DELIVERY OF POTENT ANTI-MITOTIC CHEMOTHERAPEUTIC USING HIGH ASPECT RATIO, SOFT MATTER NANOPARTICLES

by

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List of Abbreviations

ADC: Antibody drug conjugate
EPR: Enhanced permeability retention effect
FBS: Fetal bovine serum
IFRT: Involved-field radiation therapy
Lys: Lysine
NHL: Non-Hodgkin’s Lymphoma
NHS: N-hydroxysuccinimide
PEG: Polyethylene glycol
PB: Permeabilization buffer
R-CHOP: Combined therapy of Rituximab, Cyclophosphamide, Vincristine, Doxorubicin, and Prednisone
SA: Serum albumin
SATP: N-succinimidyl-S-acetylthiopropionate
TMV: Tobacco mosaic virus
TEM: Transmission electron microscopy
vcMMAE : valine-citrulline monomethyl auristatin E
VNP: Viral nanoparticle
Delivery of Potent Anti-Mitotic Chemotherapeutic Using High Aspect Ratio, Soft Matter Nanoparticles

Abstract

by

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Non-Hodgkin’s lymphoma, the most common type of blood cancer, has traditionally been treated with chemotherapeutics. The side effects of this treatment regime significantly affect quality of life for patients. As a result, researchers are developing targeted therapies that deliver the drug to the site of disease more efficiently; the mainstream of developments has focused on antibody-drug conjugates. As an alternative, nanoparticle drug therapies have shown great promise enabling drug delivery to sites of disease based on their size, shape and surface engineerability. Viral nanoparticles are biology-derived carriers that are growing in popularity due to their simple genetic and chemical modification, size tunability from spherical to high-aspect ratio, and biocompatibility. These attributes give a wide range of tools for engineers to design therapeutics with specific toxic loads and surface chemistries to efficiently navigate the body. In this thesis, I developed a viral nanoparticle using the nucleoprotein component from the tobacco mosaic virus (TMV) as the carrier for delivery of the anti-mitotic drug valine-citrulline monomethyl auristatin E (vcMMAE). I demonstrate
successful synthesis of the formulation, and effective cell killing of Non-Hodgkin’s lymphoma in vitro.

**Introduction**

According to the Leukemia and Lymphoma Society, over 700,000 Americans are currently living with lymphoma.\(^1\) Lymphoma is a neoplastic disorder of the lymphatic system, and occurs when lymphocytes, or white blood cells, grow and multiply uncontrollably. Lymphoma can be divided into two categories: Hodgkin’s lymphoma, and Non-Hodgkin’s lymphoma.

Hodgkin’s disease is a specific kind of lymphoma commonly diagnosed by the appearance of Reed-Sternberg cells in tissue biopsies. Fortunately, Hodgkin’s lymphoma is one of the most curable forms of cancer, and patients with this disease have a 5-year survival rate of 85%.\(^1\),\(^2\) However, Non-Hodgkin’s lymphoma is the more common type of lymphoma, and covers a range of lymphatic cancers, including: diffuse large B-cell lymphoma, follicular lymphoma, small lymphocytic lymphoma, mantle cell lymphoma, marginal zone B-cell lymphoma, burkitt lymphoma, lymphoplasmacytic lymphoma, hairy cell leukemia, primary central nervous system lymphoma, precursor T-lymphoblastic lymphoma and peripheral T-cell lymphomas.\(^3\)

The most common form of non-Hodgkin’s lymphoma is diffuse large B-cell lymphoma. Patients with this disease have poor survival with a 5 year survival rate as low as 58%.\(^3\) The treatment success rates and prognosis heavily depend on the stage at which the disease is diagnosed. Both, Hodgkin’s and non-Hodgkin’s lymphoma are staged according to the Ann Arbor staging system, which stages a cancer from I to IV. The
number represents the extent to which the cancer has spread, with stages I-III representing one to three cancerous lymph nodes, respectively. Stage IV marks disseminated disease that has spread into secondary organs away from the main sites of disease. Depending on the disease staging at the time of diagnoses, the treatment regime differs: For example, for patients with non-Hodgkin’s lymphoma stage I/II, the clinical protocol calls for a combination therapy of Rituximab, cyclophosphamide, vincristine, doxorubicin, and prednisone (R-CHOP) for 3-4 cycles. In the advanced stages III and IV, R-CHOP is administered for 6 cycles, sometimes with involved-field radiation therapy (IFRT). In cases of relapse, the patient may be administered platinum based chemotherapy, radio-immunotherapy, and higher doses of previous chemotherapeutics.

It is clear that novel and innovative therapeutic strategies are needed to increase the survival rates as well as to mitigate the adverse side effects associated with many of the treatment regimes described above. Key strategies include to device methods to lower the effective dose of systemically administered toxic drugs – this can be achieved through targeted drug delivery strategies thereby increasing the partitioning of the drug to the site of the disease.

One avenue of disease-specific drug targeting is found in the application of antibodies, and more specifically antibody-drug conjugates (ADCs). Antibodies can be selected to virtually any disease target; the antibody itself can be therapeutic or can carry a therapeutic cargo. Drug-targeting my means of antibody target-antigen specificity holds great potential for cancer therapy. Tumor neoantigens have been discovered allowing the selection of target-specific antibodies, therefore allowing drug targeting. Immunotherapies are gaining momentum not only in clinical trials, but such biological
therapies have become a clinical reality. A notable example is the monoclonal antibodies therapy rituximab. Rituximab targets CD20, which is a protein expressed on most non-Hodgkin’s B-cell lymphoma. The CD20 target is not normally found in circulation, therefore making this therapy highly specific. Rituximab is the standard for patients diagnosed with non-Hodgkin’s B-cell lymphoma, yet the survival rates are only 58%, indicating there is still room for improvement.[6]–[8]

Another avenue toward targeted therapies is the development of nanoparticles. Nanoparticles typically measure between 10-500 nm and are thus small enough to efficiently navigate circulation, traffic through tissues and target and enter cells.[9]–[10] Nanoparticles are larger than antibodies and offer multivalency; i.e., while an IgG antibody offers two binding sites for antigen binding, a nanoparticle has the potential to bind to multiple hundred-to-thousands of binding sites. The multivalency provides a mechanism to increase target specificity through added avidity effects. Furthermore, multifunctional designs are possible, where toxic payloads and/or contrast agents are loaded into the nanoparticle while targeting ligands enable tissue-specific delivery with increased payload delivery.[11]–[13]

Nanoparticles take advantage of their size and shape to gain increased uptake into tumor vasculature. Rapid angiogenesis occurs to supply the tumor with nutrients and oxygen and support the increased growth – as a result the neovasculation is leaky with a porous endothelium. This leaky vasculature with pores at the nano-to-micron size, create the perfect entry ways for nanoparticles to enter the tumor.[9],[14] Simultaneously, the microenvironment created by the angiogenesis causes local compressive forces, which in turn lead to poor lymphatic drainage. This effect is known as the enhanced permeability
and retention effect (EPR). Simply by flowing through the bloodstream, the nanoparticles are likely to extravasate into the tumor, and stay in that environment due to the EPR effect. Some nanoparticles, such as doxil, a liposomal formulation of doxorubicin, have been clinically approved for treatments in ovarian cancer, AIDS-related Kaposi sarcoma, and multiple myeloma.\textsuperscript{[15]} Nevertheless, while the research development pipeline is moving rapidly, nanoparticle therapies have not yet made it into the standard of care for non-Hogkin’s-lymphoma.\textsuperscript{[16]} Of course, the EPR effect does not hold count for blood cancers such as non-Hogkin’s-lymphoma. Alternative methods must be developed to target potent therapies to this disease.

Toward this goal, in this work, I describe a first study of the development and testing of an anti-mitotic soft matter virus-based nanoparticle (VNP) using the nucleoprotein components of the tobacco mosaic virus (TMV, Figure 1). TMV is a plant virus that has several properties that it a favorable nanoparticle choice for biomedical applications. TMV offers a stable, monodisperse, and biocompatible protein-based scaffold.\textsuperscript{[17],[18]} The TMV structure is known to an atomic resolution, this allows the biomedical engineer and chemist to identify specific reactive groups to be targeted for bioconjugation of medical cargo such as drug or contrast agents and display of targeting ligands or shielding molecules.\textsuperscript{[17]} To be specific, TMV is a 300 nm by 18 nm rigid hollow nanotube with a 4 nm-wide interior channel (see Figure 1). The rod shaped, soft matter nanoparticle consists of 2130 identical coat proteins. The reactive groups on the exterior or interior channel are known: with tyrosine 139 on the exterior, and glutamine 97 and glutamine 106 on the interior. Furthermore, genetic engineering, allows the introduction of new functionality. One example of this is the lysine-added mutant ‘TMV-
lysine' that displays reactive lysine groups on the exterior protein shell, and is easily modifiable using well known N-Hydroxysuccinimide (NHS) chemistry (TMV-lys, Figure 1). To conclude, TMV offers a modifiable, uniform, and organic nanoparticle scaffold to build a therapeutic nanoformulation.

Figure 1) Structure of tobacco mosaic virus (TMV). Chimera image showing the 300 nm by 18 nm rod with top and side views. Coat protein subunit in lower right highlights the reactive groups, glutamines on the interior, and lysine on the exterior.

