Video Article

Viral Nanoparticles for *In vivo* Tumor Imaging

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Abstract

The use of nanomaterials has the potential to revolutionize materials science and medicine. Currently, a number of different nanoparticles are being investigated for applications in imaging and therapy. Viral nanoparticles (VNPs) derived from plants can be regarded as self-assembled bionanomaterials with defined sizes and shapes. Plant viruses under investigation in the Steinmetz lab include icosahedral particles formed by *Cowpea mosaic virus* (CPMV) and *Brome mosaic virus* (BMV), both of which are 30 nm in diameter. We are also developing rod-shaped and filamentous structures derived from the following plant viruses: *Tobacco mosaic virus* (TMV), which forms rigid rods with dimensions of 300 nm by 18 nm, and *Potato virus X* (PVX), which form filamentous particles 515 nm in length and 13 nm in width (*the reader is referred to refs.* [1] *and* [2] *for further information on VNPs)*.

From a materials scientist's point of view, VNPs are attractive building blocks for several reasons: the particles are monodisperse, can be produced with ease on large scale *in planta*, are exceptionally stable, and biocompatible. Also, VNPs are "programmable" units, which can be specifically engineered using genetic modification or chemical bioconjugation methods [3]. The structure of VNPs is known to atomic resolution, and modifications can be carried out with spatial precision at the atomic level[4], a level of control that cannot be achieved using synthetic nanomaterials with current state-of-the-art technologies.

In this paper, we describe the propagation of CPMV, PVX, TMV, and BMV in *Vigna ungiuculata* and *Nicotiana benthamiana* plants. Extraction and purification protocols for each VNP are given. Methods for characterization of purified and chemically-labeled VNPs are described. In this study, we focus on chemical labeling of VNPs with fluorophores (*e.g.* Alexa Fluor 647) and polyethylene glycol (PEG). The dyes facilitate tracking and detection of the VNPs [5-10], and PEG reduces immunogenicity of the proteinaceous nanoparticles while enhancing their pharmacokinetics [8,11]. We demonstrate tumor homing of PEGylated VNPs using a mouse xenograft tumor model. A combination of fluorescence imaging of tissues *ex vivo* using Maestro Imaging System, fluorescence quantification in homogenized tissues, and confocal microscopy is used to study biodistribution. VNPs are cleared via the reticuloendothelial system (RES); tumor homing is achieved passively via the enhanced permeability and retention (EPR) effect[12]. The VNP nanotechnology is a powerful plug-and-play technology to image and treat sites of disease *in vivo*. We are further developing VNPs to carry drug cargos and clinically-relevant imaging moieties, as well as tissue-specific ligands to target molecular receptors overexpressed in cancer and cardiovascular disease.

Video Link

The video component of this article can be found at https://www.jove.com/video/4352/

Protocol

1. VNP (CPMV, BMV, PVX, and TMV) Propagation

1. Set the indoor plant chamber controls to 15 hr of day (100% light, 25 °C, 65% humidity) and 9 hr of night (0% light, 22 °C, 60% humidity).
2. Inoculate plants according to the timeline in **Table 1**.

CPMV	**PVX, TMV, and BMV**
Day 0: Plant 3 cowpea seeds/pot.	***Day 0:*** *Plant ~30 N. benthamiana seeds/pot. Fertilize once a week with 1 tablespoon fertilizer/5 L water.*
	Day 14: *Re-pot N. benthamiana at 1 plant/pot.*
Day 10: Infect leaves primary leaves with CPMV (5 µg/50 µl/leaf) by mechanical inoculation using a light dusting of carborundum.	***Day 28:*** *Infect three to five leaves with PVX, TMV, or BMV (5 µg/50 µl/ leaf) by mechanical inoculation using a light dusting of carborundum.*

Day 20: Harvest leaves and store in -80 °C.	***Day 42:** Harvest leaves and store in -80 °C.*

Table 1. Timeline for growing, infecting, and harvesting leaves.

Note: only CPMV propagation is demonstrated as an example.

2. VNP (CPMV, BMV, PVX, and TMV) Purification

Note: All steps are carried out on ice or at 4 °C.

1. Homogenize 100 g of frozen leaves in a standard blender using 2 volumes of cold buffer (see **Table 2**). Filter through 2-3 layers of cheesecloth.
2. For PVX, adjust pH to 6.5 using 1 M HCl. Add 0.2% (w/v) ascorbic acid and 0.2% (w/v) sodium sulfite.
3. Centrifuge crude plant homogenate at 5,500 x g for 20 min. Collect supernatant.
4. For BMV, layer 25 ml of supernatant over 5 ml of 10% (w/v) sucrose solution. Centrifuge at 9,000 x g for 3 hr and resuspend pellets in 38.5% CsCl solution (w/v). Mix by shaking for 5 hr, then continue with step 2.12.
5. Extract plant material by adding 0.7 volumes of 1:1 (v/v) chloroform:1-butanol. Stir mixture for 30-60 min.
6. Centrifuge solution at 5,500 x g for 20 min. Collect the upper aqueous phase.
7. Add NaCl to 0.2 M and 8% (w/v) PEG (MW 8,000). For TMV, also add 1% (v/v) Triton X-100. Stir for at least 1 hr, then let sit for at least 1 hr.
8. Centrifuge solution at 15,000 x g for 15 min. Resuspend pellet in 10 ml of buffer. For PVX, add 0.1% β-mercaptoethanol and urea to 0.5 M.
9. Centrifuge at 8,000 x g for 30 min and collect supernatant.
10. Ultracentrifuge supernatant at 160,000 x g for 3 hr. Resuspend pellet in 5 ml buffer overnight.
11. Prepare a 10-40% sucrose gradient using equal volumes of 10%, 20%, 30%, and 40% sucrose in buffer (heaviest first). Allow the gradient to equilibrate overnight at room temperature.
12. Ultracentrifuge resuspended pellet over sucrose gradient at 100,000 x g for 2 hr (24 hr for BMV).
13. Collect light scattering band and dialyze against buffer.
14. Characterize the VNPs (below) and store at 4 °C. For long-term storage, store at -80 °C.

