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Supporting Information

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Genome-Free Viral Capsids as Multivalent Carriers for Taxol Delivery

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Supporting Information

General Procedures and Materials

Unless otherwise noted, all chemicals were obtained from commercial sources and used without further purification. Analytical thin layer chromatography (TLC) was performed on EM Reagent 0.25 mm silica gel 60-F_{254} plates with visualization by ultraviolet (UV) irradiation at 254 nm and/or staining with potassium permanganate. Purifications by flash chromatography were performed using EM silica gel 60 (230-400 mesh). The eluting system for each purification was determined by TLC analysis. Chromatography solvents were used without distillation. All organic solvents were removed under reduced pressure using a rotary evaporator. Water (ddH_2O) used in biological procedures or as a reaction solvent was deionized using a NANOpure^{TM} purification system (Barnstead, USA) purchased from Aldrich. Solid phase extraction (SPE) columns were purchased from Phenomenex, Inc. (www.phenomenex.com) and used as specified, unless otherwise noted. Monoclonal goat Anti-mouse−Peroxidase (sc-205) and Mouse Anti-Taxol IgG antibodies (sc-69899) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used without further purification. Polyclonal goat anti-rabbit antibodies (ab6721) were obtained from Abcam Inc. (Cambridge, MA) and used without further purification. Polyclonal rabbit anti-MS2 antibodies were obtained from a previously reported procedure.[1] All cell culture reagents were obtained from Gibco/Invitrogen Corp (Carlsbad, CA) unless otherwise noted.

Instrumentation and Sample Analysis Preparations

NMR. ^{1}H and ^{13}C spectra were measured with a Bruker AV-500 (500 MHz), or a Bruker DRX-500 (500 MHz) spectrometer, as noted. ^{1}H NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to $CDCl_3$ (δ 7.26, singlet), or methanol-d_4 (δ 3.31, pentet).

Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), or m (multiplet). Coupling constants are reported as a *J* value in Hertz (Hz). The number of protons (n) for a given resonance is indicated as nH, and is based on spectral integration values. ^{13}C NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to $CDCl_3$ (δ 77.2, triplet), or methanol-d_4 (δ 49.00, septet).

Mass Spectrometry. High resolution Electrospray (ESI) and Fast Atom Bombardment (FAB^+) mass spectra were obtained at the UC Berkeley Mass Spectrometry Facility. Electrospray LC/MS analysis was performed using an API 150EX system (Applied Biosystems, USA) equipped with a Turbospray source and an Agilent 1100 series LC pump. Protein chromatography was performed using a Phenomenex Jupiter™ 300 5μ C5 300 Å reversed-phase column (2.0 mm x 150 mm) with a MeCN:ddH_2O gradient mobile phase containing 0.1% formic acid (250 μL/min). Protein mass reconstruction was performed on the charge ladder with Analyst software (version 1.3.1, Applied Biosystems).

High Perfomance Liquid Chromatography. HPLC was performed on an Agilent 1100 Series HPLC System (Agilent Technologies, USA). Size exclusion chromatography was accomplished on an Agilent Zorbax® GF-250 with isocratic (0.5 mL/min) flow or a Phenomenex PolySep-GFC-P 5000 (PS5K) column (300 x 7.8 mm, flow rate 1.0 mL/min) using an aqueous mobile phase (10 mM Na_2HPO_4, pH 7.2) . Reversed-phase liquid chromatography on protein samples was accomplished on a Agilent Poroshell 300 SB-C18 column (2.1 x 75 mm) using a MeCN:ddH_2O gradient mobile phase containing 0.1% trifluoroacetic acid. Semi-preparatory scale purification was performed using a Agilent Zorbax 300 SB-C18 column (9.4 mm x 25 cm). Sample analysis for all HPLC experiments was achieved with an inline diode array detector (DAD) and an inline fluorescence detector (FLD).

Gel Analyses. Sodium dodecyl sulfate-poly(acrylamide) gel electrophoresis (SDS-PAGE) was accomplished on a Mini-Protean apparatus from Bio-Rad (Hercules, CA) with 10-20% gradient polyacrylamide gels (BioRad, CA), following the protocol of Laemmli.[2] All electrophoresis protein samples were mixed with SDS loading buffer in the presence of dithiothreitol (DTT) and heated to 100 °C for 10 min to ensure reduction of disulfide bonds and complete denaturation unless otherwise noted. Commercially available molecular mass markers (Bio-Rad) were applied to at least one lane of each gel for calculation of the apparent molecular

masses. Gel imaging was performed on an EpiChem3 Darkroom system (UVP, USA).

