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# Self-Assembling Light-Harvesting Systems from Synthetically Modified Tobacco Mosaic Virus Coat Proteins

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***Abstract:*** A new protein-based approach has been developed for the construction of light-harvesting systems through self-assembly. The building blocks were prepared by attaching fluorescent chromophores to cysteine residues introduced on tobacco mosaic virus coat protein monomers. When placed under the appropriate buffer conditions, these conjugates could be assembled into stacks of disks or into rods that reached hundreds of nanometers in length. Characterization of the system using fluorescence spectroscopy indicated that efficient energy transfer could be achieved from large numbers of donor chromophores to a single acceptor. Energy transfer is proposed to occur through direct donor−acceptor interactions, although degenerate donor-to-donor transfer events are also possible. Three-chromophore systems were also prepared to achieve broad spectrum light collection with over 90% overall efficiency. Through the combination of self-organizing biological structures and synthetic building blocks, a highly tunable new method has emerged for the construction of photovoltaic device components.

## Introduction

Light-harvesting systems in nature absorb light and transfer the energy to photosynthetic reaction centers, facilitating the conversion of collected sunlight into chemical bonds.[1] The high efficiency of the natural system is achieved through a precisely spaced array of chromophores that can transport energy over long distances through a series of fluorescence resonance energy transfer (FRET) events. In addition to increasing the effective extinction coefficient of the reaction center, light-harvesting assemblies incorporate multiple types of chromophores that collect light over a wide spectral bandwidth. In bacteria, it has been shown that over 200 bacteriochlorophylls can supply energy to a single reaction center, allowing a dramatic enhancement in the overall rate of the subsequent redox processes involved in photosynthesis.[2]

Outside of the cellular context, light-harvesting systems could be used to sensitize solar cells, drive photocatalysts, and serve as components of optical sensors. To this end, several strategies have been explored for the synthesis of artificial light-harvesting systems, with the goal of producing stable arrays with tunable absorption characteristics.[3] Successful scaffolds have been provided by dendrimers,[4] multilayer polymer films,[5] direct porphyrin assemblies,[6] and semisyntheic metalloprotein complexes.[7] Each of these systems provides an elegant approach to the challenging task of positioning multiple chromophores with well-defined spatial relationships.

Self-assembling protein scaffolds offer an attractive alternative for the construction of arrays of regularly spaced chromophores reminiscent of the natural system.[8,9] In addition to offering advantages of synthetic ease, the rigidity of protein-based platforms would maintain the close chromophore spacing

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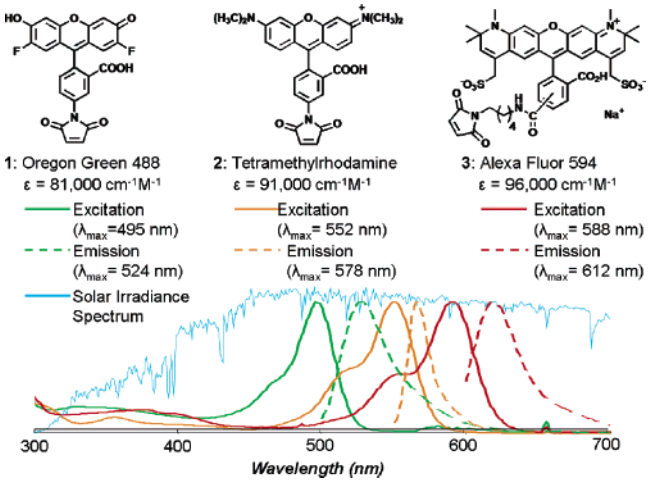


***Figure 1.*** Donor (**1** and **2**) and acceptor (**3**) chromophores used in this study. Normalized fluorescence excitation and emission spectra are plotted against the solar spectrum.[14]

required for optimal energy transfer while preventing energy loss through contact quenching. Here, we report an example of such a system, built from chromophore-labeled monomers of the tobacco mosaic virus coat protein (TMVP). Previous studies have highlighted the utility of intact viral capsids for the construction of inorganic[10] and organic materials.[11] In constrast, the current study relies on the self-assembly of functionalized protein subunits. Simple adjustments in pH and ionic strength cause these protein conjugates to assemble into disk- and rod-shaped materials capable of efficient energy transfer between chromophores. The capability of the system to integrate different types of chromophores is demonstrated, allowing light collection over a broad range of wavelengths in the visible spectrum.