CPMV and TMV	**0.1 M potassium phosphate buffer (pH 7.0)** 38.5 mM KH_2PO_4 61.5 mM K_2HPO_4
PVX	**0.5 M borate buffer (pH 7.8)** 0.5 M boric acid Adjust pH with NaOH
BMV	**SAMA buffer (pH 4.5)** 250 mM sodium acetate 10 mM $MgCl_2$ 2 mM β-mercaptoethanol (add fresh)

Table 2. Buffers and their recipes for each VNP.

Note: only CPMV propagation is demonstrated as an example.

3. VNP (CPMV, BMV, PVX, and TMV) Characterization

1. Perform UV/visible spectroscopy to determine the concentration of VNPs.
    1. Measure the absorbance of 2 µl of sample using a NanoDrop spectrophotometer.
    2. Determine the concentration of particles and dyes using the Beer-Lambert law (A=εcl, where A is the absorbance, ε is the extinction coefficient, c is the concentration, and l is the path length). The path length is 0.1 cm for the NanoDrop.
    The VNP-specific extinction coefficients are:
    CPMV: 8.1 $cm^{-1}mg^{-1}ml$ (at 260 nm)
    PVX: 2.97 $cm^{-1}mg^{-1}ml$ (at 260 nm)
    TMV: 3.0 $cm^{-1}mg^{-1}ml$ (at 260 nm)
    BMV: 5.15 $cm^{-1}mg^{-1}ml$ (at 260 nm)

2. Analyze particles by size-exclusion fast protein liquid chromatography (FPLC).
    1. Using a Superose 6 size-exclusion column and the ÄKTA Explorer, load 50-100 µg of VNPs in 200 µl of 0.1 M potassium phosphate buffer (pH 7.0).
    2. Set detectors to 260 nm (nucleic acid), 280 nm (protein), and the excitation wavelength of any dyes attached.
    3. Run at a flow rate of 0.5 ml/min for 72 min.
    4. The elution profile and A260:A280 nm indicates whether the VNP preparation is pure and whether particles are intact and assembled. The following A260:280 ratios indicate a pure VNP preparation:
    CPMV: 1.8±0.1
    PVX: 1.2±0.1
    TMV: 1.1±0.1
    BMV: 1.7±0.1

3. Perform denaturing (pre-cast NuPAGE) Bis-Tris polyacrylamide 4-12% gradient gel electrophoresis to analyze purity of preparation and conjugation to individual coat proteins.
    1. Add 3 ml of 4x LDS sample buffer to 10 µg of the particles in 9 µl of potassium phosphate buffer. Add an additional 1 µl of 4x LDS sample buffer and 3 µl of β-mercaptoethanol to BMV to reduce the high number of disulfide bonds.
    2. Incubate in heat block for 5 min at 100 °C.
    3. Load samples onto an SDS gel.
    4. Run samples at 200 V for 1 hr in 1x MOPS running buffer.
    5. Document the gel under UV light to visualize fluorescent coat proteins.
    6. For non-fluorescent protein, stain with Coomassie blue (0.25% (w/v) Coomassie Brilliant Blue R-250, 30% (v/v) methanol, 10% (v/v) acetic acid) for 1 hr.
    7. Destain with 30% methanol, 10% acetic acid overnight. Change the solution if required.
    8. Document the gel under white light.

4. Analyze integrity of particles by transmission electron microscopy (TEM).
    1. Dilute samples to 0.1-1 mg/ml in 20 µl of DI water.
    2. Place 20 µl drops of the samples on Parafilm.
    3. Cover drops with a TEM grid and let sit for 2 min. Wick off the excess solution on the grid with filter paper.
    4. Wash grid by placing on a drop of DI water then wicking dry.
    5. Stain grid by placing on a 20 µl drop of 2% (w/v) uranyl acetate for 2 min. Wick off the excess stain with filter paper.
    6. Wash grid once more in water.
    7. Observe grid under a transmission electron microscope.

