Supplementary information for

3D Bioprinting Cowpea Mosaic Virus as an Immunotherapy Depot for Ovarian Cancer Prevention in a Preclinical Mouse Model

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Materials and Methods

Production and Characterization of CPMV and CPMV-Cy5. CPMV was propagated in black eyed pea No. 5 plants and purified as previously reported[1, 2]. CPMV-Cy5 was synthesized by attaching sulfo-Cy5 NHS ester (777.95 g/mol, Lumiprobe) linkers to solvent-exposed lysine residues on the CPMV surface. CPMV-Cy5 was prepared by mixing a 3000-molar excess of sulfo-Cy5 NHS ester per CPMV with 2 mg/mL (final concentration) of CPMV (molecular weight = 5.6×10^6 g/mol) in PBS and incubated for 2 h at room temperature. To remove unconjugated sulfo-Cy5 NHS ester, the reaction mixtures were loaded onto Amicon Ultra-0.5 mL centrifugal filters with a 100-kDa cutoff (Millipore Sigma) and washed four times with PBS. Purified CPMV-Cy5 were stored at 4 °C.

UV-Vis Spectroscopy

The concentrations of CPMV and CPMV-Cy5 were determined by UV-Vis (NanoDrop 2000, Thermofisher Scientific) using the extinction coefficient (ε) of CPMV at 260 nm = 8.1 mL / (mg x cm). The number of conjugated sulfo-Cy5 per CPMV particle was determined using the molar extinction coefficient (ε) for sulfo-Cy5 (271000 L / (mol x cm)) at 647 nm.

NuPAGE

CPMV and CPMV-Cy5 were characterized by NuPAGE. For sample preparation, CPMV and CPMV-Cy5 were mixed with 4x lithium dodecyl sulfate buffer (Life Technologies) and 10x reducing agent (Invitrogen) and heated at 95°C for 8 min. 20 µL of each sample were loaded onto a 4-12% NuPAGE gel (ThermoFisher Scientific) and run at 200 V, 120 mA, and 25 W for 35 min using 1x MOPS buffer (ThermoFisher Scientific). For the Cy5 labeled samples, Cy5 fluorescence was first imaged using the MultiColor red filter. Then all gels were stained with Coomassie blue for protein imaging. All images were recorded using a ProteinSimple FluorChem R imager.

Transmission electron microscopy (TEM)

CPMV and CPMV-Cy5 were analyzed by TEM. First, all samples were diluted to 0.5 mg/mL in PBS. 4 µL of each sample was applied to a glow-discharged carbon film with a 300-mesh Cu grid for 30 s, blotted using filter paper, washed using Milli-Q water for 30s, blotted using filter paper, and then stained with 4 µL 1% (w/v) uranyl acetate solution (Electron Microscopy Sciences) for 30 s, followed by blotting with filter paper, grids were then air dried for imaging using a ThermoFisher Talos Transmission Electron Microscope at a nominal magnification of 120,000x.

Preparation of implantable hydrogel with CPMV and CPMV-Cy5

The implantable hydrogel was prepared as previously described[3]. Briefly, gelatin methacrylate (GelMA) was synthesized in a carbonate-bicarbonate buffer (pH = 9.2) with gelatin Type A (Sigma-Aldrich) and methacrylic anhydride (Sigma-Aldrich) per the protocol reported[4]. Polyethylene glycol diacrylate (Mn 700, PEGDA700) was purchased from Sigma-Aldrich. The materials were dissolved in PBS to reach the designated concentration to prepare the bioink. The digital-light-processing (DLP) printing was

mediated by photopolymerization which is initiated by lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP). The reagent was purchased from TCI Chemicals and added to the bioink to a final concentration of 0.6% (w/v). CPMV or CPMV-Cy5 were first resuspended in PBS, then added to the bioink to the designated concentration. The DLP printing was performed on an in-house developed bioprinter in a layer-by-layer manner, where a 405nm blue light source at 79.4 mW/cm^2 was used to illuminate bioink with a rectangular pattern delivered by a digital micro-mirror device. The thickness of each layer and number of layers were adjusted by a motorized stage to control the thickness of the implantable hydrogel. After printing, the implantable hydrogels were rinsed with PBS twice to remove excessive bioink and CPMV on the surface and subjected to characterization and animal studies immediately.