In a previous study, that I co-authored, we observed that TMV was taken up by the cervical cancer cell line HeLa; uptake was by endocytosis targeting the TMV-based nanoparticle into the endolysosomal compartment. In this low pH environment with highly reactive protease and hydrolase, the particles were left intact over the time course studied (1 week), but the chemically bio-conjugated molecules on the exterior and interior were rapidly cleaved. Based on these previous findings, I hypothesized that we could make use of the natural cancer cell uptake and fate of the TMV carrier to deliver an
anti-mitotic drug cargo that could be activated and released within the cellular endolysosome. Therefore, when in the conjugated form the drug would be rendered non-active and therefore safe – and would only induce toxicity upon cancer cell targeting and activation.

I selected the anti-mitotic drug: vcMMAE, valine-citrulline monomethyl auristatin E (see Figure 2). vcMMAE consists of a lysosomally cleavable dipeptide, valine-citrulline, and the anti-mitotic agent, MMAE. Once cleaved, the MMAE drug is cytotoxic through inhibiting the polymerization of tubulin, which stops the cell from replicating.\textsuperscript{[20]}

Thus far VNP technologies have primarily focused on the delivery of drugs targeting solid tumors— as a new direction, I set out to develop these soft matter nanoparticles for applications targeting lymphoma and delivering vcMMAE.
Methods:

Propagation and Purification of TMV-lysine

TMV-lysine (TMV-lys) is a genetically modified TMV where reactive lysine groups were introduced on the ends of the coat proteins.\cite{21} TMV-lys was propagated using *Nicotiana benthamiana* plants (a tobacco plant species).\cite{22} The virus was isolated via established purification procedures yielding up to 100 milligrams of TMV-lys per 100 grams of infected tobacco plant leaves.\cite{22}

UV/VIS Spectroscopy:

A Thermo Scientific NanoDrop 2000 Spectrophotometer was used to measure the concentration of TMV using the TMV specific extinction coefficient (1.36 M\(^{-1}\) cm\(^{-1}\)), the known path length (0.1 cm\(^{-1}\)) and the Beer-Lambert equation. The Beer-Lambert law is defined as \(A = \varepsilon \cdot d \cdot c\), where \(\varepsilon\) is the extinction coefficient, \(A\) is the absorbance measured at a defined wavelength, \(d\) is the path length, and \(c\) is the concentration. To solve for concentration we rearrange the equation as \(A/(\varepsilon \cdot d)\).\cite{23}

Chemical Conjugation:

TMV-lys was reacted with 10 molar excess of N-succinimidyl-S-acetylthiopropionate (SATP) (Thermo Fisher) with 10% (v/v) dimethyl sulfide (DMSO) in 10 mM potassium phosphate buffer (pH 7.0). The resulting TMV-SATP complex was purified via ultracentrifuge, reacted with 10% deacetylation solution (v/v), (5 M
Hydroxylamine, 25mM EDTA in PBS, pH 7.4), for two hours, and then purified with a PD-10 desalting column (GE healthcare life sciences). Next, the TMV-SATP solution was reacted with 10 molar excess of vcMMAE (Med Chem Express) overnight and purified via ultracentrifuge. Yields after ultracentrifuge purification range from 70%-90%, and yields after desalting column range from 30%-50%.

**SDS-PAGE:**

Coat proteins were analyzed using 4-12% NuPAGE gels (Invitrogen) using 1x (N-morpholino) propanesulfonic acid (MOPS) running buffer (Invitrogen). 20 µg of protein with 4X loading LDS dye was added, and denatured through heating at 100°C for 5 minutes. After separation, the gel was first placing the gel in destain solution (10% acetic acid, 50% methanol, and 40% H₂O) for 30 minutes. 10 mL of the used destain solution was then diluted with 40 mL H₂O and 50 µL of Coomassie Blue R250. The gel was placed in this solution for 30 minutes to stain for protein, then immediately photographed using an AlphaImager (Biosciences) imaging system.

**Transmission electron microscopy (TEM)**

2 µL drops of 0.1 mg/mL TMV-vcMMAE in H₂O was placed onto TEM grids and allowed to dry. The grid was then washed in DI water, and stained with 2% (w/v) uranyl acetate for two minutes. After drying, samples were examined by Dr. Pitek using a Zeiss Libra 200FE transmission electron microscope operated at 200 kV.
**Cell culture:**

Karpas 299 cells (ATCC) were maintained in RPMI-1640 at 37° C in a 5% CO₂ humidified atmosphere. The medium was supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS), and 1% (v/v) penicillin–streptomycin. All reagents were obtained from Gibco.

**Cell viability assay:**

Cells at 10,000 cells/mL were seeded in a sterile, tissue culture-treated, 96-well clear bottom plate for 24 hours at 37° C in a 5% CO₂ humidified atmosphere. Cells were then incubated with fresh media with 0.02 nM to 200 nM VcMMAE for 72 hours. At 92 hours, Alamar Blue (ThermoFisher) was added, and cell viability was measured at 96 hours according to the manufacturer’s instructions using a fluorescence plate reader with emission at 540 nM and excitation at 610 nM.

**Confocal Microscopy**

Karpas 299 cells were seeded at a density of 500,000 cells/well in a 96 well plate in fresh RPMI and incubated with TMV at 1,000,000 particles/cells for 8 hours at 37°C in a 5% CO₂ humidified atmosphere. Cells were spun down at 500 g and washed two times with CELL buffer (0.1 mL 0.5 M EDTA, 0.5 mL fetal bovine serum, 1.25 mL 1 M HEPES pH 7.0, 48.15 mL PBS), and then fixed using 2% (v/v) paraformaldehyde in CELL buffer, then washed an additional two times in CELL buffer. Cells were washed in PB buffer.
(0.2% (v/v) Triton X-100 in DPBS) twice, and incubated with rabbit anti-TMV (1:500) (Pacific Immunology) and mouse anti-human LAMP-1 (1:500) (Sigma Aldrich) in CELL buffer for an hour. The cells were then washed two more times with PB buffer and spun down on cover slips at 2,000 rpm for 5 minutes. Then the cells were incubated with secondary antibodies using AlexaFluor 488-labeled goat anti-mouse antibody and AlexaFluor 555 anti-rabbit antibody (Invitrogen) in PB with 5% (v/v) goat serum for one hour. Lastly, the slips were washed two more times with CELL buffer, and then stained with DAPI (Sigma Aldrich) and imaged at 40X on an Olympus Fluo-ViewTM FV1000 LSCM microscope.
Results and Discussion

TMV-lys was first propagated in *N. benthamiana* plants. TMV-lys is a lysine-added mutant of TMV which displays a lysine residue instead of a serine at amino acid position 158; the amine-functional lysine group is therefore solvent-exposed and located at the C-terminus of the coat protein (see Figure 1).\(^{[24]}\) TMV-lys was extracted in yields of 1 mg of pure virus per gram of infected leaves using established virus purification techniques.\(^{14}\)

To obtain vcMMAE-loaded TMV, the anti-mitotic drug was bioconjugated using a three-step reaction (Figure 2). First, the amine handle of the TMV-lys was converted into a acetate-protected sulfhydryl group using the bi-functional linker N-succinimidyl-S-acetylthiopropionate (SATP), using a ratio of SATP:TMV of 15:1 (Figure 2). The reaction was purified via ultracentrifugation over a 40% (w-v) sucrose cushion. The TMV-SATP ending sulfhydryl group was then deprotected using hydroxylamine and purified using a PD-10 desalting column, yielding a free thiol handle for conjugation with the maleimide-terminated vcMMAE through thiol-Michael reactions; using a ratio of vcMMAE:TMV of 10:1. The reaction mix again was purified by ultracentrifugation over sucrose cushion and the final formulation was characterized by UV-VIS, SDS-gel, and TEM (Figure 3).
Figure 2) Chemical structures and bioconjugation reaction. A) Bioconjugation scheme steps: First TMV-lysine reacts with SATP via NHS chemistry, next TMV-SATP’s thiol group is deprotected using hydroxylamine, followed by reaction of TMV-SH b with the maleimide group of vcMMAE, resulting in TMV-MMAE. B) Chemical structure of vcMMAE.
UV/VIS spectroscopy was conducted to analyze the amount of protein recovered. Reactions starting with 5 mg of TMV-lys resulted in yields of approximately 3.7 mg of TMV-SATP, and yields of 1.3 mg of TMV-vcMMAE. This means that 75% was recovered in step 1 and only 35% of particles were recovered in step 2.

The particles were characterized after each conjugation step using SDS-PAGE (Figure 3). Using this technique, the TMV particle is denatured and disassembled into its coat proteins, which are analyzed by electrophoresis through a polyacrylamide matrix. Using this method, the coat proteins are separated by electrophoretic mobility based on molecular weight. Differences in the bands pattern indicate differences in the molecular weight of the various compositions of coat protein (CP) vs. CP-SATP vs. CP-vcMMAE. The band pattern reveals the molecular weight of the CPs and therefore indicates whether the drug cargo is attached.