4. Chemical Conjugation of VNPs with PEG and Fluorophores, Purification, and Characterization

1. For calculations for the reactions below, the molar mass of the VNPs are:
   CPMV: 5.6 x 10^6 g/mol
   PVX: 35 x 10^6 g/mol
   TMV: 41 x 10^6 g/mol
   BMV: 4.6 x 10^6 g/mol
2. Conjugate dyes and PEG to surface lysines of CPMV and PVX using a one-step *N*-hydroxy succinimide coupling reaction: Add 2,500 molar equivalents (all molar excesses refer to molar excess per VNP) of Alexa Fluor 647 succinimidyl ester and 4,500 equivalents of NHS-PEG (M.W. 5,000) dissolved in DMSO to CPMV in 0.1 M potassium phosphate buffer. When working with PVX, add a 10,000 molar excess of NHS-dye and NHS-PEG. Adjust the buffer and DMSO volumes such that the final concentration of CPMV and PVX is 2 mg/ml and DMSO content is 10% of the total reaction volume. Incubate the reaction mixture overnight at room temperature protected from light. CPMV and PVX have 300 and 1,270 addressable lysines, respectively. (*The reader is referred to the following references for further reading on chemical modification of CPMV and PVX:* [13-15]).
3. Conjugate dyes and PEG to tyrosines of TMV by diazonium coupling followed by copper(I)-catalyzed azide-alkyne cycloaddition.
    1. Prepare diazonium salt (alkyne) by mixing 400 µl of 0.3 M *p*-toluenesulfonic acid monohydrate, 25 µl of 3.0 M sodium nitrite, and 75 µl of 0.68 M distilled 3-ethynylaniline dissolved in acetonitrile at 4 °C for 1 hr.
    2. Add 3.3 ml of borate buffer, pH 8.8, containing 100 mM NaCl to 1.25 ml of TMV (20 mg/ml stock solution).
    3. React the TMV with 450 µl of the diazonium salt (alkyne) solution in an ice bath for 3 hr to add an alkyne ligation handle to TMV by diazonium coupling. The solution will turn into a light brown color. TMV has 2,140 available tyrosines for conjugation.
    4. Purify the final product using a sucrose cushion as described in step 4.4.
    5. Attach azide-functional Alexa Fluor 647 and PEG-azide (M.W. 5,000) using copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC). Add 2 equivalents of dye- and PEG-azide per coat protein and incubate with 1 mM $CuSO_4$, 2 mM AMG, and 2 mM sodium ascorbate at room temperature for 15 min. Adjust the buffer volume such that the final reaction concentration of TMV is 2 mg/ml. (*The reader is referred to the following references for further reading on chemical modification of TMV:* [16,17]).

4. Conjugate dyes to lysines and PEG to cysteines of BMV cysteine mutant (cBMV):
    1. Add 2,000 molar equivalents of Oregon Green 488 succinimidyl ester dissolved in DMSO to cBMV in 0.1 M TNKM buffer (50 mM Tris base, 50 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, pH 7.4). Adjust the buffer and DMSO volumes such that the final concentration of BMV is 1 mg/ml and DMSO content is 10% of the total reaction volume. Incubate the reaction mixture overnight at 4 °C protected from light.
    2. Purify particles using centrifugal filters as described in step 4.4.
    3. Add 2,000 molar excess of PEG-maleimide (M.W. 2,000) using the same reaction conditions as before and incubate the reaction mixture for 2 hr at 4 °C. cBMV has 180 reactive lysines and cysteines. (*The reader is referred to the following references for further reading on chemical modification of BMV:*[18]).

5. **Purification:** Pass the solution through a 40% (w/v) sucrose cushion at 160,000 x g for 2.5 hr. Re-dissolve the pellet in buffer. Alternatively, dialyze against appropriate buffer using 10 kDa cut-off spin filters.
6. **Characterization:** PEGylated and fluorescently-labeled VNPs are analyzed using the above-described methods: UV/visible spectroscopy, SDS gel electrophoresis, FPLC, and TEM (*not shown, however, refer to* ***Figures 6 and 7***) .

5. Tumor Targeting and Imaging using a Mouse Xenograft Model

1. Culture HT-29 human colon cancer cells in RPMI medium supplemented with 5% FBS, 1% penicillin-streptomycin, and 1% L-glutamine at 37 °C in 5% CO_2 using 175 cm^2 cell culture flasks.

2. Wash cells twice with sterile PBS and harvest by incubating with 5 ml of trypsin-EDTA at 37 °C for 5 min. Inactivate the trypsin with 5 ml of RPMI medium. Collect cells by centrifuging at 500 x g for 5 min. at 4 °C and resuspend in fresh RPMI at $5x10^6$ cells/50 µl medium (determine total cell count using trypan blue and a hemocytometer). Mix with an equal volume of matrigel prior to injection (keep all solutions and reagents sterile).
3. Procure six-week old NCR nu/nu mice and maintain them on an alfalfa free diet for 2 weeks. [Note: all animal procedures must be IACUC approved.] Induce tumor xenografts by subcutaneous injection of $5x10^6$ cells/100 µl/tumor in the flanks (2 tumors/mouse) using an 18 1/2 gauge sterile needle. Monitor the animals regularly. Measure the tumor size using calipers and allow the tumors to grow to an average volume of 20 mm^3 (within the next 12 days). Assign mice to two different groups randomly: PBS and VNP (n = 3 animals/group/time point). Using a 1 ml 28 gauge insulin syringe, administer by intravenous tail vein injection 100 µl of sterile PBS or 10 mg/kg VNP formulation.

Note: Tissue culture experiments and studies with live animals will not be demonstrated. Hands-on demonstration will be limited to tissue processing and data acquisition. For a reference on the HT-29 tumor xenograft model, the reader is referred to ref. [19]

Three techniques are used to evaluate tumor homing of VNPs:

