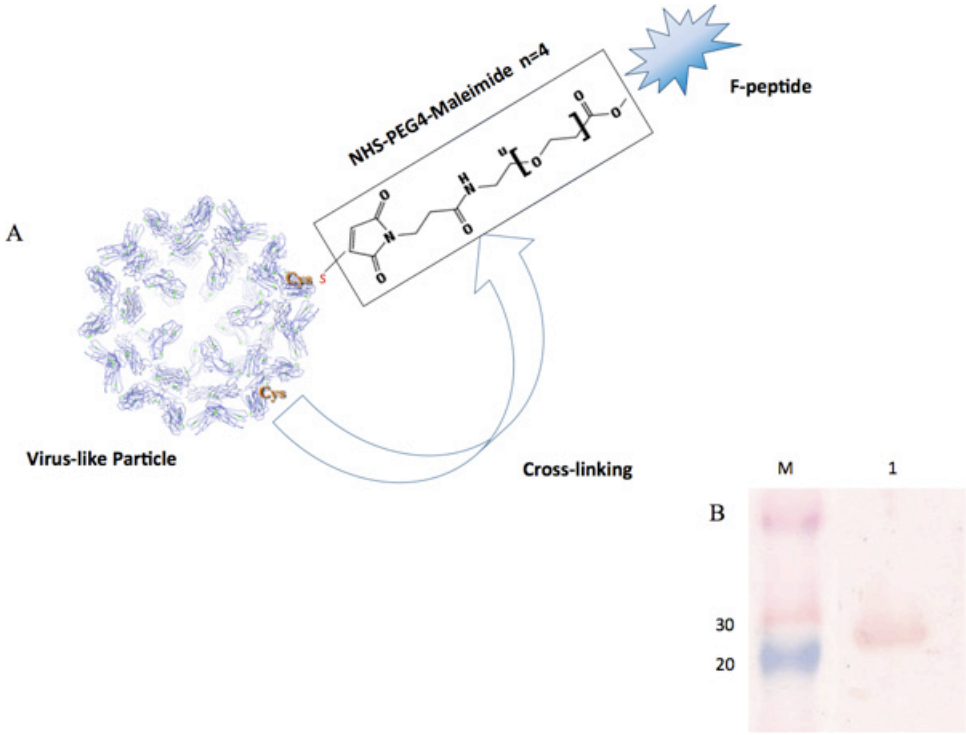

Figure 6. Diagram illustrating protein cross-linking between Cys 2-VLPs and F peptide **(A)** and Western blot **(B)** of Cys-VLPs-F probed with specific antibodies to F peptide. M: Prestained broad range protein marker (Bio-Rad, Hercules, CA), 1: Cys-VLPs-F produced in step 4. Click here to view larger figure.

Discussion

The method presented here enables a very sensitive and rapid analysis of reactive cysteines present on the surface of plant-produced VLPs as well as on other protein complexes. Maleimides are thiol-specific reagents, which react with free sulfhydryl-containing molecules to form stable thioether bonds. This method draws on the specificity of the maleimides to react with sulfhydryl groups not involved in interactions with other amino acids. Preserving the native structure of the VLPs is very important through the entire process. In the application described, the reactions are performed in native conditions and at pH 7 to maintain the native structure of the VLPs. At neutral pH, maleimides have optimal reactivity with free sulfhydryl groups. This allows labeling of the VLPs with either fluorescein or biotin to cysteine accessible to modifications.

Mild pH (6.5-7.5) and buffer conditions are also employed in the cross-linking reactions. The number of functional groups on the VLPs determines the appropriate ratio between cross-linker and VLPs. Lower cross-linker to protein ratios can be used in cases where there are many functional groups. Conversely, a higher cross-linker to protein ratio is employed with fewer available functional groups.

An array of factors including space arm, cleavability, chemical composition, dimension, and solubility ultimately determine the selection of cross-linkers used. In addition, the choice of the cross-linker used is also a function of the size of the two proteins (in this case VLPs and peptide) involved in the conjugation. Hetero-bifunctional cross-linkers are ideal for protein-peptide or protein-protein cross-linking. They allow more controlled two-step reactions that minimize the undesired self-conjugation and polymerization which occurs when homo-bifunctional NHS-ester reagents are used. In the first step, the NHS ester reacts with primary amines forming amino bonds. In the second step, the maleimide reacts with sulfhydryl groups forming thioester bonds.

Disclosures

Mention of the trade names of commercial products in the publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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