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Chemical Modification of the Inner and Outer Surfaces of Tobacco Mosaic Virus (TMV)

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Abstract

Viral nanoparticles derived from tobacco mosaic virus (TMV) have broad applications in various fields. We report the purification and chemical engineering of TMV which is a hollow rod-shaped plant viral nanoparticle with modifiable interior and exterior surfaces. Here, we describe methods to isolate TMV from its tobacco plant host for spatially controlled interior and exterior chemical modification and to characterize the resulting TMV hybrid materials.

Keywords

Bioconjugation chemistry; CuAAC (click) chemistry; Tobacco mosaic virus (TMV); Viral nanoparticles (VNPs)

1 Introduction

Tobacco mosaic virus (TMV) was discovered more than 110 years ago and has been a model for molecular plant pathology since the 1920s [1, 2]. Many research breakthroughs in plant and general virology as well as physical virology are attributed to research on TMV [2]. More recently, this stiff rod-shaped nanoparticle has been utilized as a material for a variety of applications in nanotechnology [3, 4]. Chemically and genetically engineered TMV particles are being developed and tested for applications as light harvesting systems [5, 6], energy storage [7], sensing [8], cell growth [9, 10], and magnetic resonance imaging contrast enhancement [11].

The physical and biological characteristics of TMV are well known. Native TMV infects 200+ plant species [12], several of which can be used for propagation of the viral

nanoparticle. Using *Nicotiana benthamiana* (tobacco) plants, as high as 4.5 mg TMV per gram infected leaf material can be purified. Similar yields can be obtained from *Nicotiana rustica* and other tobacco species. Native TMV particles are stable at temperatures over 60 °C for 30 min, pH values ranging from 2 to 10, and organic solvents up to 80 % by volume [13]. TMV is made up of 2,130 identical coat proteins with a molecular weight of 17,534 Da arranged helically around a single-strand, positive-sense RNA molecule. The 6,395 bp genomic RNA dictates the length of the 300 nm hollow rod with an 18 nm outer diameter and 4 nm channel and a total particle weight of 3.94×10^7 Da [14]. The coat protein and RNA structure has been refined to 2.9 Å (PDB ID: 2TMV, Fig. 1) [15], which allows us to pinpoint the location of specific amino acids and predict chemical reactivity by using protein modeling software such as PyMol.

The exterior surface of TMV has available tyrosine residues (Tyr139) that can be labeled with small molecules, polymers, and peptides [8, 10, 16]. Alternatively, fluorescent dyes, MRI contrast agents and therapeutics can be loaded onto interior glutamic acids 97 and 106 residues [11, 17]. For chemical modification of TMV, a two-step bioconjugation strategy is applied: First, terminal alkyne handles are chemically conjugated to the interior and exterior surfaces. Interior carboxylic acids, glutamic acids 97 and 106, react with an 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDC) activated propargyl amine to form an amide bond. On the exterior surface, an electrophilic substitution reaction with an in situ generated diazonium salt (3-ethynyl aniline) labels the *ortho* position of the phenol group of tyrosine 139. In the second step, the terminal alkynes are functionalized using a copper-catalyzed azidealkyne cycloaddition (CuAAC) reaction, also termed “click” reaction [18], to attach a wide variety of azido-functionalized molecules (Fig. 2). Following chemical modification, the structural integrity of the particles is confirmed with transmission electron microscopy (TEM) and size exclusion chromatography (SEC). Further, conjugation efficiency is determined using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), SEC on a fast protein liquid chromatography (FPLC) system, and ultraviolet–visible (UV–Vis) absorption spectroscopy.

2 Materials

All solutions are prepared using ultrapure water. All buffer and TMV solutions are stored at 4 °C. Chemicals were purchased through Sigma unless specifically noted.

2.1 Purification of TMV

1. *Nicotiana benthamiana* seeds (seeds are collected from noninfected plants).
2. Carborundum.
3. Cheesecloth.
4. Kimwipes (11.8 × 11.8 in.).
5. Ziploc bags.
6. Blender.