4. ***Fluorescence imaging using Maestro Imaging System:*** Sacrifice mice at different time points (2, 24, and 72 hr) using CO_2 gas. Dissect the animals and excise all major organs (brain, heart, lungs, spleen, kidneys and liver) along with the tumors on the flanks, place the tissues on parafilm, and analyze with fluorescence imaging instrument using yellow excitation and emission filters (800 ms exposure) to detect the presence of fluorescent signals in the tissues (derived from A647 label conjugated to the VNPs). Save the images and analyze fluorescent intensities using ImageJ 1.44o software (http://imagej.nih.gov/ij). Compare the pattern of uptake of the VNPs in tumors with other major tissues with time.
5. After imaging, cut each tissue in half and embed one half in OCT compound for cryo-sectioning and confocal analysis. Collect the other half in pre-weighed cryo-vials and immediately freeze them using liquid N_2. Store at -80 °C until ready for further processing.
6. ***Fluorescence quantification:*** Record tissues weights. Thaw frozen tissues at room temperature and place them in separate 50 ml Falcon tubes containing 1 ml of PBS. Using a handheld tissue homogenizer, homogenize the tissues for 2-3 min in PBS then transfer the homogenate to microfuge tubes. Centrifuge the homogenates for 10 min at 13,000 x g to remove non-homogenized tissue.
7. Pipet 100 µl of the supernatant from the tissues from each group (PBS and VNP formulations/time points) into a 384 well black UV plate. Evaluate fluorescence intensity (Ex/Em wavelengths 600/665) using a plate reader. Normalize the obtained fluorescent values by the tissue weights.
8. ***Immunohistochemistry:*** Prepare cryo-microtome sections (10 µm) and store at -20 °C. Stain tissue sections for cell nuclei (DAPI) and endothelial cell marker (FITC-labeled anti-mouse CD31 antibody). Carry out confocal microscopy analysis to map the vascular and intra-tumoral localization of fluorescently-labeled VNPs.

Note: This procedure will not be demonstrated, representative data are shown in **Figure 8**. For a reference on immunohistochemistry and the described staining methods, the reader is referred to ref. [19]

Representative Results

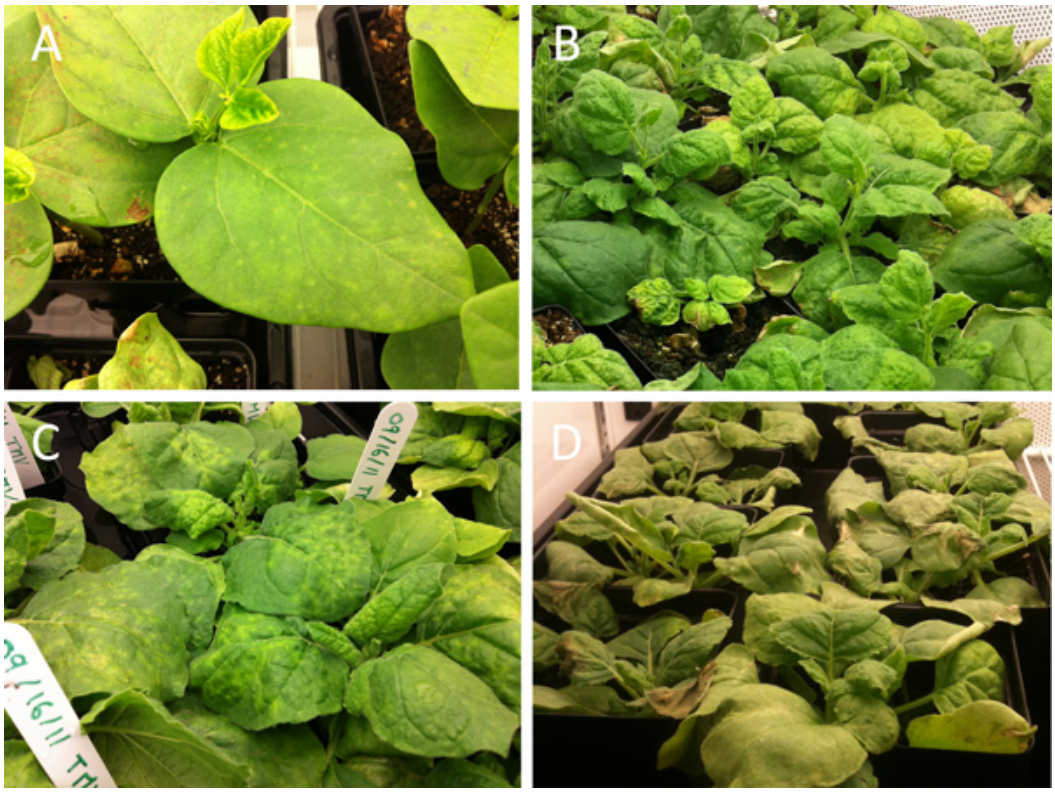

Figure 1. Plant virus-infected plants. *Vigna unguiculata* plants infected with CPMV (A). *Nicotiana benthamiana* plants infected with PVX (B), TMV (C), and BMV (D). The pictures were taken about 10 days post infection by mechanical inoculation.

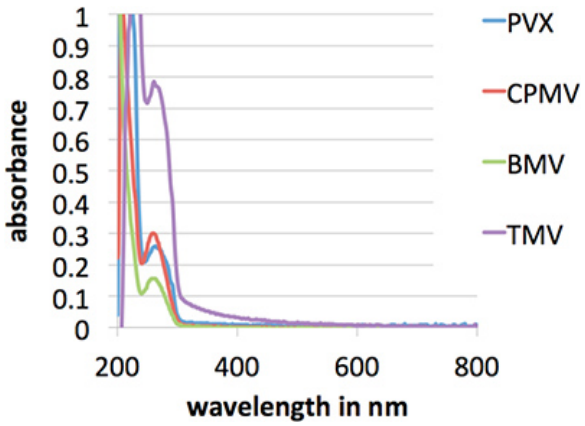

Figure 2. UV/visible spectra of CPMV, PVX, TMV, and BMV. Absorbance at 260 nm is proportional to the nucleic acid concentration, and absorbance at 280 nm is proportional to the protein concentration. The A260:A280 ratio can be used to assess purity of VNP preparations. The characteristic ratio for CPMV is 1.8, for PVX is 1.2, for BMV is 1.7, and for TMV is 1.1.