The TMV coat protein measures 17.5-kDa and is detectable on the SDS-PAGE (Lane 1, Figure 3). A small percentage of coat protein dimers are also detectable at ~ 40-kDa molecular weight standard. The dimers are a results of inter-coat protein crosslinking based on disulfide bridges. Shifts in the band pattern indicate successful conjugation of SATP and the drug vcMMAE (Figure 3 AB). The SATP linker has a molecular weight of 245.25 g/mol; a negligible shift toward higher molecular weight bands indicates successful conjugation, however, it should be noted that the SDS PAGE methods does not provide the resolution to resolve the small difference in the molecular weight change of 0.5 kDa comparing the CP vs. CP-SATP. The drug, vcMMAE, has a molecular
weight of 1316.64 g/mol; a shift towards the higher molecular weight band indicates that conjugation was successful, and the CP-vcMMAE band is detectable at 20 kDa.

The SDS-gel displayed distinct bands comparing lane 1, TMV, and lane 5, TMV-vcMMAE, indicating that a higher molecular weight complex was present. Densinometric analysis conducted using ImageJ showed ~100% band separation. The uniform shift of the band indicates complete conversion of the amines to thiols, followed by drug loading, i.e. the SDS-PAGE indicates that each of the 2,130 available sites were labeled with the therapeutic cargo (Figure 3B).

Finally, transmission electron microscopy (TEM) revealed that the TMV-vcMMAE formulations remained intact; imaging of negatively-stained TEM grids showed structurally sound TMV nanoparticles with dimensions of 300x18 nm (Figure 3C). TEM images showed that the TMV rods were intact and non-aggregated after the bioconjugation and purification processes.

To conclude the synthesis of the vcMMAE-conjugated TMV: I have developed a protocol allowing efficient drug loading to TMV. The chemical reaction reached completion with each of the 2,130 coat proteins being modified with a drug molecule. On a %-molecular weight basis, this translates to a drug loading efficiency of ~7% wt per TMV. This is comparable to immunotherapies which have drug loading of up to ~7% wt per IgG, and up to ~10% wt per diabody in antibody conjugated MMAE studies. Nevertheless, antibody-drug conjugates are limited to only carrying up to 8 drug molecules per antibody, which is far fewer than the load which TMV can carry, namely ~2,000 copies of the payload.
Next, I set out to study the *in vitro* properties of the drug candidate using Karpas 299 cells, a human derived non-Hodgkin’s large B cell lymphoma. First, I studied the fate of the TMV formulation in these cells, and second, drug efficacy.

A cell uptake and colocalization study was carried out to evaluate whether TMV would target and be taken up by Karpas 299 cells, and whether the particles would be
targeted to the endolysosomal compartment, as I have reported in my previous paper.\textsuperscript{12} First, Karpas 299 cells were incubated with TMV for 8 hours using one million TMV particles per cell. Then the cells were washed to remove excess TMV, followed by staining of TMV using TMV-specific antibodies and secondary dye-labeled antibodies. At the same time, the endolysosome was stained using LAMP-1 staining. The cells were then fixed, mounted and imaged by fluorescence microscopy (Figure 4).

As seen in Figure 4, the red fluorescent antibodies tagging TMV overlap with the green antibodies tagging the endolysosome. Thus the confocal microscopy imaging confirmed that the Karpas 299 cells uptake TMV, and that the nanoparticles traffic to the endolysosome. Based on these findings, I hypothesize that the TMV-vcMMAE would also be taken up by the Karpas 299 cells targeting the endolysosome. The vcMMAE drug is designed to be activated and cleaved within the endolysosomal compartment. Specifically the valine-citruline (vc)-linker is a protease-specific cleavable linker, resulting in release of the active MMAE component.\textsuperscript{12}
Figure 4) Karpas 299 Cell Interactions with TMV. DAPI: DAPI stain showing the nuclei in blue. Lamp1: Endolysosomes stained with mouse anti-human Lamp-1 antibody, and secondary Alexa Fluor 488 goat anti-mouse antibody in green. TMV: TMV particles stained with primary rabbit antibody alpha-TMV, and anti-rabbit-555 in red. Overlay: All three labels simultaneously shown, with the colocalization of TMV and the endolysosome in yellow.

To evaluate the efficacy of TMV-vcMMAE, cell viability assays were performed using Alamar Blue assay. Alamar Blue reagent is primarily made of resazurin, a non-toxic cell permeable non-fluorescent blue molecule. Upon entering viable cells, resazurin is reduced to resorufin, which is a bright red fluorescent molecule.²⁶,²⁷
Karpas 299 cells were seeded for 24 hours and then incubated with free vcMMAE or TMV-MMAE for 72 hours. At 92 hours, Alamar Blue was added and the cells fluorescence was measured after 4 hours of incubation at 540 nm excitation and 610 nm emission. The results for both free vcMMAE and TMV-MMAE are plotted in Figure 4, and the IC$_{50}$ values were calculated. The IC$_{50}$ value defines the drug concentration where half of the cells are killed. The IC$_{50}$ for free vcMMAE was determined to be about 25.8 nM, and the IC$_{50}$ for TMV-MMAE was found to be 256.1 nM (Figure 5).

![Karpas 299 Dose Response](image)

**Figure 5) Cell viability assay.** Cell viability assay using Karpas 299 cells after treatment with vcMMAE (purple) and TMV-vcMMAE (blue). Cell viability was determined after 72 hours using Alamar Blue assay. Error bars were calculated by standard deviation (experiments were done in triplicates). Data was analyzed and graphed using Prism® v6.0b software.
IC₅₀ values of the free vcMMAE drug vary in the literature depending on the set up of the assay, e.g. time course and cell lines used, and are found in the low nanomolar range at around 5 nM. IC₅₀ values of antibody drug conjugates are generally found ranging from 2 nM to 42 nM. In our results we see a similar trend, with a low IC₅₀ of the free drug, and an order of magnitude higher IC₅₀ using our VNP loaded with drug. The lower IC₅₀ values of the nanoparticle conjugates may be explained by lower cell uptake kinetics, as has been reported with other systems. Nevertheless, I hypothesize that the nanoparticle-assisted targeted drug delivery approach will outperform the in vivo profiles of the free drug through enhanced biodistribution and pharmacokinetics.
Conclusions and Future Directions:

I demonstrated that TMV could be loaded with a high payload of vcMMAE, with all of its coat proteins labeled with drug for treatment of lymphoma. The particles are taken up by cancer cells and are targeted to the endolysosomal compartment, where the drug is then cleaved from the VNP. I found that TMV-delivered vcMMAE was effective in killing cancer cells in vitro and demonstrated an IC$_{50}$ in the nanomolar range. We found that the TMV-vcMMAE complex had a higher IC$_{50}$ value than free vcMMAE. One explanation why the efficacy of the viral nanoparticle may be lower is due to lower cell uptake kinetics – future experiments could assess this through time course studies.

One of the next steps for the further development and optimization of the TMV-vcMMAE therapeutic would be to give the particles selectivity. This can be achieved through conjugation of targeting peptides or antibodies. For example, direction the drug-loaded nanoparticles with specificity for markers of diffuse large B cell lymphoma is expected to increase cell uptake rates and selectivity. CD20, CD22, and CD40 have been identified as candidate targets and nanoparticle formulations with selectivity for these targets are under development for treatment of B-cell lymphomas.$^{[30],[31],[40],[32]}$

Antibodies with specificity toward these markers are also in development, with some formulations already being used in the clinic for treatment of lymphoma. However, antibodies are limited in only being able to carry up to 8 drug molecules. TMV provides advantages, because this nanoparticle formulation can carry more than 2,000 drugs on its exterior. Furthermore, the interior channel can also be used for conjugation of drugs or contrast agents – toward the development of theranostics. This high number of reactive sites opens up greater flexibility compared to antibody drug conjugate therapies. Bio-
conjugation of antibodies to the TMV-vcMMAE formulation is expected to be a powerful tool for targeted drug delivery targeting Non-Hodgkin’s lymphoma.

Another step for further development of the TMV-vcMMAE therapeutic would be to give the particles shielding in order to increase circulation time. Unlike in vitro models, animal and human models have immune systems that clear out foreign particles. In in vivo studies, unconjugated TMV has been seen to be cleared by the immune system within 25 minutes. Attaching polyethylene glycol (PEG) polymer chains to the exterior of nanoparticles is a common method of shielding against immune recognition. In one viral nanoparticle, potato virus X, branched PEG was conjugated to the exterior and the particles saw increased pharmacokinetic profiles and reduced immune recognition. However, since PEG is also commonly used in commercial uses such as skin creams and toothpastes, some anti-PEG antibodies have been found. When present, these antibodies cause the PEG shielding to be ineffective, since the PEG itself would cause an immune response. An alternative candidate for shielding is serum albumin, a protein found in human plasma. TMV coated with serum albumin (SA) was observed to have better camouflaging from the immune system than TMV-PEG. Since these TMV-SA coatings were highly effective in reducing antibody recognition in vivo, bioconjugation of SA to TMV-vcMMAE would likely be a promising method for shielding the therapeutic from the immune system in order to increase the circulation time.