## Results and Discussion

Our design is based on the use of recombinant TMVP monomers[12] bearing a reactive cysteine residue for chromophore attachment. After expression of both the wild-type protein and the S123C mutant protein in *Escherichia coli*, the identities of the desired proteins were confirmed through electrospray ionization−mass spectrometry (ESI-MS) analysis.

The thiol-reactive chromophores selected for this study include Oregon Green 488 maleimide (**1**) as the primary donor, tetramethylrhodamine maleimide (**2**) as an intermediate donor, and Alexa Fluor 594 maleimide (**3**) as the acceptor (Figure 1). These chromophores were selected on the basis of their high extinction coefficients (80,000−100,000 $cm^{-1}$ $M^{-1}$), relative stability against photobleaching, and high degree of overlap with the solar spectrum.[13,14] Appreciable overlap between the absorption and emission bands of the donor chromophore allows donor-to-donor FRET to occur, and the overlap between the donor emission band and the acceptor absorption band promotes donor-to-acceptor FRET. After reaction with reagents **1**−**3** at pH = 7.0 for 15 min, the mass of cysteine mutant **4** increased by the molecular weight of the maleimide derivatives (Figure 2a, b). The mass of the wild-type TMVP remained unchanged under similar reaction times, indicating selective modification of the engineered cysteine. The site selectivity of this labeling reaction was also supported by trypsin digest analysis (Supporting Information, Figure S1). The use of a similar cysteine-based strategy has recently been reported for the attachment of pyrene to the inner cavity of TMV rods.[15]

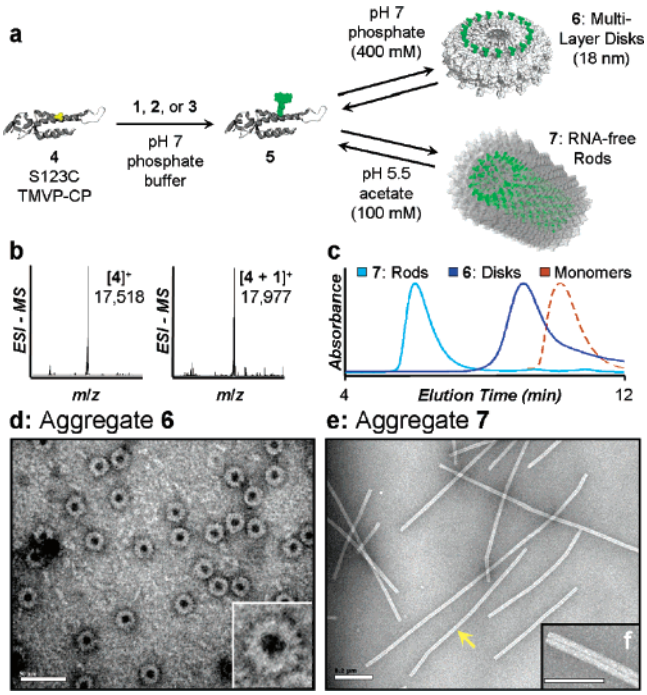


***Figure 2.*** (a) Self-assembly of modified TMV coat proteins into disk and rod structures. Disks are composed of stacked layers, each containing 17 monomers. (b) Chromophore attachment results in complete labeling, as observed by ESI-MS. Modified monomers assemble into multilayer disk (**6**) and rod (**7**) structures, as characterized by (c) SEC traces monitoring tryptophan fluorescence ($\lambda_{ex}$ = 295 nm, $\lambda_{em}$ = 330 nm) and TEM (d−f). The scale bars represent 50 nm in d, 200 nm in e, and 100 nm in f. The 900-nm rod indicated by the arrow in e contains over 6300 chromophores.