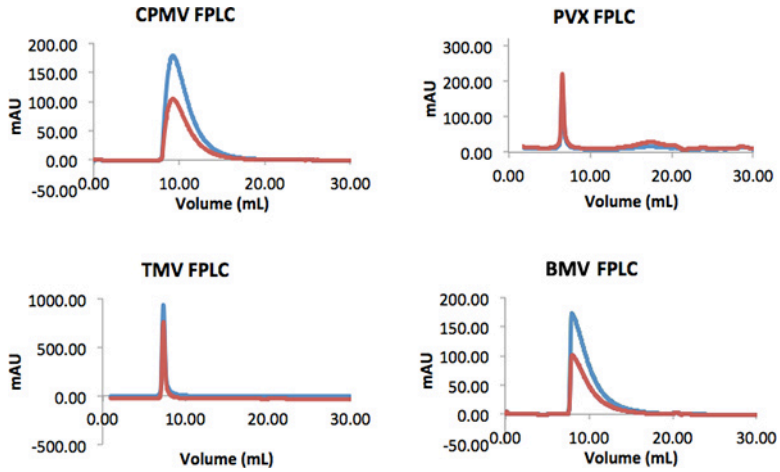

Figure 3. FPLC of CPMV, PVX, TMV, and BMV. Size-exclusion elution profiles of VNPs were obtained by using a Superose 6 column and Äkta purifier. Blue = A260 nm; red = A280 nm. As with the UV/visible spectra, the A260:A280 ratio can be used to assess purity of VNP preparations. In addition, the elution profile can be used to determine purity of the particles. CPMV typically elutes from the column at 9-10 ml and BMV around 8 ml. Due to their large size, PVX and TMV elute at the void volume of the column (approximately 7 ml). Impurities or free coat proteins would result in peaks at higher elution volumes.

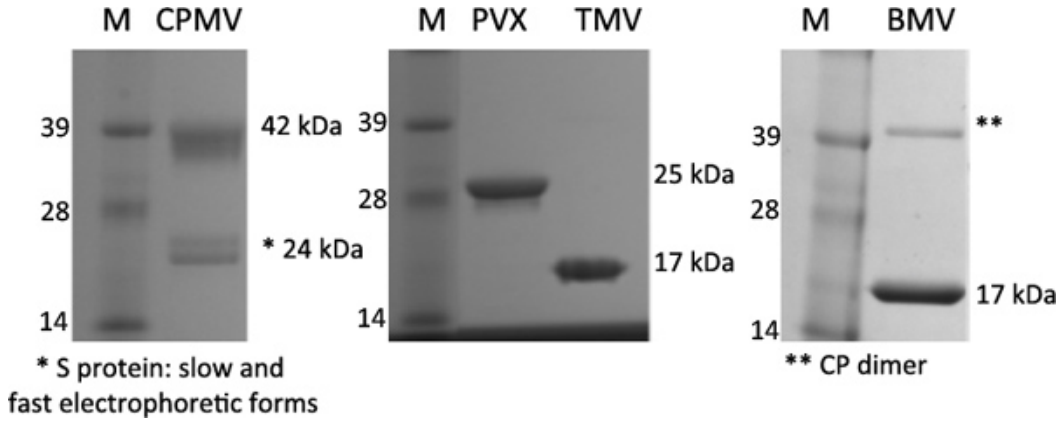

Figure 4. SDS gels of CPMV, PVX, TMV, and BMV after Coomassie staining. SDS gel electrophoresis is used to separate denatured coat protein subunits. CPMV particles are formed by two proteins, a large protein (42 kDa) and a small protein (24 kDa); the small protein has two electrophoretic forms. The PVX coat protein is 25 kDa in size, and TMV consists of a 17 kDa protein. The BMV coat protein is 17 kDa in size. M = SeeBlue Plus2 protein marker.