A final viral nanoparticle therapeutic is likely to involve all of the different conjugation proteins discussed. The high number of functional sites on TMV provides ample conjugation locations for attaching a toxic drug load, shielding, targeting proteins, and imaging agents. Future studies will reveal the efficacy and optimal formulations for
each area. In this manuscript, I described the first stage of development—the TMV-vcMMAE complex. The current therapeutic proved effective in an *in vitro* model, but future steps in immune competent models will require additional targeting and shielding functionalization. Antibodies and serum albumin are auspicious candidates for developing the viral nanoparticle in those respective areas. In conclusion, there are many promising avenues for the further development of virus-based nanoparticle therapeutics targeting soft-cancer applications.
Appendix


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Interface of Physics and Biology: Engineering Virus-Based Nanoparticles for Biophotonics

Amy M. Wen, Melissa Infusino, Antonio De Luca, Daniel L. Kern, Anna E. Czaplarska, Giuseppe Strangi, and Nicole F. Steinmetz

ABSTRACT: Virus-based nanoparticles (VNP)s have been used for a wide range of applications, spanning basic materials science and translational medicine. Their propensity to self-assemble into precise structures that offer a three-dimensional scaffold for functionalization has led to their use as optical contrast agents and related biophotonics applications. A number of fluorescently labeled platforms have been developed and their utility in optical imaging demonstrated, yet their optical properties have not been investigated in detail. In this study, two VNPs of varying architectures were compared side-by-side to determine the impact of dye density, dye localization, conjugation chemistry, and microenvironment on the optical properties of the probes. Dyes were attached to icosahedral cowpea mosaic virus (CPMV) and rod-shaped tobacco mosaic virus (TMV) through a range of chemistries to target particular side chains displayed at specific locations around the virus. The fluorescence intensity and lifetime of the particles were determined, first using photochemical experiments on the benchtop, and second in imaging experiments using tissue culture the samples. The virus-based optical probes were found to be extraordinarily robust under ultraviolet, pulsed laser light conditions with a significant amount of excitation energy, maintaining structural and chemical stability. The most effective fluorescence output was achieved through dye placement at optimized densities coupled to the exterior surface avoiding conjugated ring systems. Lifetime measurements indicate that fluorescence output depends not only on spacing the fluorophores, but also on dimer stacking and conformational changes leading to radiationless relaxation—and these processes are related to the conjugation chemistry and nanoparticle shape. For biological applications, the particles were also examined in tissue culture, from which it was found that the optical properties differed from those found on the benchtop. Data indicate that fluorescent cargos are released in the endosomal compartment of the cell targeted by the virus-based optical probes. These studies provide insight into the optical properties and fates of fluorescent proteinaceous imaging probes. The cellular release of cargo has implications not only for virus-based optical probes, but also for drug delivery and release systems.

INTRODUCTION

Fluorescent nanomaterials including nanostructures formed by viruses and protein cages have become versatile tools as photonic materials for a variety of applications, such as sensing, light harvesting, and optical imaging. Virus-based nanostructures are self-assembling systems that are highly symmetrical, dynamic, polyvalent, and monodisperse, rendering them one of the most advanced nanomaterials produced in nature. The proteinaceous capsid’s function is to protect the nucleic acid cargo; hence, their structures are extremely robust. Many structures have been solved to near-atomic resolution, allowing chemists, engineers, and physicists to tailor materials with atomic precision. For example, structure-based engineering allows the placement of metals with spatial control at the atomic level through genetic control, yielding unique plasmonic nanomaterials. The propensity to self-assemble into higher-order structures is another interesting feature, for example, hybrid virus-like nanoparticles encapsulating gold nanoparticles inside the virus shell were shown to crystallize into lattices exhibiting properties of plasmonic metamaterials. The structure of viral nanoparticles (VNPs) can be modified in several ways to allow for the loading of photonic and plasmonic materials and the modification of plasmonic properties, including the internal cavity, conjugated structures, or surfaces. Within the medical sector, virus-based probes combined with optical dyes are frequently used to study biodistribution and to evaluate cellular internalization and localization. Fluorescently labeled virus-based materials can also be used in drug delivery and release systems.

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Fluorescent Labeling of CPMV and TMV. The surface chemistry of CPMV and TMV is well understood. The 30-nanometer-sized CPMV displays 300 addressable lysine side chains per virus particle (Figure 1A), all of which are addressable using N-hydroxysuccinimide (NHS)-activated esters at large molar excess and overnight incubation. TMV forms a hollow 100-nanometer tube measuring 300 × 18 nm with a 4-nanometer-wide interior channel. Its surface chemistry inside and out is well established. Native TMV contains solvent-exposed and addressable interior glutamic acids, Glu97 and Glu106 residues, which can be modified using carbodiimide coupling reactions. The exterior surface contains a solvent-exposed tyrosine side chain, Tyr139, which can be targeted and functionalized using diaminobis coupling reactions. For our studies, we also considered a 199 lysine mutant, TMV199, which displays a genetically encoded lysine residue that replaces threonine at amino acid position 199. TMV199 consists of 2130 identical copies of its 192-residue coat protein, which means that overall TMV displays 4260 199 solvent-exposed glutamic acids on the interior of the particle and 2130 addressable tyrosines on its exterior surface; the 195 TMV199 mutants offers an additional 2130 surface-exposed 199 lysine side chains on the exterior particle surface (Figure 1B). CPMV was labeled with cyanine dye sulfo-Cy5 using NHS-activated esters targeting surface lysines (Figure 1C). CPMV was incubated with NHS-Cy5 at various molar excesses to yield CPMV-Cy5 conjugates with varying densities of dye per particle. After completion of the reaction, the resulting CPMV-Cy5 conjugates were purified by dialysis using 10-kilodalton centrifu- 232 gal filter units. The conjugates were characterized using a combination of UV–vis spectroscopy, native and SDS denaturing gel electrophoresis, and transmission electron microscopy (TEM).

UV–vis spectroscopy was used to determine the degree of labeling. Absorbance was measured and the Beer–Lambert law was applied.
Figure 2. Lifetime and fluorescence characterization of CPMV-Cy5 formulations. (a) Fluorescence lifetime decay measurements as a function of dye number for CPMV-Cy5. (b) Fluorescence intensity measurements normalized for dye concentration (left) and protein concentration (right).

Figure 3. Lifetime and fluorescence characterization of TMV-Cy5 for different formulations. (a, c, e) Lifetime decay measurements as a function of dye number for TMV-Cy5, TMV-eCy5, and TMV(tam)-Cy5, respectively. (b, d, f) Fluorescence intensity measurements normalized for dye concentration (left) and protein concentration (right) for TMV-Cy5, TMV-eCy5, and TMV(tam)-Cy5, respectively.

and the fluorophore- and CPVM-specific extinction coefficients were used to determine the number of dyes per particle formulation. Molar excesses ranging from 1000 to 8000 dyes per particle were used to obtain formulations with 8, 20, 27, 40, 48, 55 scy5 per CPVM particle (Supporting Information Figure S1). Native and denaturing gel electrophoresis confirmed covalent attachment of the dyes, and TEM imaging confirmed that the particles remained structurally sound after chemical modification (see below).

We next turned to TMV and its mutant TMV(tam), which offer attractive platforms to evaluate how spatial dye placement, conjugation chemistry, and microenvironment would affect the fluorescence properties of the nanoparticle probes. The conjugation with scy5 was achieved using a two-step protocol: first, alkyne ligation handles were introduced, and second, copper-catalyzed azide–alkyne cycloaddition (“click” chemistry) was carried out to introduce scy5 azide. To decorate the interior surface, a terminal alkyne was incorporated into the interior channel of TMV by targeting glutamic acid residues designated TMV-eAlk. To decorate the exterior TMV surface, tyrosine residues were targeted with the diazonium salt generated from 3-ethylsalicylic acid to yield TMV-eAlk. Similarly, to target exterior lysine residues using the TMV(tam) mutant, an NHS-active ester was used to introduce alkynes to yield TMV(tam)-eAlk. The reaction schemes are shown in Figure 1D–G.

Alkyne labeling was carried out under forcing conditions, i.e., a large molar excess of alkyne to TMV and/or overnight incubation, to yield maximum conversion of the carbonyl group in hydroxylalkyl ring or amine functional groups into alkynes. In brief, 25 molar excess of propargylamine per coat protein was reacted using EDC coupling overnight to produce TMV-eAlk.
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35 equiv of ethylenediamine diurea salt was reacted for 30 min to yield TMV-alk, and 10 mol excess of NHS-alkyloxyacrylate was reacted overnight with TMV-alk, to form TMV-alk-NH-alk. Citrate conjugates to form the TMV nanoparticles. TMV-alk-NH-alk, TMV-alk-NH-alk-NH-alk, and TMV-alk-NH-alk-NH-alk were incubated with scy5., tert, and TMV-alk-NH-alk-NH-alk conjugates with varying densities of dye per particle. After completion of the reaction, the resulting TMV-alk-NH-alk-NH-alk conjugates were purified by ultrafiltration.

UV-vis spectroscopy, native and denaturing gel electrophoresis, and transmission electron microscopy (TEM) (see below).

As in the case of CPMV, UV-vis spectroscopy was used to determine the degree of labeling, with the minor adjustment of using TMV-specific extinction coefficients to determine the number of dyes per particle formulation. For TMV-alk-NH-alk-NH-alk, dye excesses from 0.2 to 6 dyes per protein (420 to 12,780 per particle) were used to obtain 68, 97, 222, 399, 403, 498, and 525 scy5 per TMV particle; for TMV-alk-NH-alk-NH-alk, dye excesses from 0.2 to 6 dyes per protein resulted in 124, 164, 324, 396, 509, and 633 scy5 per particle. For TMV-alk-NH-alk-NH-alk, dye excesses ranging from 0.02 to 2 scy5 per protein were used to obtain 81, 165, 234, 302, and 365 dyes per particle.