7. Phosphate buffers: Prepare 0.1 M solutions of monobasic and dibasic potassium phosphate (KP) buffers and add dibasic solution to monobasic until desired pH is reached. A pH of 7.4–7.8 is common for purifications and reactions. For 0.01 M phosphate, simply dilute 100 mL of 0.1 M buffer in 900 mL H₂O.
8. B-mercaptoethanol, PEG MW 8 k, chloroform, NaCl, and *n*-butanol, and Triton-X 100 were used as received.
9. Sucrose gradient (10–40 %): Two methods can be used to prepare the sucrose gradient.
 - a. The first method uses a Gradient Master (BioComp Instruments, Inc.) to form the gradient. 40 % (w/v) sucrose in 0.01 M KP buffer are added to the bottom half of the ultraclear centrifuge tube (Fisher Cat. No. NC9640905) and 10 % (w/v) sucrose in 0.01 M KP buffer is carefully added on top. The tubes are then run in the gradient maker using the 10–40 % (w/v) program provided by BioComp.
 - b. Alternatively, prepare a 40 % (w/v) solution of sucrose in 0.01 M KP buffer and freeze in ultraclear centrifuge tubes at –20 °C and thaw at room temperature. A gradient will form during the thaw process (*see* Note 1).
10. Suggested centrifuges and rotors.
 - a. Beckman Coulter Optima L-90 K Ultracentrifuge.
 - b. Beckman Coulter SW 32 Ti.
 - c. Beckman Coulter Type 50.2 Ti.
 - d. Beckman Coulter Avanti J-E High Performance Centrifuge.
 - e. Beckman Coulter JLA16.250.
 - f. Beckman Coulter JLA 10.500.
 - g. Beckman Coulter Microfuge 16 Centrifuge.
 - h. Beckman Coulter FX241.5P.

2.2 Chemical Modification TMV

All solutions are prepared fresh for each chemical reaction. Buffers are sterile filtered (using a 0.22 µm filter, e.g., Millipore) after being adjusted to the appropriate volume and pH. Buffers and solutions are stored at 4 °C (*see* Note 2).

2.2.1 Diazonium Coupling to Exterior Tyrosine Residues—

1. *P*-toluenesulfonic acid monohydrate (pTSA): 0.3 M (57 mg/mL in H₂O).
2. Sodium nitrite (NaNO₂): 3.0 M (207 mg/mL in H₂O).

¹One freeze–thaw cycle is adequate for TMV purification. It should be noted that additional freeze–thaw cycles improve the quality of the gradient.

²Buffers are kept until they appear to have particulates floating in solution.

3. 3-Ethynylaniline: 0.68 M (79 mg/mL in acetonitrile).
4. Borate buffer: 0.1 M pH 8.8 with 0.1 M NaCl. Dissolve 6.18 g boric acid and 5.84 g of sodium chloride in 800 mL H₂O. Adjust pH to 8.8 with 10 M NaOH, then dilute to 1 L with H₂O.

2.2.2 EDC-Mediated Conjugation of Interior Glutamic Acid Residues—

1. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer: 0.1 M pH 7.4. Dissolve 23.8 g HEPES in 800 mL H₂O. Adjust pH to 7.4 with 10 M NaOH then dilute to 1 L with H₂O.
2. Propargyl amine: 0.1 M stock solution (5.5 mg/mL in H₂O).
3. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC): 0.1 M stock solution (15.5 mg/mL in H₂O).
4. Hydroxybenzotriazole (HOBt): 0.1 M stock solution (13.5 mg/mL in H₂O) or added directly to the solution.

2.2.3 Click Reaction on Alkyne-Labeled TMV Particles—

1. Sulfo-Cy5 azide (Lumiprobe): 0.01 M stock solution. Dissolve the 5 mg of dye in 642 μ L of DMSO in the tube it arrived in. Keep in -20°C for future use.
2. Aminoguanidine: 0.2 M stock solution (22 mg/mL in H₂O).
3. Ascorbic acid sodium salt: 0.2 M stock solution (39 mg/mL in H₂O).
4. Copper (II) sulfate pentahydrate: 0.1 M stock solution (25 mg/mL in H₂O) (*see* Note 3).
5. Ethylenediaminetetraacetic acid (EDTA): 0.5 M stock solution. Add 18.6 g EDTA to 80 mL H₂O. Add about 2 g NaOH to adjust pH to 8.0. Add the last few pellets slowly trying not to overshoot the pH (*see* Note 4).