Characterization of bioprinted hydrogel

Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR analysis was conducted on as-printed scaffolds which underwent a lyophilization process and manually compressed to form a thin film. The analysis was performed using the PerkinElmer Spectrum Two FT-IR spectroscopy, equipped with a universal attenuated total reflectance (UATR) crystal. To ensure an optimal signal-to-noise ratio of this composition of samples, data was collected accumulating 32 scans.

Mechanical testing

The mechanical properties of the 3D printed scaffold were assessed in terms of Young's modulus using a microsquisher (CellScale) in compression mode. To suit the microsquisher's settings, the test samples were prepared as cylinders with a uniform height and diameter of 1.5 mm, replicating the material composition and printing conditions of the implantable hydrogels. The testing involved force measurement in response to displacement using the ramp function. The samples underwent a compression of 20% of their original height, with both the loading and recovery phases set to a duration of 0.125 seconds per um.

Scanning Electron Microscopy (SEM)

To investigate the internal micro-structure of the as-printed scaffolds, the samples were first lyophilized and manually sectioned. These slices were then affixed onto SEM stages with conductive tape and sputter-coated with iridium using Emitech K575X sputter coater. SEM imaging was performed with the FEI Apreo HiVac microscope operating at voltage of 3.00kV.

In vitro release study

CPMV implants were inserted into 1.7-ml Eppendorf tubes containing 1 ml of PBS and incubated at 37°C for a duration of 30 days, with continuous agitation at 100 rpm. Throughout this incubation period, we retrieved 1 ml aliquots of the samples on days 1, 3, 7, 10, 14, 20, and 30. To ensure a consistent release of particles, we supplemented each aliquot with 1 ml of fresh PBS. Quantification of the released CPMV particles was conducted utilizing a CPMV ELISA kit (CD Biosciences). This quantification involved referencing a standard curve established from pure samples at concentrations of 0.05,

0.1, 0.15, 0.25, and 0.5 µg/ml. Before testing, all the released CPMV samples were diluted 100 times in PBS. MaxiSorp 96-well plates (Thermo Fisher Scientific) were prepared by coating them with 100 µl of capture antibody, which were allowed to incubate overnight. Subsequently, the plates were washed five times with 200 µl of PBS containing 0.05% Tween-20 (PBST). Each well was then loaded with 100 µl of the CPMV standards and released CPMV samples, followed by an incubation period at room temperature for 2.5 hours. The plates were subjected to seven additional washes with 200 µl of PBST. Next, 100 µl of the conjugating enzyme was introduced into each well, and the plates were once again incubated at room temperature for 2.5 hours. After another seven washes as described above, 100 µl of detection substrate was added to each well and left to incubate at room temperature for 10 minutes. Finally, 50 µl of stop solution was introduced into each well, and the absorbance was measured at 405 nm using a Tecan plate reader. The recorded values were then matched against the standard curve to determine the quantity of CPMV particles present in each sample.

Cell culture

ID8-Defb29/Vegf-A-Luc murine ovarian cancer cells were cultured in RPMI 1640 medium with L-glutamine (Corning) supplemented with 10% (v/v) fetal bovine serum (FBS) (VWR), 1% (v/v) penicillin/streptomycin (Pen/Strep) (Cytiva), 1 mM sodium pyruvate (Thermo Fisher Scientific), and 0.05 mM β-mercaptoethanol (Thermo Fisher Scientific) in an incubator at 37 °C with a 5% CO_2 atmosphere. To inoculate tumors, ID8-Defb29/Vegf-A-Luc cells were harvested using Trypsin-EDTA (Corning), washed 3 times using PBS (Corning), then resuspended in PBS for tumor inoculation.