The assembly properties of the TMVP−chromophore conjugates (**5**) were next investigated.[16] Disk structures (**6**) were formed upon raising the potassium phosphate concentration from 0.1 to 0.4 M (pH = 7). The extent of the assembly was monitored by size-exclusion chromatography (SEC, Figure 2c). Characterization by transmission electron microscopy (TEM, Figure 2d) revealed disks with a characteristic central pore and an outside diameter of 18 nm.[17] In a previous report, native recombinant TMVP was observed to assemble into four-layer stacked disks.[18] However, under our buffer conditions, the

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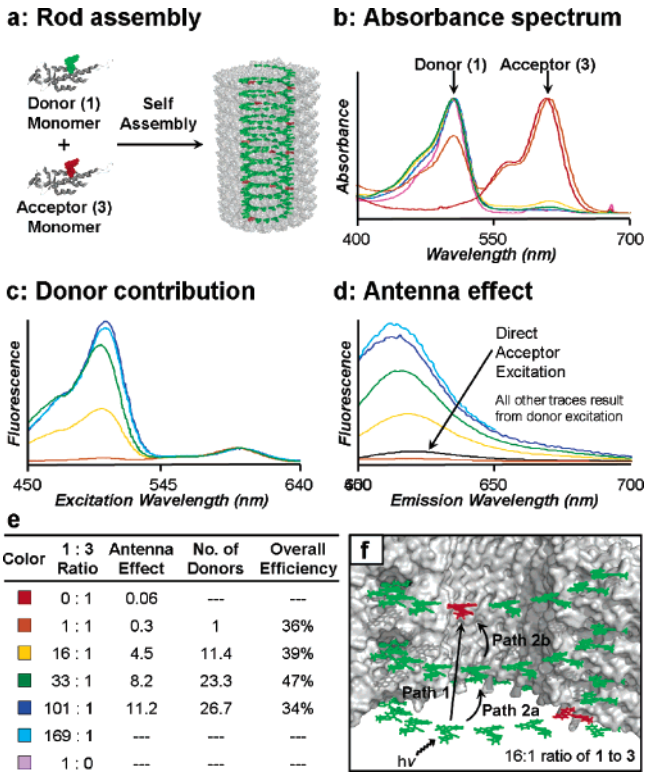
S123C mutant yielded a population of disks ranging from 2 to 6 layers, as determined from the measured thicknesses of several oblique samples (Supporting Information, Figure S2). While these data indicate that a variety of disk aggregates are present, the figures in this report will depict the double-layer disks, as these appear to be the smallest aggregates in solution under these conditions.

Despite the steric bulk of the attached chromophores, large rod aggregates were formed upon dialysis against 100 mM sodium acetate (pH = 5.5). SEC analysis indicated that >94% of the protein monomers were incorporated into these assemblies, and TEM anlaysis revealed that the rods could extend over hundreds of nanometers (Figure 2e). The central channel of the assembly was visible in samples stained with uranyl formate (Figure 2f), and the diameter of the rods matched that of samples prepared from unlabeled monomers. As the presence of the chromophores leads to no apparent distortion of the assembly, it is likely that the flat aromatic groups occupy the RNA-binding channel that is present in the native virus (see Figure 3f). The cysteine attachment site is well-positioned for this to occur. These assemblies contain 700 chromophores per 100 nm of rod length with an estimated horizontal spacing of 1.8 nm and a vertical spacing of 2.3 nm. Both of these distances are within the expected Förster radius for energy transfer between these dyes (see Supporting Information for an estimation of this value).