Fluorescence Properties of CPMV- and TMV-Dye Conjugates. To evaluate the photophysical properties of CPMV and TMV-dye conjugates, steady-state and time-resolved fluorescence measurements were carried out (Figures 2 and 3).

Fluorescence spectra of CPMV-alk-NH-alk-NH-alk with dye loading of 8 and 85 dyes per CPMV were compared (data were normalized for dye content). The sample with highest fluorescence intensity (FI) was the sample with highest dye content. CPMV displaying 27 scy5 dyes gave a fluorescence reading of 4760 cts, which is over 2.5 times the fluorescence intensity of 1795 cts per CPMV with 55 dyes at the same particle concentration. This indicates that maximum fluorescence intensity is achieved at sparse labeling with ~30 dyes per particle, as the dye density increases. Fluorescence quenching occurs, reducing the overall emission intensity. It is well-known that fluorescent molecules in close proximity (< ~10 nm) undergo interprotein interactions resulting in a decrease of their emission intensity. A reduction in the emission intensity of the fluorescent labels is observed, and this is significantly correlated with the partial overlap of their absorption and emission curves.24-26 This phenomenon is referred to as Förster resonant energy transfer (FRET) and involves excited donor energy transfer to an excited acceptor electronic state, which may transfer its excitation energy to another acceptor chromophore in a nonradiative way through long-range dipole–dipole interactions. FRET processes are associated with the observation of fluorescence quenching and red shifts in the emission decay times. It is also known that FRET strongly depends on the distance between donor and acceptor molecules and scales with R-6, where R is the molecular distance. Increasing the number of scy5 dye molecules per CPMV results in a decrease of the distance between dye molecules and consequently leads to stronger dipolar coupling between them (see Supporting Information Figure S2). The spacing between dye molecules was calculated based on the average distance between each dye's nearest neighbor in multiple simulations where the dyes were randomly positioned on the available lys side chains (the coordinates were determined from the structure of CPMV, which is available at viderb.scripps.edu, file IYNT).

The proximity of excitons is commonly considered to be responsible for fluorescence quenching. While the proximity does contribute to the overall optical properties of CPMV-alk-NH-alk-NH-alk based probes, additional factors must be considered. In particular, the experimental evidence suggests that three radiationless processes govern the disposition of the electronic excited state energy in this type of system: (i) energy transfer between dye molecules, (ii) trapping by dimers, and (iii) radiationless relaxation of the dye excited state. Along with the intrinsic spectral overlap between absorption and emission bands, the creation of the effective FRET.

In the case of CPMV, fluorescence intensity measurements show an initial monotonic increase in emission as a function of the dye molecules for CPMV, and the maximum value is obtained for 27 dyes/CPMV. At this density, the average interdye separation is estimated to be about 8 nm and the lifetime decay was measured to be longer than 1 ns, indicating the absolute absence of coupling between molecules. Above this level of loading the average separation distance between dye molecules becomes short enough to induce strong molecular coupling and to trigger FRET processes, causing a reduction in the emission. This was accompanied by a reduction of the fluorescence decay time by 43%, confirming that dye coupling and energy transfer are behind this effect (see Figure 2). However, this fluorescence lifetime reduction is not compatible with the formation of trapped dimers, which usually show much shorter decay times (<100 ps). This indicates that dimers undergo rapid configurational changes, which enhance the population of radiationless relaxation rates.

With regard to the fluorescence, similar trends were observed when comparing the TMV samples: the underlying photophysical factors, however, differed. Each sample reached a plateau at a 275 specific dye-to-TMV ratio, and this was dependent on the spatial placement and independence of the chromophore processes. Specifically, for either of the externally labeled TMV samples, the maximum F1 was reached upon attachment of 165 scy5 dyes to either tyrosine or lysine side chains. On the other hand, much higher dye loading was required to reach a maximal fluorescence intensity (F1) of 774 cts for TMV-alk-NH-alk-NH-alk with 402 scy5 (Figure 3). When measuring the F1 value of the 165 exteriorly labeled particles with the same density of 165 dyes attached, we found that TMV-alk-NH-alk-NH-alk reached an intensity of 1616 cts, which was 4.5 times higher compared to the F1 value of 354 cts observed for TMV-alk-NH-alk-NH-alk. A discussion of a lower
quantum yield for TMV-eCy5 can be related to the formation of dimers. In fact, it is important to note that lifetime measurements show very short-lived excited states (τ ≤ 100 ps), indicative of dimer trapping, above 300 dyne·TMV. It is possible that the planar shape of the tyrosine residue results in 291 studying of the Cy5 dye on the high aspect ratio of the TMV structure, therefore resulting in dimer trapping. In contrast, steady-state fluorescence data for TMV-iCy5 and TMV-eCy5 show higher values of F1 with respect TMV-eCy5 (see Figure 3). Such evidence of the importance of conjugation chemistry is additionally corroborated by comparable decay times (τ ≥ 400 ps) for the systems showing higher F1.

It was interesting to note that stark differences were observed when comparing the various formulations. Normalized for particle concentration, the brightest CPMV sample (CPMV-eCy5 with 27 dyes) resulted in fluorescence intensities four times higher (FI = 4768 cts) than the brightest TMV sample (TMV-eCy5, FI = 1616 cts), despite both samples having comparable dye concentrations in the range of 0.2 to 0.3 mg/mL. For the brightest TMV samples, TMV-iCy5 had an intermediate FI about twice that of TMV-eCy5, while the brightest TMV sample across the board was TMV-eCy5 reaching a FI another factor of 2 times that of TMV-eCy5. These data indicate that not only spatial placement (inside versus outside), conjugation chemistry (see Figure 1), and dye density, but also nanoparticle shape and microenvironment dictate the fluorescence properties of (virus-based) nanoparticles. Time-resolved fluorescence measurements play a key role in gaining further insight in the processes behind the change of the radiative emission rate of the fluorophores. The measurements allow us to understand whether fluorescence quantum yield is affected by the chemical microenvironment or excited state energy transport occurs due to dipole–dipole interactions between the dye molecules, and the energy transport causes fluorescence depolarization effects while not affecting the fluorescence quantum yield.

The lowest F1s, accompanied by corresponding shortest fluorescence decay times (τ ≤ 100 ps), were observed when studying the TMV-eCy5 samples. This can be explained by the conjugation chemistry and the formation of dimers. In this case, the fluorophores were placed on the aromatic tyrosine residues via diazirine bonds, and electron delocalization in the conjugated ring systems induce a reduction of the spontaneous emission rate. It is generally more effective to conjugate dyes via nonaromatic systems to avoid quenching. On the other hand, the formation of dimers, as proven by the very short-lived states, represents a configuration with the fastest channel to release the excitation energy nonradiatively because of their extremely rapid conformational changes.

TMV-eCy5 reached higher F1s compared to those obtained for the TMV-iCy5; this may be explained by spatial localization: internal placement results in crowding of the dyes. The average distance of dyes in the TMV-iCy5 sample containing 402 dyes is 3 nm, compared to 8 nm for TMV-eCy5 and TMV-iCy5 with 165 dyes (see Supporting Information Figure S3). As 8 nm was also the interdye distance found for CPMV-Cy5 with 27 dyes, this is likely the optimal distance for Cy5 dyes spread along the exterior viral capsid wall. Instead, the rate of energy transfer is inversely proportional to the sixth power of the distance between the donor and acceptor. Therefore, the efficiency of the transfer rapidly declines to zero at distances larger than the Förster radius. Förster radii have been experimentally determined for each specific donor–acceptor pair, and the majority of fluorophore pairs fall within the 5–10 nm range. Since the interior channel of TMV is only 4 nm wide, decreasing the dye labels to 165 dyes for TMV-iCy5 only increases the distance between.
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356 dyes to about 3.6 mm. The balance between improving the 357 fluorescence per particle through less per particle to achieve 358 the amount of quenching and increasing the fluorescence per 359 particle by having more dye labels per particle is therefore 360 altered for TMV-s-Cys compared to the other particles. 361 Fluorescence lifetime data corroborates this explanation. In fact, 362 the decay times of both TMV-s-Cys and TMV-s-Cys 363 decrease linearly with the dye molecule number, whereas the 364 decay times of TMV-s-Cys shows a saturation effect by 365 reaching a plateau around 400 ps at a loading value of 222 dyes 366 per particle.

367 Overall, these data indicate that design principles beyond 368 intermolecular spacing must be considered to develop optical probes 369 with maximized fluorescent output: interdye spacing can be 370 calculated based on the averaged distance between each dye’s 371 nearest neighbors; dye-loading can be optimized through 372 adjustment of the conjugation protocol (excess reagents used 373 and incubation time). To avoid dimer trapping, dyes should be 374 placed to avoid crowding and/or attachment via conjugated 375 ring systems. To overcome losses due to configurational 376 interactions leading to enhanced radiationless relaxation rates, 377 one might consider alternative linker chemistries that may affect 378 the fluorophore in stringent chemical positions.