In vivo studies

Mice studies were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego (UCSD) and were approved by the Animal Ethics Committee of UCSD. 7-weeks old female BALB/c and C57BL/6J mice were purchased from Jackson Laboratories.

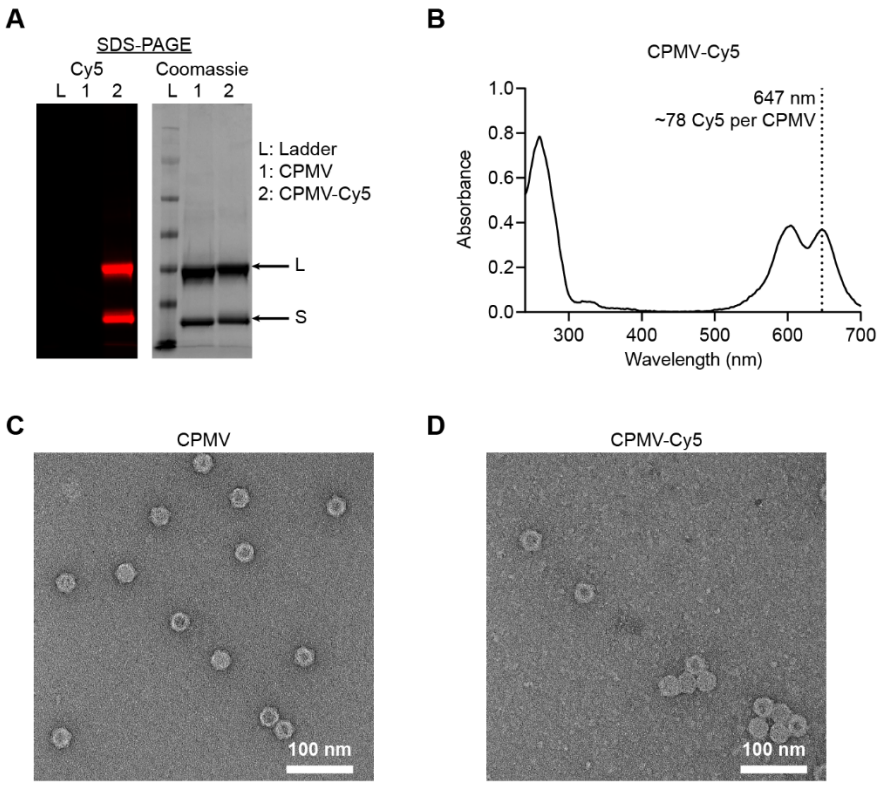
CPMV-Cy5 in vivo release study

BALB/c mice were fed with alfalfa free diet for 10 days prior to surgical implantation of the hydrogels to avoid autofluorescence. On day 0, hydrogels that contains 600 µg CPMV-Cy5 were implanted into the i.p. space (n=4). 600 µg soluble CPMV-Cy5 in 200 µL PBS was i.p. injected to serve as control. All mice were imaged using a Xenogen IVIS 200 imaging system to quantify Cy5 fluorescence within the i.p space.