A key attribute of light-harvesting systems is the number of donor chromophores that contribute to acceptor emission. To determine this, rod assemblies with varying donor-to-acceptor ratios were formed. Chromophores **1** and **3** were chosen for this purpose, as their excitation and emission spectra were well-resolved and therefore could be used to determine these parameters accurately. Monomers modified with either **1** or **3** were combined in the appropriate stoichiometric ratios (on the basis of protein concentration, as measured in stock solutions using a Bradford assay[19]), and were allowed to equilibrate before buffer exchange into rod-forming conditions (Figure 3a). Absorbance measurements of the resulting assemblies are shown in Figure 3b.

Fluorescence excitation measurements indicated that donor excitation is by far the major contributor to acceptor emission in systems with high donor-to-acceptor ratios (Figure 3c). To ensure that residual donor fluorescence was not being collected, the reported spectra were collected at 650 nm, rather than at the acceptor emission maximum of 612 nm. However, spectra collected at 650 nm revealed analogous trends. A comparison of rods containing 33 donors per acceptor to systems containing equal amounts of the two chromophores indicated that at least 20 donor chromophores can funnel energy to a single acceptor. This is likely to be a lower estimate, as some losses will occur with each energy-transfer event.

Since a single acceptor molecule can have a maximum of eight neighboring donors (Figure 3f), energy transfer must also




e

| Color | 1 : 3 Ratio | Antenna Effect | No. of Donors | Overall Efficiency |
|---|---|---|---|---|
| | 0 : 1 | 0.06 | --- | --- |
| | 1 : 1 | 0.3 | 1 | 36% |
| | 16 : 1 | 4.5 | 11.4 | 39% |
| | 33 : 1 | 8.2 | 23.3 | 47% |
| | 101 : 1 | 11.2 | 26.7 | 34% |
| | 169 : 1 | --- | --- | --- |
| | 1 : 0 | --- | --- | --- |




***Figure 3.*** Self-assembly of light-harvesting structures. (a) Mixtures of TMVP monomers labeled with **1** and **3** were assembled into rods. (b) Absorbance spectra confirmed the chromophore ratios listed in e. (c) For light-harvesting systems, fluorescence excitation spectra were obtained by monitoring the fluorescence of acceptor **3** at 650 nm and normalizing at the maximum excitation wavelength of acceptor **3** (597 nm). (d) The antenna effect for each spectrum ($\lambda_{ex}$ = 495 nm) is shown relative to the sample's acceptor emission by direct excitation ($\lambda_{ex}$ = 588 nm). (e) Tabulated data for these systems. The antenna effect (column three) was normalized to direct acceptor excitation. The number of donors contributing to the emission of a single acceptor chromophore in each system (column four) represents the integration of the excitation spectrum from 450 to 545 nm, relative to the 1:1 chromophore system. The overall efficiency of each system (column five) was calculated by comparing the peak at 495 nm in excitation and absorbance spectra normalized at 597 nm. (f) For systems with large numbers of donors, energy can be transferred to acceptor chromophores through direct FRET (path 1) or via multiple donor-to-donor transfers (path 2a and 2b).

be occurring from nonadjacent donor chromophores. Assuming a random orientation of the chromophores and an aqueous refractive index, the Förster radii can be estimated at 4.0 nm for donor-to-acceptor transfers and at 4.4 nm for donor-to-donor transfers (see Supporting Information for calculation details). Thus, it is possible that direct energy transfer can occur from donors up to two positions away from each acceptor group (Figure 3f, Path 1). Energy transfer can also occur through multiple degenerate donor-to-donor events (Figure 3f, Path 2a and 2b). On the basis of distance and spectral overlap calculations, the latter pathway should occur approximately 20 times more rapidly. Both possibilities are being considered in ongoing experiments to elucidate this process.