2.3 Characterization of Modified TMV

2.3.1 Size Exclusion Chromatography—

1. Superose 6 10/300GL size exclusion column on an AKTA explorer FPLC system (GE Lifesciences).

2.3.2 Transmission Electron Microscopy—

1. Uranyl acetate: 2 % (w/v) (20 mg/mL in H₂O) (*see* Notes 4 and 5).
2. Carbon-coated grids (*see* Note 6).

³Copper (II) sulfate anhydrous can also be used.

⁴This solution can be stored for an extended period of time.

⁵For safety reasons, shield uranyl acetate solution by wrapping containers in aluminum foil.

⁶The choice of grids is often personal. We use Carbon type-B with Formvar on a 300-mesh copper grid (Cat. No. 01813).

2.3.3 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry—

1. Millipore ZipTip pipet tips with micro bed of C₁₈ media (Cat No. ZTC18M096).
2. Acetonitrile.
3. Guanidine hydrochloride: 6 M stock solution (573 mg/mL in H₂O) (*see* Note 4).
4. Trifluoroacetic acid (TFA): 0.1 % v/v in H₂O (10 µL concentrated TFA in 990 µL H₂O).
5. Sinapinic acid matrix solution: 70 % acetonitrile with 0.1 % TFA (700 µL acetonitrile, 290 µL H₂O, and 10 µL TFA). Add sinapinic acid solid and dissolve until the solution becomes saturated.

3 Methods

3.1 Purification of TMV

1. Grow *Nicotiana benthamiana* plants for 6–8 weeks.
2. Inoculate each leaf by sprinkling a small amount of carborundum over the plants then gently rubbing 100 µL of 0.01 mg/mL TMV in KP buffer per leaf (*see* Note 7).
3. Collect infected leaves 7–10 days post-infection when mosaic patterns present themselves and before the leaves die.
4. Immediately freeze leaves and store in Ziploc bags at –80 °C (*see* Note 8).
5. When ready, pulverize frozen leaves by squeezing bag (*see* Note 9).
6. Homogenize leaves in 3 volume of prechilled (4 °C) 0.1 M KP buffer (~300 mL), pH 7.4 and 0.2 % (v/v) β-mercaptoethanol (~600 µL).
7. Filter homogenate through 2 layers of cheesecloth; squeeze cheesecloth to collect all of the filtrate.
8. Centrifuge the filtrate for 20 min at 11,000 × *g* (10,500 rpm when using Beckman Coulter JLA-16.250 rotor).
9. Carefully pour the supernatant through 4 layers of Kimwipes.
10. To the supernatant (approximately 300 mL) add equal volume of 1:1 chloroform:*n*-butanol (150 and 150 mL).
11. Stir for 30 min on ice (avoid foaming by controlling the stirring speed).
12. Centrifuge for 10 min at 4,500 × *g* (6,000 rpm when using Beckman Coulter JLA-10.500 rotor).

⁷Keep lights off for 8–12 h after inoculation with TMV.

⁸Label bags of infected leaves with date collected, bag weight, and “TMV.”

⁹The volumes and amounts used in this purification method are based on extraction of TMV from 100 g infected leaf material.