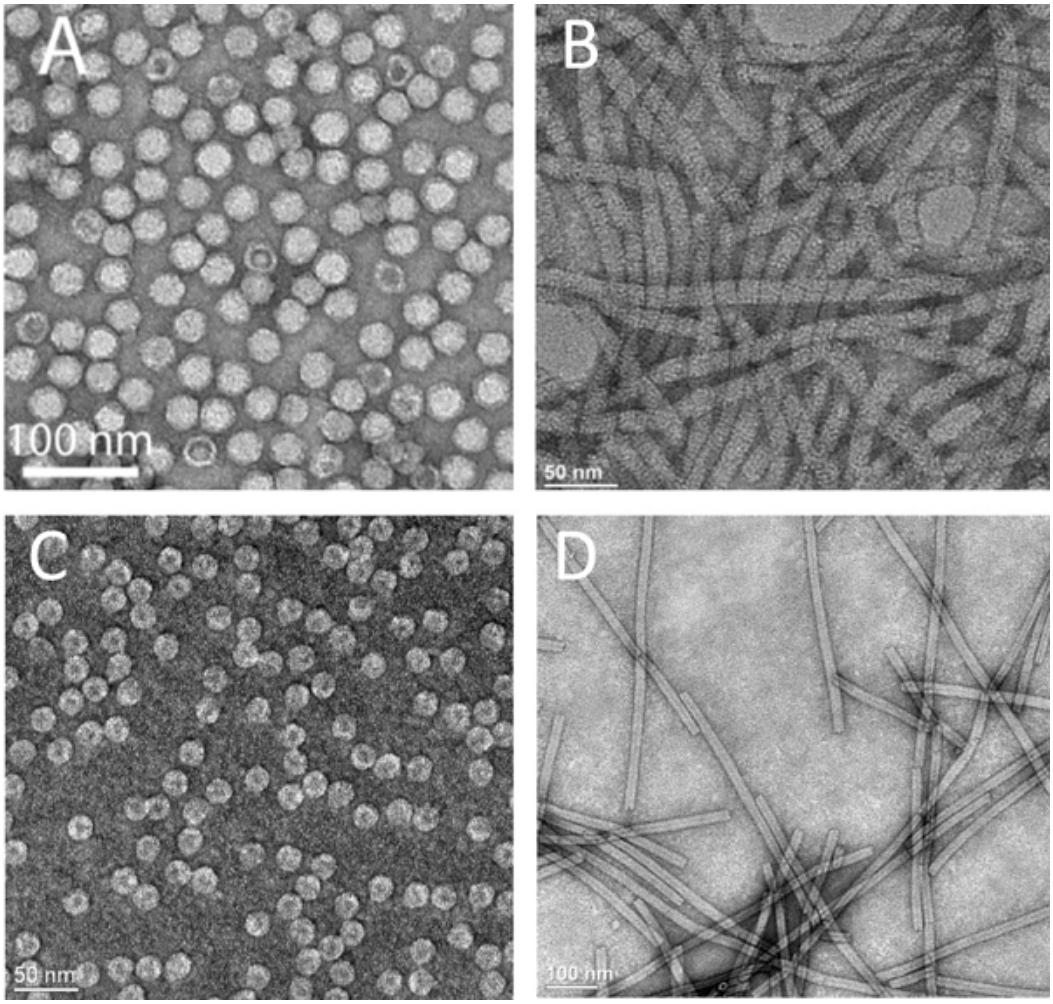

Figure 5. TEM images of virus particles. TEM grids were negatively stained with 2% uranyl acetate, so the particles appear light on a dark background. Images are of 30 nm-sized CPMV (A), 515x13 nm-sized PVX (B), 30 nm-sized BMV (C), and 300x18 nm-sized TMV (D).

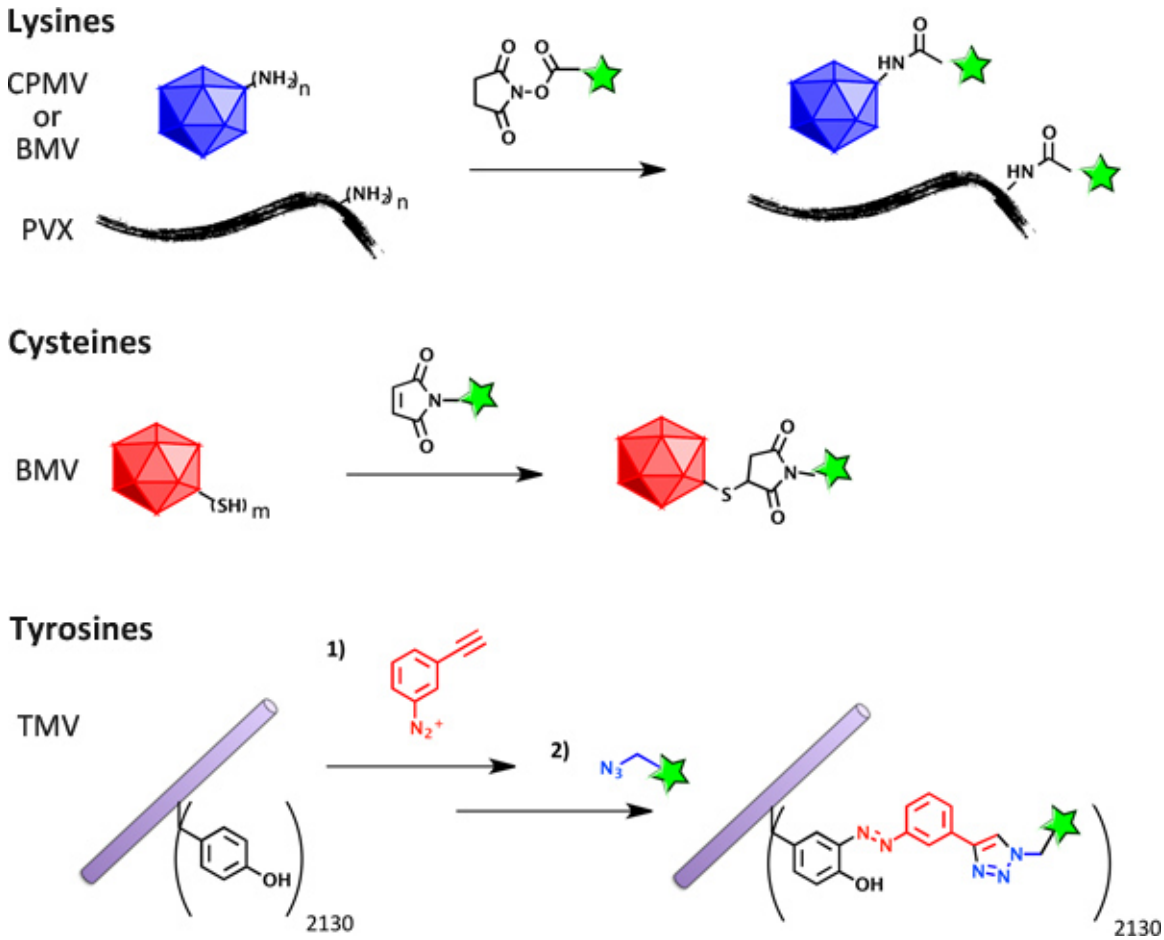

Figure 6. Reaction scheme for conjugation to VNPs. Three bioconjugation methods are used to conjugate PEG and dyes to the VNPs. CPMV, BMV, and PVX have lysines that can be used to react with functionalized NHS esters.[20] BMV cysteine mutants also have reactive thiols that can be chemically modified using a maleimide group.[18] Lastly, the tyrosines of TMV can be functionalized through a two-step reaction, where an alkyne handle is first installed via diazonium coupling and then CuAAC is performed with an azide-modified fluorophore or PEG.[16]