An alternative way to evaluate the light-harvesting capabilities of the system is to determine the degree to which donor excitation results in amplified acceptor emission, previously defined as the "antenna effect" in reports of dendritic systems.[20]

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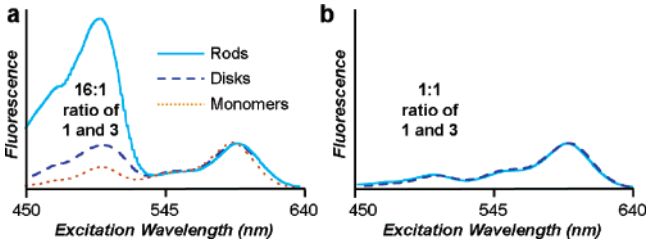


***Figure 4.*** Comparison of disk and rod assemblies for light harvesting. For large donor/acceptor ratios, some disk structures will lack acceptor chromophores and thus cannot participate in light harvesting. This effect can be observed by comparing the fluorescence excitation spectra for disk and rod aggregates with (a) 16:1 and (b) 1:1 donor to acceptor ratios. Emission is monitored at 650 nm, and spectra are normalized to the acceptor excitation maximum (597 nm).

For each donor:acceptor ratio, this value was calculated using the following equation:

$$\text{antenna effect} = I_{\text{A(ex495)}}/I_{\text{A(ex597)}}$$

where $I_{\text{A(ex597)}}$ is the acceptor emission resulting from direct acceptor excitation and $I_{\text{A(ex495)}}$ is the acceptor emission resulting from donor excitation (Figure 3d). In the absence of donor chromophores, this value is only 6%. For rods assembled from 1:1 donor-to-acceptor solutions, a value of 30% was obtained (compare the black and orange curves). However, the acceptor emission increased significantly as the relative number of donor dyes was increased. An overall 4-fold amplification of acceptor emission was observed in constructs containing a 16-fold excess of donors (yellow curve); further increase to one acceptor per 33 donors (green curve) resulted in an 8-fold increase of acceptor fluorescence. Energy transfer was eventually saturated at one acceptor per 101 donors, resulting in an 11-fold increase, and an effective extinction coefficient of over $1 \times 10^6$ cm$^{-1}$ M$^{-1}$ for the acceptors at 495 nm. These data are summarized in Figure 3e.

The efficiency of energy transfer showed a strong dependence on the assembly state. Control experiments indicated that minimal energy transfer occurs between monomers and oligomeric aggregates, Figure 4a (see also Supporting Information, Figure S4). Disks containing 16 donors per acceptor showed marked improvement upon assembly into rods. One explanation for this behavior is the presence of a statistical population of disks that lack an acceptor dye, effectively removing the contribution of these donors from the acceptor fluorescence (Figure 4a). These isolated donor systems do not occur upon incorporation into the larger rod assemblies, allowing the full set of donor dyes to participate in light harvesting. In line with this explanation, energy transfer in disks containing one donor per acceptor does not increase upon assembly into rods (Figure 4b).

The overall efficiency of the 2-chromophore system was measured by comparing the excitation spectra to the absorbance spectra, normalized at the acceptor wavelength (597 nm) (Supporting Information, Figure S5).[21] This analysis indicated that, depending on the chromophore ratio, 34−47% of the light absorbed by the system can be funneled to the acceptor group (Figure 3e). As this low efficiency was likely due to the less