379 These quantitative data were correlated with results from gel 380 electrophoresis analysis of CPMV- and TMV-dye conjugates. 381 Samples with highest FI were compared to samples with higher 382 dye loading but lower FI. Specifically, CPMV and CPMV-sCys 383 with 17 and 35TMV, and TMV-sCys 384 e-sCys containing 165 and 365 dyes were analyzed by gel 385 electrophoresis. First, CPMV samples were separated using 386 native agarose gels (TMV cannot be analyzed under these 387 experimental conditions, and therefore only denaturing gel 388 analysis was performed). After electrophoretic separation of 389 intact CPMV in native agarose gels, the particles were visualized 390 under UV light as well as Maestro 2D fluorescence imaging 391 system with a yellow (635 nm) long-pass filter and then stained 392 with Coomassie blue followed by imaging under white light 393 (Figure 4A). The mobility of CPMV in the native gel increases 394 upon dye conjugation; the sCys dye is a negatively charged 395 molecule, so as more dyes are conjugated to CPMV, the more 396 negative the surface properties of CPMV (see also Supporting 397 Information Figure S4). This enhances the mobility of CPMV- 398 sCys toward the positively charged anode as a function of dye 399 loading. CPMV-sCys with 55 sCys molecules has the highest 400 mobility compared to CPMV-sCys with 27 sCys dyes, and 401 native CPMV has the slowest mobility. Analysis of the 402 fluorescence signal intensity (Figure 4A, heat map) showed 403 that CPMV-sCys with 27 sCys dyes has a higher fluorescence 404 intensity compared to its counterpart displaying 55 dyes per 405 particle, therefore further confirming the fluorescence lifetime 406 and intensity measurements (see Figure 2).

407 While native gels separate intact viral nanoparticles, in 408 denaturing gels the coat proteins are separated. CPMV consists 409 of 60 copies each of a small (24 kDa) and large (42 kDa) 410 protein, and 21,30 copies of a single 15 kDa protein form TMV 411 particles. Both the small and large coat proteins of CPMV are 412 detectable after electrophoretic separation and show the 413 expected sizes; it is apparent that fluorophores were conjugated 414 to both coat protein subunits. We hypothesize that the sCys 415 dyes are located around a fivefold axis of CPMV near the 416 interface of the small and large coat proteins where the most 417 reactive lysines (Lys358 and Lys99) are located (see Figure 4A). 418 (Lai, 19, 20 The protein concentration between each sample was 419 consistent, which is confirmed by comparable signal intensities 420 in the Coomassie-stained gels, and the lack of the fluorophore- 421 consistent signals is consistent with UV/vis quantification of dye loading. 422 As CPMV-sCys with 55 sCys dyes displays a higher dye per 423 protein density compared to CPMV-sCys with 27 sCys dyes, 424 since denatured proteins are intact and these gels and 425 quenching effects are lost (Figure 4B). Similarly, fluorescence 426 signal from denaturing gel electrophoresis of TMV samples also 427 indicates a higher dye-to-protein ratio for TMV samples labeled 428 with more dyes (a brighter signal is observed for TMV-sCys 429 e-sCys containing 365 dyes versus 165 dyes and TMV-sCys 430 is not fluorescent, Figure 4B).

Stability of Fluorescently Labeled CPMV and TMV. We 431 assessed the stability of CPMV and TMV conjugates, 432 specifically addressing the question of whether CPMV and 433 TMV-dye conjugates remain structurally sound and whether 434 the dyes would remain covalently attached during fluorescence 435 and lifetime measurements. After lifetime measurements, the 436 CPMV- and TMV-sCys conjugates were evaluated using 437 TEM and native gel electrophoresis (Supporting Information 438 Figure S4). The combination of methods provides insights into 439 the structural integrity as measured by direct TEM imaging, and 440 chemical stability as measured by detection and quantification 441 of the covalent modifications. After completion of electro- 442 phoretic separation, CPMV bands were visualized under UV 443 light. The electrophoretic mobility of the bands did not alter 444 before and after measurements, indicating that the particles 445 remain structurally sound and therefore only denaturing gel 446 analysis was performed. The samples analyzed, therefore indicating that chemical stability is 447 maintained. These data were in good agreement with TEM 448 analysis of the samples, with the CPMV and TMV samples all 449 found to be intact (Supporting Information Figure S4).

450 In addition, unlabeled CPMV samples were exposed to 451 various pulse and power settings using various laser beams, 452 system was tested to stability under optical excitation. Different parameters, such as pulse duration, energy per pulse, repetition rate, wavelength, and average power, were selected to mimic various scenarios relevant to photonics and plasmonic applications (future direction), detailed procedures are described in the Supporting Information (see also Supporting Information Figure S5 and S6). In short, both CPMV and TMV were found to be very 453 robust and stable; being able to endure significantly high 456 excitation energy fluence for a high range of wavelengths makes 458 them suitable candidates for biophotonic applications. Optical 459 excitation by means of trains of ultrashort pulses (about 100 fs) 460 represents a very strict physical requirement to understand 461 radiative and nonradiative mechanisms between excitonic 462 molecules. The virus particle stability has been investigated 463 under severe optical excitation upon varying several physical 464 parameters (wavelength, pulse duration, energy/pulse, and 465 repetition rate) with the precise aim to evaluate stability and 466 tolerances to be used for optical spectroscopies.

467 Fluorescently Labeled CPMV and TMV in Cell Imaging 468 Applications. We then set out to determine whether the 469 properties observed in the test tube would translate into cell 470 imaging studies. We continued with both CPMV and TMV to 471 additionally evaluate shape-dependent effects, specifically 472 choosing to study TMVp, since it had superior fluorescent 473 properties to internally and externally labeled wild-type 474 particles. Fluorescence and lifetime measurements indicated 475 that a CPMV formulation labeled at external lysine side chains 476
Figure 5. Cell interactions of CPMV-sCy5 and TMV<sub>x</sub>-sCy5. (a) Flow cytometry studies with mean fluorescence intensity from CPMV antibody staining with Alexa Fluor 555 secondary (striped bars) and from sCy5 labels on the particles (solid bars). (b) Flow cytometry quantifying the percent of cells positive for TMV uptake determined from TMV antibody staining with Alexa Fluor 555 secondary (striped bars) and from sCy5 labels on the particles (solid bars), obtained from the indicated regions in the histograms to the left. (c,d) Confocal imaging of HeLa cells showing cellular uptake of CPMV-sCy5 and TMV<sub>x</sub>-sCy5 after 3 h. Nuclei are shown in blue, endolysosomes stained with mouse anti-human Lamp-1 antibody and secondary Alexa Fluor 488 goat anti-mouse antibody are shown in green, and CPMV-sCy5 and TMV<sub>x</sub>-sCy5 are shown in red. Co-localization is shown in yellow, with associated Manders' coefficient as indicated. Scale bars = 20 µm.

with 27 sCy5 is brighter (FI ~ 4766 cts) compared to a 38 CPMV sample labeled at external lysines with 55 sCy5 (FI ~ 1795 cts). Similarly, TMV labeled with 165 sCy5 dyes on its 493 genetically introduced, external lysine side chain was brighter 496 (FI ~ 1616 cts) compared to TMV samples labeled with 365 497 sCy5 dyes conjugated using the same chemistry (FI ~ 539 cts). To 498 test the hypothesis that CPMV-sCy5<sub>27</sub> and TMV<sub>x</sub>-sCy5<sub>165</sub> 499 would outperform their counterparts with higher dye loading 500 (but reduced fluorescence intensity due to quenching), we 501 performed quantitative flow cytometry studies using HeLa cells;

these studies were complemented by confocal imaging studies 504 (Figure 5).

In brief, CPMV or TMV-based sensors were incubated with 490 HeLa cells at a concentration of 5 x 10<sup>3</sup> particles per cell 491 and were collected at 30 min and 3 h post incubation with CPMV 492 or TMV, fixed, permeabilized, and stained with anti-CPMV or 493 anti-TMV antibodies and fluorescently labeled secondary 494 antibodies. This allowed detection of the signals derived from 495 the fluorophores conjugated to the plant virus-based nano- 496
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particles as well as imaging of the nanoparticles through antibody staining.

In the case of CPMV, antibody staining indicated that the more dyes are conjugated, the more efficiently CPMV nanoparticles are being internalized by the cells, and this effect is more profound at longer incubation times (Figure 5A). It should be noted that flow cytometry does not differentiate between bound and internalized nanoparticles; however, cell uptake was confirmed by confocal microscopy (Figure SC). Increased cell uptake with increasing numbers of dyes was also observed when studying the TMV formulations (Figure 5B). Increased cell uptake as a function of dye-loading could be explained by Cy5 dye—cell membrane interactions. It may also be possible that altered surface charges contribute to the cell uptake efficiency; however, conjugation of the Cy5 dye contributes to an increased negative charge of the plant virus—based nanoparticles (e.g., see Figure 4); first, the Cy5 itself is negatively charged, and second, conjugation to the surface lysine residues reduces the positive charge contributions. Cell membranes are negatively charged, and therefore positively charged materials interact more strongly with cells.36–38 We hypothesize that the planar, hydrophobic structure of the Cy5 dye interacts directly with the lipid bilayer of the cell membrane to promote cell binding, which then may trigger endocytosis of the nanoparticles. We had previously observed that when potato virus X (PVX) nanoparticles are modified with PEG of a molecular weight of 2000 Da and Alexa Fluor 647, cell interactions are enhanced compared to non-PEGylated PVX. In contrast, cell interactions are reduced when PVX is labeled with the same PEG coating but displays Oregon Green 488 (instead of the Alexa Fluor 647 dye), suggesting the hypothesis that the fluorophore may play a role in mediating cell interactions and uptake.