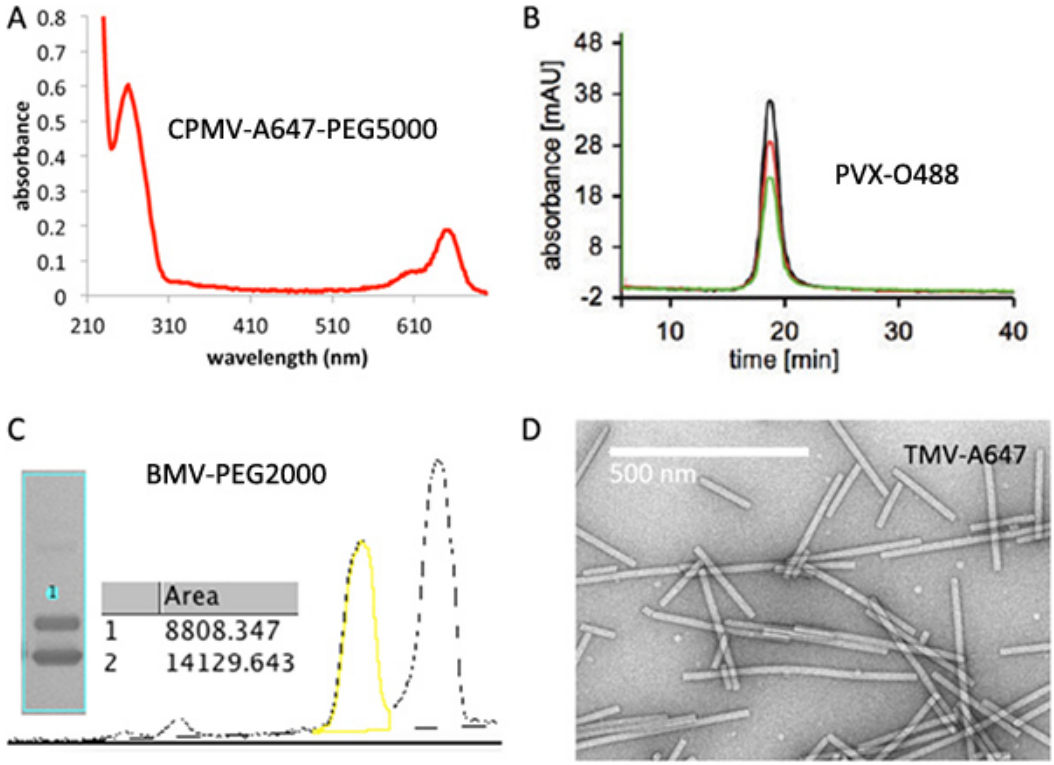

Figure 7. Representative data of fluorescently-labeled and PEGylated VNPs. UV/visible spectroscopy of CPMV-A647-PEG5000 (A). The absorbance peak at 650 nm is indicative of conjugated A647. The concentration of CPMV and A647 can be calculated using the Beer-Lambert law, from which the number of dyes per particle can be determined (about 60). FPLC of fluorescently-labeled PVX (here Oregon Green 488) (B). Black = A260 nm; red = A280 nm; green = A496 nm. Co-elution of PVX and dye confirmed covalent attachment. SDS gel of cBMV and cBMV-PEG2000 (C). PEG was conjugated to the cBMV cysteines. PEGylated CPs have lower mobility due to higher molecular weight, so the upper band is the conjugated CPs. ImageJ software and band analysis tool was used to quantify the number of PEG chains per cBMV. Approximately 40% (*i.e.*, 70 CPs per cBMV) of the CPs were labeled with PEG. Negatively-stained TEM of TMV-A647 (D). The majority of particles are shown to be intact after modification.