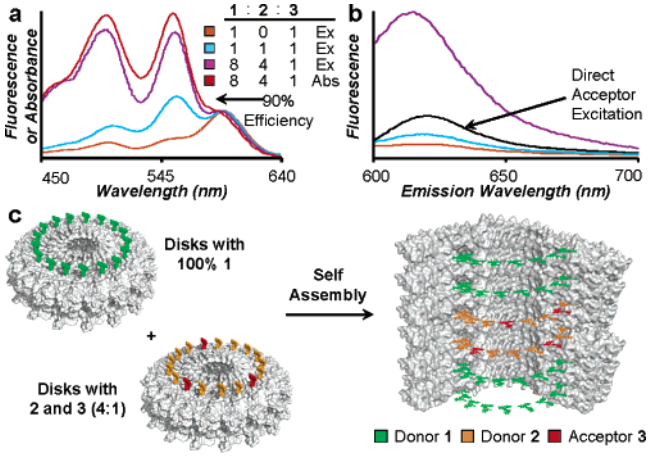


***Figure 5.*** Three-chromophore systems for broad-spectrum light harvesting. TMVP monomers labeled with **1**, **2**, or **3** were combined in the ratios indicated in a. (a) Fluorescence excitation spectra (Ex), normalized at the acceptor excitation at 597 nm, indicated light harvesting over a wide range of wavelengths. The absorbance spectrum (Abs) for the 8:4:1 system is shown in red. (b) The antenna effect for each spectrum ($\lambda_{ex}$ = 495 nm) is shown relative to the sample's acceptor emission by direct excitation ($\lambda_{ex}$ = 588 nm). (c) Spatial distribution of chromophores for the 8:4:1 system.

than ideal spectral overlap between **1** and **3**, a third chromophore was added to the system to facilitate energy transfer. Monomers labeled with **2** were added to solutions of monomers labeled with **1** and **3** before rod assembly. Since the absorbance spectra of the chromophores may vary depending on pH and protein attachment,[22] all monomer concentrations were determined using Bradford assays[19] before initiating assembly. In addition to introducing a strong absorption band at 552 nm, the new chromophore was found to enhance the efficiency of energy transfer from **1** to **3** (Supporting Information, Figure S5 and S6). This can be seen by the increased contribution of chromophore **1** to acceptor fluorescence in rods containing equal amounts of the three chromophores, relative to that observed in a system lacking **2** (Figure 5a, orange and light-blue curves).

Since energy back transfer from **2** to **1** is inefficient, assemblies comprising larger numbers of donor chromophores were prepared such that chromophore **2** had access to a continuous pathway to acceptor **3**. This was accomplished by first preparing two independent samples of disks: one labeled homogeneously with donor **1**, and a second with a 4:1 ratio of **2** and **3**. These separate samples were then combined and assembled into striated rods containing **1**, **2**, and **3** in a final ratio of 8:4:1 (Figure 5c). The resulting system exhibited efficient transfer of light collected over a broad set of wavelengths (Figure 5a, b) and an antenna effect of 4.6 at 495 nm. The overall efficiency of the system was determined to be over 90% (Figure 5a) by comparison of the absorbance and excitation spectra. This behavior was confirmed by the observation of virtually complete donor quenching (Supporting Information, Figure S7).[23,24] For comparative purposes, an assembly containing an identical 8:4:1 ratio of chromophores was prepared from disks displaying a random mixture of chromophores. Even this

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system was capable of achieving an overall efficiency of 84% (Supporting Information, Figure S8).

## Conclusions

By taking advantage of the robust nature of TMVP self-assembly and the rigid positioning that the resulting scaffolds provide, this new light-harvesting system provides a useful building block for optical device construction. Its modular nature allows the composition of chromophores to be changed easily to match virtually any spectral input. To incorporate these assemblies into photovoltaic and photocatalytic devices, current efforts are focusing on the use of additional chemical modifications to attach synthetic functionalities to the inside and outside of the light-harvesting rods.[11f] In terms of fundamental research, this system could be used as a scaffold for modeling the multidonor energy transfer that occurs in natural systems. With this goal in mind, time-resolved fluorescence studies are in progress to gain further understanding of the energy-transfer characteristics of this system.

## Materials and Methods

**General Procedures.** Unless otherwise noted, all chemicals and solvents were of analytical grade and were used as received from commercial sources. Water (dd-$H_20$) used in biological procedures or as reaction solvents was deionized using a NANOpure purification system (Barnstead, United States). The centrifugations required in spin concentration steps were conducted using an Allegra 64R Tabletop Centrifuge (Beckman Coulter, Inc., United States).