13. Carefully collect the aqueous phase (~300 mL, top layer that contains the TMV) using a pipette and store on ice (*see* Notes 10 and ¹¹).
14. Add NaCl to 0.2 M (3.5 g), PEG 8 k to 8 % (w/v) (24 g), Triton-X 100 surfactant to 1 % (v/v) (3 mL) to the collected supernatant and put the mix on ice.
15. Stir on ice for 30 min, then store in refrigerator for at least 1 h.
16. Centrifuge for 15 min at $22,000 \times g$ or max speed of centrifuge/rotor) (15,000 rpm when using Beckman Coulter JLA-16.250 rotor) (*see* Note 12).
17. Resuspend the pellet in 15 mL 0.1 M phosphate buffer (~0.05 mL/g leaf) by carefully pipetting up and down or by incubating on a shaker for 4 h to overnight at 4 °C (*see* Note 13).
18. Centrifuge for 15 min at $9,000 \times g$ (9,500 rpm when using Beckman Coulter JLA-16.250 rotor).
19. Layer the supernatant on a sucrose gradient (*see* Subheading 2) and centrifuge in a swing bucket rotor for 2 h at $96,000 \times g$ (28,000 rpm when using Beckman Coulter SW 32 Ti rotor).
20. Collect the light scattering region (*see* Fig. 3) and dilute with 0.01 M phosphate buffer to fill ultracentrifuge tube.
21. Centrifuge in a fixed angle rotor for 2.5 h at $160,000 \times g$ (42,000 rpm when using Beckman Coulter type 50.2 Ti rotor).
22. Discard supernatant and resuspend pellet in 0.01 M KP buffer overnight.
23. Centrifuge the resuspended pellet for 15 min at $7,500 \times g$ (10,000 rpm when using Beckman Coulter FX241.5P rotor) in a table top centrifuge (e.g., Beckman Coulter Microfuge 16) and save supernatant: pure TMV solution.
24. Test concentration using UV–Vis absorbance (e.g., Nanodrop). TMV has an $A_{260 \text{ nm}} = 0.3$ for a 1 mg/mL solution and 1 cm path length. An A_{260}/A_{280} ratio equal to 1.2 indicates intact TMV particles.
25. Test purity using SEC with a Superose 6 10/300GL column by monitoring the absorbance at 260 nm.

3.2 Modification of TMV

All reactions are done at 2 mg/mL TMV protein concentration at 1 mL total volume. Covalent attachment of Sulfo-Cy5-azide (Lumiprobe) dyes to the interior and exterior surfaces will be described for appropriate biomedical relevance (*see* Note 14).

¹⁰We recommend using an electronic pipette for the bulk of the solution and a 1.5 mL glass pipette for final 5–10 mL. Be careful not to disturb or collect the organic layer.

¹¹Maximizing the amount of aqueous layer collected here will significantly improve the overall yield.

¹²Save the supernatant and recentrifuge in case yields are low.

¹³It is common to resuspend overnight for this step.

¹⁴Modification of TMV particles with positively charged molecules usually causes TMV aggregation. For dual functionalized TMV, modify the exterior surface first, then the interior surface. Modifying the exterior last can cause aggregation.

3.2.1 Diazonium Coupling to Exterior Tyrosine Residues—

1. Begin by cooling 400 μL of a 0.3 M pTSA solution on ice.
2. Add 75 μL of 0.68 M 3-ethynylaniline and 25 μL of 3.0 M NaNO_2 and mix for 1 h on ice, protected from light (*see* Note 15).
3. Add 100 μL TMV (20 mg/mL stock solution) to 862 μL 0.1 M borate buffer pH 8.8 on ice.
4. Add 38 μL of in situ formed diazonium salt (35 eq (*see* Note 16)) to TMV solution.
5. Briefly mix solution with vortex or by hand, then place in ice bath for 30 min, protected from light (*see* Notes 17 and 18).
6. Purify the modified TMV sample by ultracentrifugation for 2.5 h at $160,000 \times g$ over a 40 % (w/v) sucrose cushion (*see* Note 19).
7. Resuspend the pellet in 0.01 M KP buffer pH 7.4 and store at 4 °C (*see* Note 20).

3.2.2 EDC-Mediated Conjugation of Interior Glutamic Acid Residues—

1. Add 100 μL TMV (20 mg/mL stock solution) to 674 μL 0.1 M HEPES buffer pH 7.4 at room temperature. Briefly mix solution with vortex or by hand.
2. Add 130 μL of 0.1 M propargyl amine (120 eq per coat protein), 3.0 mg of HOBt (*see* Note 21) and mix. Then add three times 32 μL of 0.1 M EDC (30 eq) in the following time frame (to give a total of 90 eq total): The first addition is at the beginning of the reaction; the second and third additions are at 6 and 18 h, respectively.

¹⁵The final concentration of aniline is 0.1 M.

¹⁶The term eq refers to molar equivalents in relation to concentration of coat proteins. The equation to determine this volume is here:

$$\left(\left(\left(\frac{[\text{TMV}]}{MW_{\text{TMV}}} \right) \times \# \text{coat proteins per VNP} \right) \times \text{equivalents} \right) \frac{\times \text{Reaction volume}}{\text{Stock concentration}} = \left(\left(\frac{2 \text{mg}^{-1} \text{mL}^{-1}}{3.94 \times 10^7 \text{Da}} \right) \times 2, 130 \text{coat proteins} \right) \times 35 \text{equivalents} / 0.1 \text{M} \times 1 \text{mL} = 0.038 \text{mL} = 38 \mu\text{L} \text{diazonium salt}$$

¹⁷The reaction should turn a light brown color. Reacting for more than 1 h will cause aggregation.