Next, cell uptake (as determined based on antibody staining) was compared with the signal strength when cells were imaged based on the conjugated Cy5 dye: the more dyes that are conjugated the brighter the signals obtained from the cells (Figure 5A). The CPMV-Cy5_1 formulation had a fluorescence signal approximately 1.5 times as strong as that of the CPMV-Cy5_2 formulation (1.5 at 30 min and 1.6 at 2 h). On the other hand, cell uptake (based on the antibody staining) of the CPMV-Cy5_2 formulation was even higher, with mean antibody fluorescence 2.5 times as strong at that of the CPMV-Cy5_1 formulation. Taking the ratio (2.5:1.5), we find that the CPMV-Cy5_2 formulation indeed is still brighter, with a 1.5-fold higher fluorescence intensity per particle. Since the test tube fluorescence intensity of CPMV-Cy5_2 was about 2.6-fold stronger compared to that of CPMV-Cy5_1, this indicates that the somewhat unexpected signal increase for the CPMV-Cy5_2 formulation can only be in part explained by the increased cell uptake. Other factors may contribute to the fluorescence signal enhancement. Confocal microscopy imaging and colocalization studies indicate that CPMV samples are taken up by cells via endocytosis and colocalize with the endolysosome (Lamp-1 marker, Mander’s colocalization coefficient 0.83, Figure SC). The endolysosome is a low pH compartment with high protease and hydrolase activity, which may at least in part lead to the dissociation or degradation of the capsid proteins within the 3 h time frame; these structural changes may relax the conformation and packing of the dyes, therefore overcoming quenching and enhancing the fluorescence properties.

In the case of TMV, cell uptake was generally less efficient and only apparent after a 3 h incubation period using TMV displaying 365 Cy5 dyes per particle (Figure 6B). Cell uptake is and colocalization with the endolysosome was also confirmed. Protein A and B conjugated to Cy5 dye was used as negative control.

Figure 6. Chemical stability of TMV_5c-e-A488 in lysosomal extract over time. (a) Representative SDS-PAGE results; data shown are obtained after 1 day incubation of TMV_5c-e-A488 in lysosomal extract. After electrophoretic separation, gels were imaged first under UV light, then stained with Coomassie staining and photographed under white light. M = SeeBlue Plus2 protein size standard, the molecular weights (in kDa) are indicated on the left. 1 = BSA protein in KP pH 7.0 (negative control, BSA protein and multimers are detected on the gel), 2 = BSA in lysosomal extract (positive control demonstrating protein degradation as expected), 3 = TMV_5c-e-A488 in KP pH 7.0, 4 = TMV_5c-e-A488 in KP pH 5 (acidity has no effect on the chemical stability), 5 = TMV_5c-e-A488 in lysosomal extract, release of the fluorophores are apparent. (b) Plots of fluorescence to protein intensity (normalized to control lane 3) as measured by lane analysis tool using ImageJ software. TMV_5c-e-A488 in KP buffer at pH 7 and pH 5 remain stable, with the A488 stably attached over time; A488 release is detected for TMV_5c-e-A488 exposed to lysosomal extracts. (Figure 6D). The shape of the nanocarrier may explain the reduced cell uptake: the high aspect ratio and stiff nature of the TMV rod (300 × 18 nm) may reduce nonspecific cell interactions and cell uptake kinetics.11 It should be noted that targeting ligands were not applied in this study; therefore, cell uptake depends on nonspecific cell interactions, at least in the case of TMV.36–38 Manchester and colleagues reported that, despite being a plant pathogen, CPMV binds to and is internalized by mammalian cells through surface-expressed vitaminin, a protein that is overexpressed on tumor endothelial cells.18,39 We demonstrated that this interaction can be exploited to target CPMV-based nanoparticles to cancer cells, including HeLa cells expressing surface vitaminin.39 Such receptor-specific interactions have not been described for TMV. Therefore, differences in surface chemistry and shape of the nanocarriers may explain their distinct cell uptake behavior. Even though TMV cell uptake is negligible, low level fluorescence signals are detectable and consistent with findings reported for CPMV, the more dye conjugated to TMV, the more efficient its cell uptake properties.
and the brighter the fluorescence obtained from the conjugated 40
515-Cy5 dye.
516 Cell studies pinpoint distinct fluorescent properties of VNPs in 517 cellular environments that do not match the properties on the benchtop. These observations indicate 518 structural changes or metabolic degradation of the carrier after 519 cell entry. To gain an initial understanding of this phenomenon, 520 we set out to determine whether chemical stability of the virus- 521 bound antibody-probes was maintained within the endosomal 522 compartment. A fluorescently labeled TMV ε-cyl-E888 sample 523 (conjugated with Alexa Fluor 488) was prepared to enable 524 visualization of fluorescent protein bands and free dye under 525 transmitted light microscopy. TMV ε-cyl-E888 was then 526 incubated in a lysosomal extract obtained from liver tissue of 527 Balb/C mice; then, its integrity over time was examined. 528 Indeed, the data indicate release of the fluorescent cargo from 529 the coat proteins, the coat proteins, however, appeared to 530 remain intact even after several days of exposure (as measured 531 by their molecular weights; Figure 6). Cargo release was also 532 evaluated by measuring the TMV ε-cyl-E888 sample under low- 533 pH conditions triggered with HCl. Release of cargo was not observed, therefore indicating that 534 enzymatic activity may cause the cleavage of the fluorescent cargo. In ongoing studies, we are elucidating the underlying 535 mechanism to determine the time course and enzymes 536 involved. The release of the fluorophores after cell entry 537 results in reduced quenching and hence increased fluorescence 538 output within the cell, which is consistent with the observations 539 made in the cell imaging study (see Figure 5).

In summary, cell studies indicate distinct behavior of the 540 CPMV and TMV carriers; CPMV exhibits favorable cell uptake 541 properties. Data indicate that dye-labeling influences cell 542 interactions; the more dyes that were conjugated, the stronger 543 the cell interactions. Fluorescent dyes are frequently used in 544 preclinical evaluation of nanocarriers for imaging or drug delivery; therefore, it is pertinent to carefully evaluate and test 545 each formulation to understand the nonspecific and potentially 546 nonspecific contributions from organic fluorophores on cell- 547 specificity and uptake rates. While fluorescence and lifetime 548 measurements of CPMV and TMV with less dye exhibit 549 enhanced fluorescence properties, this may not translate into 550 cell imaging studies in vitro; altered cell uptake properties and 551 chemical degradation of the optical within the endosome may 552 result in brighter signals from particles with increased dye 553 loading.

III. CONCLUSIONS

Using CPMV and TMV as scaffolds, we synthesized a set of 555 fluorescent-labeled virus-based nanoparticles. Our data show 556 that density, spatial placement, conjugation chemistry, and 557 microenvironment affect the optical properties of the probes. 558 The brightest probes were obtained using CPMV with sparse 559 dye labeling (CPMV-cyl-C5 with 27 dyes); its fluorescence 560 intensity was about 3x higher compared to the brightest TMV 561 sample (TMV ε-cyl-C5, FI ~ 1616), even though both 562 samples contained comparable dye concentrations with dye 563 distances estimated at ~8 nm. This dye interdistance is the 564 theoretical minimum to avoid coupling and FRET that are 565 responsible for photoluminescence quenching effects. The 566 differences between the probes may be explained by differences 567 in the microenvironment; it is possible that aromatic amino 568 acids in proximity to Lys138 on the TMV scaffold interfere with 569 emission. It is interesting to note that others have investigated 570 the fluorescence properties of dye-labeled CPMV and reported 571 that CPMV labeled with 70 dyes of A488 or 120 dyes of A555 572 did not show any apparent quenching, therefore indicating 573 that the nature of the fluorophore is another variable to 574 consider. Conjugation chemistry matters; our data confirm that 575 coupling of fluorophores to aromatic tyrosine residues via 576 diazoamine coupling results in probes the least bright, which is 577 consistent with electron delocalization in the conjugated ring 578 systems resulting in quenching. Last, placement of dyes on the 579 interior capsid surface is less efficient because of significant 580 coupling of the densely located fluorophores, and this is 581 consistent using TMV (this study) as well as CPMV. 582

Stickingly, stability investigations show that viruses (CPMV 583 and TMV) can be exposed to pulsed laser light characterized by 584 a significant amount of excitation energy fluence while 585 remaining intact. Ultrashort pulses with different wavelengths 586 and intensity, typically used for laser spectroscopy studies, leave 587 the structure of the virus unmodified because of reduced 588 absorption coefficient. The overall low absorbance is 589 responsible for moderate increase of the local temperature and 589 therefore avoiding effects of thermal denaturation of viruses. 589

Finally, cell studies indicate distinct behavior of the CPMV and TMV carriers displaying dyes at various ratios. CPMV 590 exhibits favorable cell uptake properties compared to TMV; and 591 this could be explained by a combination of nanoparticle shape 592 and molecular recognition. The spherical shape of CPMV may 593 enhance its cell uptake properties compared to the still, 594 elongated rod, further, CPMV targets surface-exposed vimentin 595 on HeLa cells, therefore mediating receptor-specific internal- 596 ization.1,3,10 Our data also demonstrate that dye-labeling 597 influences cell interactions; the more dyes that were 598 conjugated, the stronger the cell interactions, and this may be 599 a result of the dye interacting with the cell membranes. Because 600 various fluorescent dyes are utilized in preclinical imaging of 601 targeted nanoparticles, it is important to use proper controls to 602 delineate and differentiate between effects from the fluorophore 603 versus targeting ligands. Furthermore, the brightest particles on 604 the bench may not necessarily result in the brightest candidates 605 in cells; our data indicate that the changing environment may 606 affect the structural and hence the optical properties of the 607 fluorescent probes. We provide insights into the fate of the 608 optical probes in cells. Our data indicate that enzymatic 609 cleavage alters the fluorescence of the optical probes in the 610 cellular environment. Therefore, benchtop testing of optical 611 probes does not necessarily reflect their optical properties in 612 vivo.