Prior to analysis, biological samples were desalted and separated from small molecule contaminants using BioSpin G-25 centrifuge columns (Amersham Biosciences, United States) and NAP-5 and NAP-10 gel filtration columns (Amersham Biosciences, United States). Control experiments have indicated that assembled TMVP aggregates elute in the void volume of these columns, while small molecules are retained. Additionally, 3500 molecular weight cutoff Slide-A-Lyzer Dialysis Cassettes (Pierce Biotechnology, Inc., United States) were employed as indicated below.

**Instrumentation and Sample Analysis Preparations.** Electrospray LC/MS analysis was performed using an API 150EX system (Applied Biosystems, United States) equipped with a Turbospray source and an Agilent 1100 series LC pump. UV−vis spectroscopic measurements were conducted on a Tidas-II benchtop spectrophotometer (J & M, Germany). Fluorescence measurements were obtained on a Fluoromax-2 spectrofluorometer (ISA Instruments). Samples were prepared for TEM analysis by applying analyte solution (approximately 0.2 mg/mL in TMVP) to carbon-coated copper grids for 3 min, followed by rinsing with dd-$H_2O$. The grids were then exposed to a 1% aqueous solution of either uranyl acetate or uranyl formate for a short time (30 s to 1 min) as a negative stain. Transmission electron microscopy (TEM) images were obtained at the Berkeley Electron Microscope Lab using a FEI Tecnai 12 transmission electron microscope with 100 kV accelerating voltage. High-resolution images were obtained with a Tecnai 12 transmission microscope equipped with a LaB6 source. Images were recorded on a Gatan Multiscan 794 CCD camera or on Kodak ISO-163 films and then were scanned with a Nikon Super Coolscan 8000 scanner with a 12.7-$\mu$m raster size.

**Construction of TMVP Expression Plasmids.** pTMV0041, a wild-type TMV cDNA clone, was received as a gift from Dr. Dennis Lewandowski, University of Florida. Standard recombinant techniques were used to construct an expression plasmid with pET20b vector DNA (Novagen). The TMVP gene was amplified by PCR, using an upstream primer with the sequence 5′-GATTCGTTTTACATATGTCTTAC-3′ and a downstream primer with the sequence 5′-TAGTACCATG-GCATCTTGACTAC-3′. The amplification product was digested with NdeI and NcoI (NEB) before ligation into pET20b with T4 DNA ligase (NEB). A TMVP-S123C mutant construct was made using standard recombinant techniques.

**Expression and Purification of Recombinant TMV Coat Protein (TMVP).** TMVP was expressed and purified according to a modified literature procedure.[12] Tuner DE3pLysS competent cells (Novagen) were transformed with pTMVP, and colonies were selected for inoculation of Terrific Broth cultures. When cultures reached mid-log phase as determined by O.D. 600, expression was induced by addition of 10 $\mu$M IPTG (Invitrogen). Cultures were grown 14−18 h at 30 °C, were harvested by centrifugation, and were stored at −80 °C.

Induced cells were thawed and resuspended in ice-cold Buffer A (20 mM Tris, pH = 7.2; 5 mM DTT; 20 mM NaCl) containing 1 mM Pefabloc SC (Roche). Cells were lysed by sonication (Branson Ultrasonics), and the resulting lysate was cleared by ultracentriguation for 50 min at 40,000 rpm in a Beckman 40 Ti rotor. The cleared lysate was applied to a DEAE anion-exchange column (Amersham) and was eluted with Buffer A at 4 °C. Fractions were analyzed by SDS-PAGE, and fractions containing TMVP were combined, concentrated, and dialyzed against 50 mM sodium citrate buffer, pH = 3.5. The resulting TMVP precipitate was collected by centrifugation, was washed with additional citrate buffer, and was resuspended in Buffer B (100 mM Tris, pH 8). The purified TMVP was quantified by Bradford assay,[19] was flash-frozen, and was stored at −20 °C. The TMVP-S123C mutant was prepared in the same manner, except Buffer A contained 15 mM DTT and Buffer B contained 10 mM TCEP to prevent thiol oxidation.