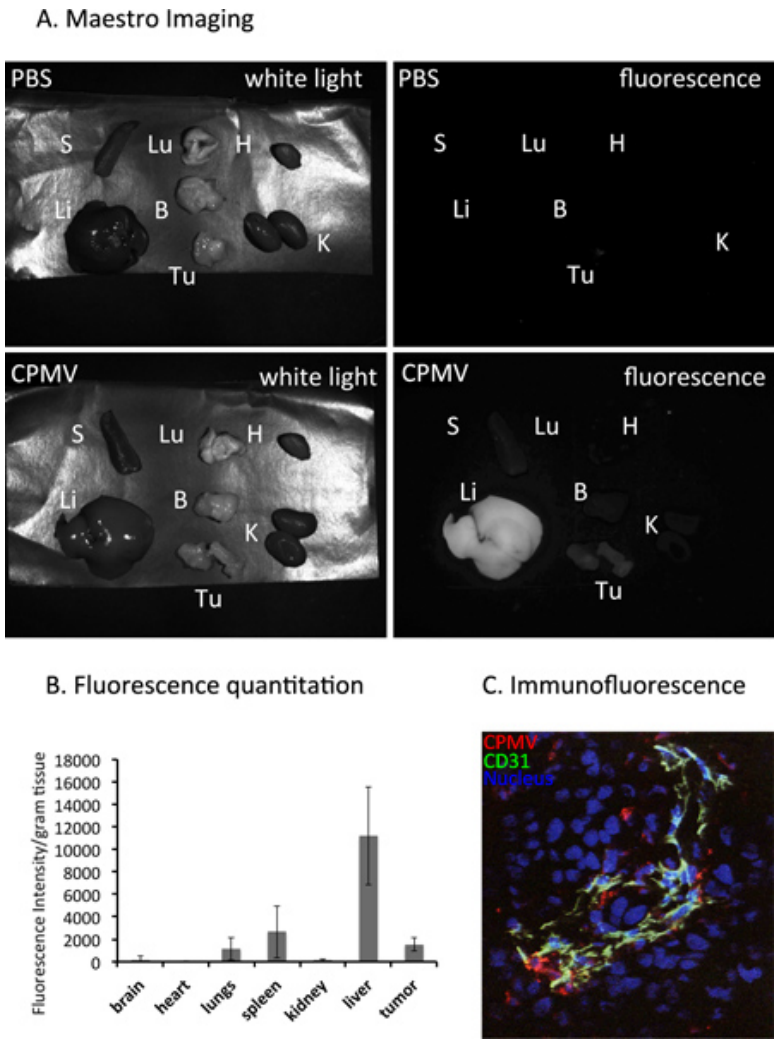

Figure 8. Biodistribution of CPMV in a mouse xenograft tumor model. Nude mice were implanted with human HT-29 xenografts. Biodistribution and tumor homing was visualized using Maestro animal imaging of tissues *ex vivo* (A). No fluorescence was observed for the PBS control, while for CPMV conjugated with A647 and PEG, there was prominent fluorescence in the liver and some in the spleen due to clearing by the RES. There was also fluorescence seen in the tumor, indicating tumor homing via the EPR effect. Quantitative data were obtained via fluorescence measurement of homogenized tissues (normalized by tissue weight) using a plate reader assay (B). As expected, the majority of the CPMV was found in the liver and spleen, but there was also a trace in the tumor. Intratumoral localization was studied using cryo-sectioned tissues and immunostaining (C). Red = fluorescently-labeled CPMV-PEG; green = CD31 marker for endothelial cells; blue = DAPI for cell nuclei. The CPMV was mainly found near the vasculature of the tumor. [20]

Discussion

This protocol provides an approach for the chemical modification of VNPs and their applications for *in vivo* tumor imaging. The animal fluorescence imaging, fluorescence quantification, and immunohistochemistry techniques presented here are useful for studying biodistribution and evaluating tumor homing. These techniques provide valuable information regarding access of the nanoparticles to the tumor via the EPR effect. By combining the results from the various analytical methods, we get a powerful approach for evaluating localization and biodistribution of the VNPs.

Before these studies can be performed however, it is essential to obtain a pure virus preparation. Apart from mechanical inoculation, a possible alternative for VNP propagation is agroinfiltration, which can have a high transformation and accumulation rate. When propagating the VNPs, care should be taken to keep plants infected with different viruses separate as to avoid cross-contamination. In particular, TMV is readily spread and transmitted mechanically. Another critical step to be mindful of is carrying out virus purification on ice or at 4 °C. All buffers should be used ice cold. The lower temperature is necessary to prevent any damage to the VNPs as a result of proteases and oxidizing enzymes present in the plant material.

Low yields of purified VNPs may arise from multiple factors. A possible cause could be the age of the VNPs used for propagation. It is preferable to use more recent virus preparations. For example, the C-terminal 24 amino acids of the S coat protein of CPMV are necessary for the suppression of gene silencing. Deletion of this surface-exposed region occurs over time due to proteolysis. Although the structure and stability of CPMV are not affected, growth retardation and delayed spread of the virus results. Another source of low yields is loss of the VNPs during

purification. Particular attention should be given to the resuspension step after PEG precipitation. Incomplete resuspension of the pellet would result in VNP lost in the pellet of the subsequent centrifugation step.

Overall, the bioconjugation chemistries presented here are straightforward.[14,17,18,21] The characterization methods described are valuable for ensuring particle integrity and determining extent of conjugation. Molar excesses can be adjusted accordingly depending on the application and the degree of functionalization desired.

When working with the mouse xenograft model, the most essential practice is following aseptic technique. In addition, the tail vein injection is a critical step to ensure a uniform sample dose for each mouse. It may help to place the tail in warm water beforehand to dilate the vein. Another consideration is autofluorescence for fluorescence imaging and measurements. Generally, tissue autofluorescence is minimized beyond 600 nm, making long wavelength dyes with emission maxima beyond 600 nm optimal as the imaging probe. However, normal mouse diets consist of chlorophyll-containing alfalfa, which also fluoresces red. As a result, it is necessary to maintain the mice on an alfalfa free diet for at least 2 weeks to reduce background signals. Finally, it should be noted that fluorescence detection using fluorescence imaging is limited due to differences in the scattering and absorption environments of the various tissues. Consequently, imaging is used for visualization and as an initial method for monitoring biodistribution, while quantification is performed using tissue homogenates.

Some advantages of using VNPs as the platform technology are their monodispersity, biocompatibility, capability for scale-up production, and amenability to multiple functionalization strategies. In addition to chemical engineering, genetic engineering is illustrated here with the introduction of cysteines to BMV as a target for bioconjugation. Genetic engineering could also be used to introduce targeting peptides or affinity tags. While the protocol described utilizes dual-labeled (dye and PEG) particles, ongoing studies are looking at incorporating additional functionalities (*i.e.*, therapeutics and targeting) to enhance tissue specificity and therapeutic efficacy. Thus, this approach lays the groundwork for future biomedical applications.

Disclosures

No conflicts of interest declared.

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