In summary, the rules for designing the brightest probes are 613 to conjugate the fluorophores to the exterior surface targeting 614 nonaromatic residues and yielding a dye density with a dye 615 distance of at least ~10 nm; further, one should consider 616 targeting various locations on the capsid surface to minimize 617 effects from the microenvironment resulting in quenching the 618 latter could be achieved with VNPs through genetic design and 619 insertion of lysine side chains at various specified locations. 70
Furthermore, while the probes may exhibit extraordinary 70 stability on the benchtop, the cellular machinery, including 70 proteases and hydrolases, may contribute to structural changes, 70 which impact the optical properties in cells. This not only has 70 implications for applications of protein-based probes in optical 70 imaging, but also provides insights for their development as 70 drug delivery vehicles with a built-in cargo release strategy. 70

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**EXPERIMENTAL SECTION**

**Materials.** Sulfo-Cys (scys) NHS ester and azide were purchased from Lumiprobe (McRandale Beach, FL). Propargylamine was supplied from Sigma-Aldrich (St. Louis, MO). 

**N-hydroxybenzotriazole (HOBr) from Chem-Impex International (Wood Dale, IL), ethylmethylpropargylcarbonate (EMPC) from Pierce Biotechnology (Rockford, IL), propargyl-NHS ester from Click Chemistry Tools (Scottsdale, AZ), and 3-ethylallylne and dimethyl sulfoxide (DMSO) from Fisher.

HeLa cells were supplied from ATCC (Manassas, VA). Cells were cultured as monolayers in DMEM (Gibco) supplemented with 10% FBS, 1% glutamine, and penicillin-streptomycin (pen-strep), as well as secondary goat anti-mouse Alexa Fluor 488 antibody purchased from Life Technologies (Grand Island, NY). Mouse anti-human LAMP-1 came from Biologend (San Diego, CA).

**Propagation and Purification of CPMV and TMV.** CPMV was propagated using Vigna mungo (black moth bean) plants, and wild-type TMV and TMVΔVT mutants were propagated using Nicotiana benthamiana plants (a tobacco species). CPMV and TMV/TMVΔVT particles were purified from infected leaves using established procedures yielding 100 mg of CPMV or TMV/TMVΔVT per 100 g infected leaf material.

**Bioconjugation.** For CPMV, reactions were carried out with 1000 to 8000 mol equiv of scys NHS ester per particle at a final concentration of 2 mg/mL CPMV in 0.1 M potassium phosphate buffer (pH 7.0) with 10% (v/v) DMSO. The resultant CPMV-scys was purified using centrifugal filter units with a 10 kDa molecular weight cutoff (Millipore). TMV and TMVΔVT, in contrast, are all two-step reactions; alkylene handles were first added to the particles, then scys azide was attached through Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC). For TMV-eALK, 25 molar excess of propargylamine per protein was reacted using EDC coupling with 45 molar excess of both EDC and HOBt at a final concentration of 2 mg/mL TMV in HEPES buffer. For TMV-eALK, 35 equiv of 3-ethynylfuran diazonium salt formed by mixing 100 mg of 0.3 M p-toluene-sulfonic acid monohydrate with 75 μL of 0.68 M 3-ethynylfuran and 25 μL of 3.0 M sodium nitrite for 5 min on ice to protect from light was added to TMV at a final concentration of 2 mg/mL TMV in borate buffer for 30 min on ice protected from light. For TMVγ'eALK, the same reaction conditions as for CPMV were used, except 10 equiv of propargyl-NHS ester per protein was coated. TMV-eALK, TMV-ealk, and TMVγ'eALK were then mixed with scys azide at a molar excess ranging from 0.2 to 600 equiv per protein for TMV-eALK, 0.3 to 6 for TMV-ealk, and 0.02 to 2 per protein for TMVγ'eALK at final concentrations of 1 mg/mL TMV in 10 mM potassium phosphate buffer (pH 7.4) for 30 min on ice with 2 mM ammonium, 2 mM ascorbic acid sodium salt, and 1 mM copper sulfate. The reaction was then stopped with 2.5 mM EDTA. TMVγ'eALK-e488 used for stability studies through exposure to lysosomal extracts was synthesized via an overnight reaction using TMVγ'eALK and Alexa Fluor 488 (A488) succinimidyl ester at a molar excess of 10:1 per protein in 0.1 M potassium phosphate buffer containing 10% DMSO by volume. All reactions were purified through ultrafiltration centrifugation. Yields after purification for each reaction step were 80–90% (as measured based on protein concentration).

**UV–vis Spectroscopy.** To determine the dye attachment density, the concentration of dye–the–particles and dye–the–particles were determined using UV–vis spectroscopy. The particle-specific extinction coefficient at 260 nm is 8.1 mg mL⁻¹ cm⁻¹ for CPMV and 3 mg mL⁻¹ cm⁻¹ for TMV, while the extinction coefficient of scys at 406 nm is 27,700 M⁻¹ cm⁻¹ and the extinction coefficient of AF488 at 495 nm is 73,000 M⁻¹ cm⁻¹. It should be noted that both TMV and CPMV are RNA viruses; the encapsulated RNA molecules result in increased absorbance at 260 nm (versus 280 nm absorbance derived from the protein shell).

**Transmission Electron Microscopy (TEM).** Carbon-coated copper TEM grids (Electron Microscopy Sciences) were placed over 20 μL drops of particles diluted to 0.1 mg/mL with deionized water. The particles were allowed to adsorb for 5 min, and then the grid was briefly rinsed with deionized water followed by negative staining with 2% (w/v) uranyl acetate for 1 min. Samples were observed using a Zeiss Libra 200 FE transmission electron microscope operated at 200 kV.

**Gel Electrophoresis.** Native particles were analyzed by 12.5% (w/v) agarose gel electrophoresis (1 h at 100 V) in 0.5× TBE running buffer with ethidium bromide. Individual coat proteins were analyzed by denaturing 4–12% NuPAGE (Invitrogen) polyacrylamide gel electrophoresis (50 min at 200 V) in 1× MOPS running buffer. The gels were stained with Coomassie for protein content. Images of the gels were taken using an Alphalmager imaging system (Biosciences) for UV and white light images and a Maestro 2D fluorescence imaging system with yellow excitation and emission filters for fluorescent images.

**Fluorescence Measurements.** 50 μL of dye-labeled CPMV and TMV diluted in 1× MOPS buffer were added in triplicate to a black 384-well plate at concentrations of 2.5 μM scys, 50 nM CPMV, or 5 nM TMV. Fluorescence intensity was measured using a Tecan Infinite 200 plate reader with excitation/emission wavelengths of 460 nm and 655 nm.

**Lifetime Measurements.** For lifetime measurements, a 605-well-established and advanced technique based on ultrafast time-correlated single-photon counting spectrophotometry (TCSPC) was used. Samples were excited by means of an ultrafast pulsed light source at 4 Mhz, with a pulse duration of 140 fs, at a wavelength of 560 nm. The exciting light was produced by a Ti:sapphire laser (model Chameleon Coherent) coupled to a second harmonic generator (SHG) module and a pulse picker. This arrangement allowed synchronization of the excitation pulses with the acquisition card of a multichannel single-photon counting spectrophotometer (Edinburgh) equipped with a last generation MCP-PMT (microchannel plate photomultiplier) for detecting fluorescence light and measuring fluorescence lifetime (time resolution ~5 ps). The decay time data were fitted with 699 multiparametric functions f(t) = 1 + γ exp(-t/τ1) + A3 + A0 exp(-t/τ3) + ..., while the average decay times were calculated by means of a weighted average τ = (τ1 + A1 × τ1 + A3 × τ3 + ...)/(A0 + A1 + A3 + ...).

**Flow Cytometry.** HeLa cells were grown in MEM supplemented with 10% (v/v) FBS, 1% (v/v) l-glutamine, and 1% (v/v) pen-strep at 37 °C in 5% CO2. The cells were seeded at a density of 5,000,000 cells/200 μL MEM/well onto an untreated 96-well v-bottom plate. Particles were added in triplicate at a concentration of 50,000 CPMV or TMV per cell and incubated for 3 h. Free particles were then removed by washing the cells twice through centrifugation at 500 × g for 4 min.
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DEDICATION

This article is dedicated to the memory of Daniela Pucci, friend and collaborator, who died on April 24th, 2014.

REFERENCES


ASSOCIATED CONTENT

Supporting Information

Additional UV–vis spectra, interodye distance calculations, and TEM images of fluorescently labeled particles. This material is available free of charge via the Internet at http://pubs.acs.org.

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