**General Procedure for Chromophore Attachment.** A thawed aliquot of TMVP (8.0 mg/mL in 100 mM HEPES buffer, pH 6.5, 1% TCEP) was exchanged into 100 mM $KH_2PO_4$, pH 7, using a NAP-10 column. The solution was diluted to 1.5 mg/mL in TMVP, and 5 equiv of maleimide-functionalized chromophore was added as a DMF solution (up to 5%, v/v). In optimization experiments, the higher DMF concentrations, increased equivalents of the dye, or extended reaction times led to overlabeling, presumably at the native C27 residue. The reaction mixture was vortexed briefly and was left at room temperature for 15−20 min. The reaction was quenched with 20 equiv of $\beta$-mercaptoethanol, and the mixture was passed through a NAP-10 column to remove excess chromophore. Conversion of TMVP to modified product was monitored by LC/ESI-MS and UV−vis spectroscopy. Reactions involving smaller molecular weight dyes, such as Oregon Green 488, required a shorter reaction time compared to those involving larger dyes, such as Alexa Fluor 594. The site selectivity of the modification reaction was confirmed through mass spectrometry analysis of peptide fragments after digestion with trypsin (see Supporting Information for spectra).

**Assembly of TMVP Disks and Rods.** Solutions of dye-modified TMVP monomers were diluted to 0.75 mg/mL and were dialyzed overnight against 25 mM phosphate buffer, pH 8, to achieve equilibration to the monomer state.[16] After dialysis, TMVP mixtures were analyzed by size-exclusion chromatography using an HPLC equipped using a Phenomenex PolySep-GFC-P 5000 column (300 × 7.8 mm, flow rate 1.0 mL/min) equilibrated with 25 mM phosphate buffer, pH 8. The concentration of dye-modified TMVP monomers was quantified using a Bradford assay.[19]

The monomers were combined in the stoichiometric ratios and were allowed to equilibrate for 3 h at room temperature to allow for exchange between any preformed small aggregates before assembly. To assemble the protein into disks, monomer solutions were exchanged into 0.4 M phosphate buffer, pH 7, using a NAP-5 size-exclusion column and were dialyzed overnight. For assembly into rods, monomer solutions were exchanged into 100 mM sodium acetate buffer, pH 5.5, and were dialyzed two days, followed by two additional days of equilibration. After dialysis, the conversion to larger structures was monitored by size-exclusion chromatography, and the assemblies were characterized visually using TEM.

**Spectroscopic Measurements.** The spectroscopic properties of all monomer, disk, and rod solutions were investigated by collecting steady-state absorbance and fluorescence measurements. Samples were diluted to 0.1 OD for fluorescence measurements. Excitation measurements of the two- and three-dye systems were collected at 650 nm rather than the emission maximum of chromophore **3** (617 nm) to minimize the direct contributions from chromophore **2** emission. Area integrations were calculated using Kaleidagraph 4.0 graphing software.

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**Supporting Information Available:** MALDI-TOF MS analysis of modified TMVP trypsin digest; characterization of the recombinant S123C coat protein under disk buffer; energy-transfer calculations for the two-chromophore system; overlays of the absorbance and excitation spectra of the two- and three-chromophore systems; absorbance spectra of rod assemblies containing various ratios of donor **1**, donor **2**, and acceptor **3**; overlay of the emission spectra of selected one-, two-, and three-chromophore systems; a comparison of rods containing **1**, **2**, and **3** (8:4:1), in which chromophores are either randomly distributed or layered through assembly from preformed disks; and emission spectra of Oregon Green 488, tetramethylrhodamine, and Alexa Fluor 594 in the monomer buffer, disk buffer, and rod buffer. This material is available free of charge via the Internet at http://pubs.acs.org.

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