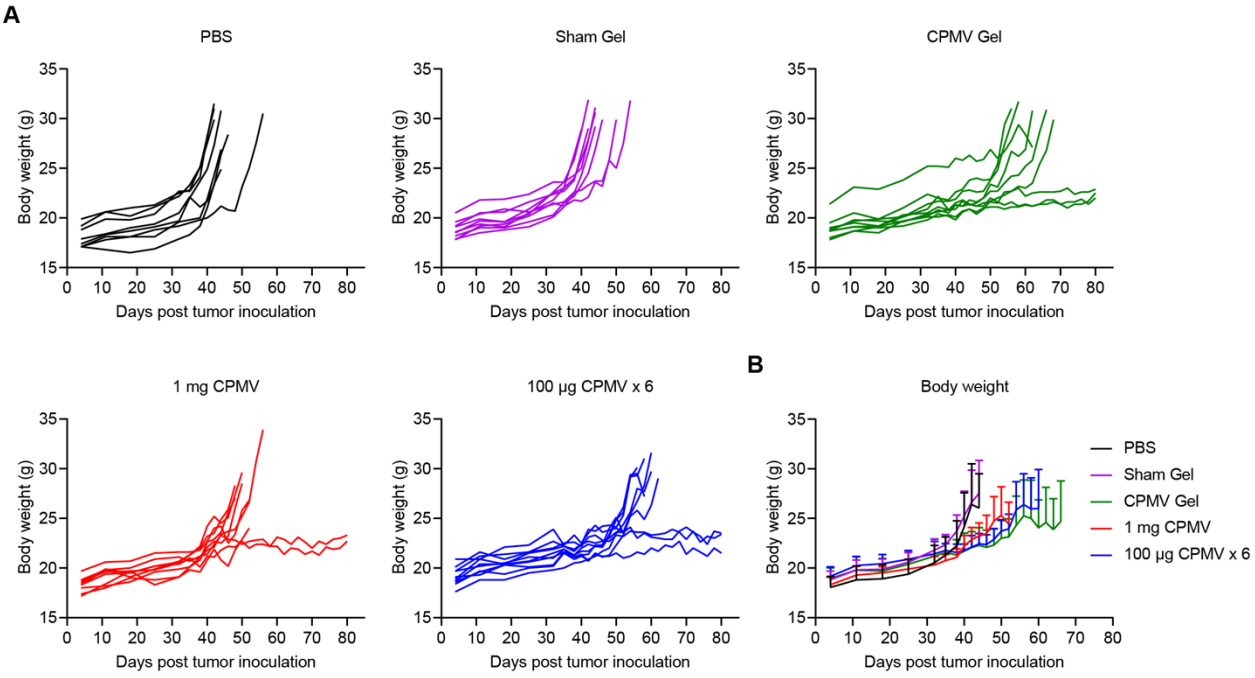
Efficacy Study

Efficacy studies were conducted using only female C57BL/6J mice because ovarian cancer only afflicts women. There were 5 treatment groups, PBS (n=8), sham gel (n=9), CPMV gel (n=9), soluble 1 mg dose of CPMV (n=9) and weekly administration of 100 µg-doses of CPMV (n=10). On day -3, mice from each group received their intraperitoneal (i.p.) implanted gels or first dosage of i.p. injection. Specifically, the PBS group received 200 µL PBS; the sham gel group received i.p. sham gel implantation through surgery; the CPMV gel group received i.p. hydrogel implantation containing 900 µg CPMV through surgery plus 100 µg soluble CPMV in 200 µL PBS; and another group received a 1 mg

bolus i.p. injection of CPMV (in 200 μL PBS). On day 0, 5×10^6 ID8-Defb29/Vegf-a-Luc cells in 200 μL PBS were i.p. injected into each mouse to inoculate ovarian tumors. For the PBS group and repetitive CPMV dosing group, mice received additional 5 weekly i.p. injections of 200 μL PBS or 100 μg CPMV in 200 μL PBS on days 4, 11, 18, 25 and 32. On day 50, all living mice that were treated with CPMV received an additional 100 μg CPMV injection in 200 μL PBS. Mice were monitored every 2 days, and the tumor burden was recorded as the increase in body weight and circumference. Mice were euthanized when their body weight reached 35 g, or their circumference reached 9 cm. In the repeated efficacy study, the experiment was carried out similar to the study above except the additional CPMV treatment on day 50, n = 8 per group.



Supplementary Figure 1. Characterization of CPMV and CPMV-Cy5. (A) NuPAGE of CPMV and CPMV-Cy5. (B) UV-Vis of CPMV-Cy5 to determine conjugated Cy5 per CPMV. TEM images of CPMV (C) and CPMV-Cy5 (D).



Supplementary Figure 2. Body weight of treated mice. (A) Individual body weight of treated mice. (B) Averaged body weight of treated mice in each group.

References

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