¹⁸If the pH of the solution is below 8, then the reaction may not occur. This happens for low stock TMV concentrations and therefore not enough buffer is used.

¹⁹A sucrose cushion is prepared (in a 25 mL tube) by adding 21 mL phosphate buffer, then carefully adding the reaction (1 mL) to the bottom of the tube with a glass pipette, then slowly and carefully adding 3 mL (roughly two full glass pipettes) 40 % (w/v) solution of sucrose to the bottom of the centrifuge tube.

²⁰We typically store modified TMV at 10 mg/mL solution.

²¹For HOBt addition, it can be added directly to the reaction instead of creating a 0.1 M solution. Creating this solution often takes a long time.

3. This reaction is performed at room temperature for 24 h.
4. Purify the modified TMV sample by ultracentrifugation for 2.5 h at 42,000 rpm ($160,000 \times g$) over a 40 % (w/v) sucrose cushion (*see* Note 19).
5. Resuspend the pellet in 0.01 M KP buffer and store at 4 °C (*see* Note 20).

3.2.3 Click Reaction on Alkyne-Labeled TMV Particles—

1. Add 200 μ L of alkyne-TMV (10 mg/mL stock) to 765 μ L ice cold 0.01 M KP buffer pH 7.4. Briefly mix solution with vortex or by hand.
2. Add 5 μ L sulfo-Cy5 azide (5 eq per coat protein) (*see* Note 22). Add 10 μ L aminoguanidine (0.002 M final concentration), 10 μ L ascorbic acid sodium salt (0.002 M final concentration), and 10 μ L copper sulfate (0.001 M final concentration) and mix (*see* Note 23).
3. React for 30 min, then add 10 μ L EDTA (0.5 M stock) for 5 min, then centrifuge for 10 min at $7,500 \times g$ (10,000 rpm when using Beckman Coulter FX241.5P rotor).
4. Purify by layering the supernatant over a 10–40 % (w/v) sucrose gradient and centrifuging for 2 h at $96,000 \times g$ (28,000 rpm when using Beckman Coulter SW32 Ti rotor).
5. Collect the light scattering region, dilute to fill ultracentrifuge tube, and centrifuge for 2.5 h at $160,000 \times g$ (42,000 rpm when using Beckman Coulter type 50.2 Ti rotor).
6. Resuspend the pellet in 0.01 M KP buffer pH 7.4 and store at 4 °C (*see* Note 24).

3.3 Characterization of TMV

3.3.1 UV–Vis Absorbance Spectroscopy—

1. Measure the absorbance of the solution and note the readings at 260 nm (RNA), 280 nm (protein), and 647 nm (Cy5). A A_{260}/A_{280} ratio of 1.2 is indicative of a pure TMV solution (*see* Note 25).
2. Use Beer's Law ($A = \epsilon cl$) to determine the dye loading per TMV. Here, A is the absorbance, ϵ is the extinction coefficient (mL/cm/mg), c is the concentration (mg/mL), and l is the path length (cm). Expanding this equation gives:

$$\left(\frac{A_{647}}{A_{Cy5}} \right) \times \left(\frac{MW_{TMV}}{\frac{A_{260}}{3}} \right) = \frac{\#Dye}{TMV}$$

²²After addition of sulfo-Cy5 azide, keep the reaction away from light.

²³Click reaction with PEG molecules will often require additional copper and ascorbic acid. Click reaction with water insoluble molecules require addition of up to 20 % DMSO in the solution.

²⁴A typical beginning to end yield for these two-step reactions are about 50 %.

²⁵Exterior modification of tyrosine residues introduces a diazonium bond that absorbs light at 324 nm and below 260 nm. This will change the A_{260}/A_{280} ratio and the extinction coefficient. Use a protein assay, such as the Modified Lowry Protein Assay Kit (Pierce), to determine the exact TMV concentration.

Here, $\epsilon_{\text{Cy5}} = 250,000$, MW TMV $\sim 4.00 \times 10^7$ Da (*see* Note 26).

3.3.2 Size Exclusion Chromatography—

1. Set up a Superose 6 10/300GL size exclusion column on an AKTA explorer FPLC system (GE Lifesciences).
2. Prepare 150–200 μL of a 1 mg/mL solution of modified TMV (and native TMV, control). Centrifuge for 10 min at $7,500 \times g$ (10,000 rpm when using Beckman Coulter FX241.5P rotor).
3. Inject solution and run column at a flow rate of 0.5 mL/min. Set the absorbance detectors for 260 nm (TMV RNA), 280 nm (TMV protein), and 647 nm (Cy5) (*see* Note 27).

3.3.3 Transmission Electron Microscopy—

1. Dilute TMV sample to a concentration of 0.1 mg/mL in water (*see* Note 28).
2. Float 20 μL of each of the following solutions on parafilm: TMV, water, water, 2 % (w/v) uranyl acetate, water.
3. Place carbon-coated TEM grid on TMV droplet for 2 min.
4. Dry TEM grid by tapping the edge on filter paper.
5. Briefly place TEM grid on two droplets of water and remove water with filter paper.
6. Place TEM grid on 2 % (w/v) uranyl acetate droplet for 2 min.
7. Rinse grid one time with last water drop, and then place in grid holder for TEM analysis.
8. Use TEM for imaging at 200 kV.

3.3.4 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)—

1. Add 3 μL of 6 M guanidinium chloride with 12 μL TMV sample.
2. With μC_{18} ZipTip, wash column bed (draw and elute solvent) 7 times with acetonitrile, then 7 times with 0.1 % (v/v) TFA, then 15 times with TMV sample. Next draw up 0.1 % (v/v) TFA and elute onto paper towel. Finally draw up 2 μL matrix solution and elute 1–2 μL onto MALDI plate.
3. Analyze sample using MALDI-TOF in linear positive mode. Figure 4 shows a representation of MALDI-TOF MS analysis of modified TMV products.

²⁶For each modification, add 0.03×10^7 Da to the molecular weight of TMV for calculation accuracy. The molecular weight of native TMV is $\sim 3.94 \times 10^7$ Da.

²⁷TMV should elute at near 7.5 mL, which is the dead volume of the column. Free proteins and dye will elute after 20 mL.

²⁸Diluting the TMV solution in buffer, especially phosphate, will leave a salt precipitation on the TEM grids. This will make it difficult to obtain high-quality images.

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Fig. 1. PyMol images depicting the morphology of tobacco mosaic virus (PDB ID 2TMV). (a) A 110 nm segment of the 300 nm TMV rod showing the helicity of the coat protein assembly. (b) A *top down* view of a single layer of coat proteins and (c) a single protein highlighting tyrosine 139 (red), glutamic acids 97 and 106 (yellow), and the single-strand RNA (black)

Fig. 2. Bioconjugation reactions showing the (*exterior*) diazonium coupling reaction to the phenol side chain of tyrosines, (*interior*) EDC-mediated coupling of a primary amine to glutamic acid residues, and CuAAC (*click*) reaction of a terminal alkyne with an azide

Fig. 3.

(a) Transmission electron microscopy of negative-stained interior Cy5-TMV. (b) Examples of sucrose gradients of native TMV pre-spin and post-spin and of Cy5-TMV. In order to see the native TMV, a flashlight is shined above the tubes. (c) UV-Vis absorbance of Native TMV, exterior alkyne-TMV, and exterior Cy5 TMV. (d) Size exclusion chromatography of exterior Cy5-labeled TMV

Fig. 4. MALDI-TOF MS analysis of modified TMV products. The values in represent the expected m/z for modified TMV products. Native TMV has a molecular weight of 17,534 Da. Exterior modification adds 128 m/z 17,662 m/z), interior modification adds 38 m/z per modification (17,572 and 17,610 m/z). The Cy5 dyes add 726 m/z per modification Exterior Cy5-labeled proteins are 18,388 m/z and interior Cy5-labeled proteins are 18,298 and 19,062

m/z . The actual data (not indicated) is within 50 m/z of the expected values and may vary based on instrument calibration

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