Immunoblot Analyses. Western blot and dot blot analyses was performed using nitrocellulose membranes (GE Osmonics, Minnetonka, MN). Membrane blocking was achieved using a solution of 2% bovine serum albumin (BSA) in phosphate-buffered saline. Chemiluminescent signal was generated from horseradish peroxidase conjugates after a 1 min incubation with Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer, USA) and captured with a Molecular Imager ChemiDoc XRS+ System (BioRad, USA).

Centrifugations were conducted with an Allegra 64R Tabletop Centrifuge (Beckman Coulter, Inc., USA). General desalting and removal of other small molecules of biological samples were achieved using NAP-5 gel filtration columns (GE Healthcare). Protein samples were concentrated by way of centrifugal ultrafiltration using Amicon Ultra-4 or Ultra-15 100 kDa molecular weight cut off (MWCO) centrifugal filter units (Millipore), or Amicon Microcon 10 kDa, 30 kDa, and 100 kDa MWCO (Millipore) centrifugal filter units.

Dynamic Light Scattering. DLS measurements were performed on a Zetasizer Nano ZS (Malvern Instruments, UK). Samples were taken in 10 mM phosphate buffer pH 7.2 at 24 ºC. Data points are calculated from an average of three measurements, each of which consists of 10 runs of 45 seconds each.

Transmission Electron Microscopy (TEM). TEM images were obtained at the UC-Berkeley Electron Microscope Lab (www.em-lab.berkeley.edu) using a FEI Tecnai 12 transmission electron microscope with 100 kV accelerating voltage.

Experimental Procedures

N87C MS2 production.

The pBAD-MS2 plasmid and protein expression has been previously reported.[3] The pBAD-MS2-N87C mutant was made by site-directed mutagenesis of the pBAD-MS2 plasmid. Position 87 was converted to a cysteine using the following forward and reverse primers:

Forward: 5'–AGCCGCATGGCGTTCGTACTTATGTATGGAACTAACCATTC–3'
Reverse: 5'–GAATGGTTAGTTCCATACATAAGTACGAACGCCATGCGGCT–3'

Growth and purification of MS2-N87C was identical to that of wtMS2. Yields are slightly less than the 100 mg/L reported for wtMS2.

(*S*)-2,5-dioxopyrrolidin-1-yl-2-(*tert*-butoxycarbonylamino)-6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanoate (2). A solution of Boc-lysine (**1**, 492 mg, 2 mmol) and maleic anhydride (215.6 mg, 2.2 mmol) in 1 mL of DMF was stirred for 2 h at RT. The reaction was then cooled to 0 ºC for 10 min, followed by the addition of DCC (906 mg, 4.4 mmol) and NHS (288 mg, 2.5 mmol). The reaction was warmed to RT and stirred overnight, resulting in a pale orange slurry. The solid was isolated via filtration and washed with approximately 100 mL of CH_2Cl_2. The combined organic filtrates were washed with three 100 mL portions of dilute aqueous $NaHCO_3$ and one portion of brine, and then dried over Na_2SO_4. The resulting solution was filtered and the solvent was removed under reduced pressure. After purification by flash chromatography (1:1 to 8:2 EtOAc:Hexanes), 380 mg of product was isolated as a white filmy solid (45%). ^{1}H NMR (500 MHz, $CDCl_3$): δ, 1.39 (m, 11H), 1.59 (m, 2H), 1.78 (m, 1H), 1.91 (m, 1H), 2.78 (s, 4H), 3.48 (t, 2H, J = 7.0 Hz), 4.57 (m, 1H), 5.18(d, 2H, J = 8.5 Hz), 6.64 (s, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ, 22.1, 25.6, 28.0, 28.3, 32.1, 37.3, 51.8, 80.4, 134.1, 154.9, 168.4, 168.8, 170.9. HRMS (FAB^+) calculated for $C_{19}H_{25}N_3O_8$ ($[M+ Li]^+$) 430.1802, found 430.1802.

(*S*)-2-(2-(*tert*-butoxycarbonylamino)-6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanamido)ethanesulfonate (3). To a solution of **2** (190 mg, 0.45 mmol) in 5 mL of DMF was added DIPEA (0.9 mmol, 161 uL) and taurine (0.45 mmol) as a 0.5 M aqueous solution. The reaction solution was stirred for 6 h. The solvent was removed under reduced pressure and the resulting yellow oil was dissolved in MeOH. Purification was achieved using a Phenomenex Strata X-AW SPE column (loaded with MeOH, washed with MeOH and water, and eluted with 2% NH_4OH in MeOH) leaving a yellow solid (111 mg, 54%). ^{1}H NMR (500 MHz, MeOD): δ, 1.28-1.40 (m, 2H), 1.44 (s, 9H), 1.59 (m, 3H), 1.76 (m, 1H), 2.95 (t, 2H, J = 6.6 Hz), 3.49 (6, 2H, J = 7 Hz), 3.58 (t, 2H, J = 6.8 Hz), 3.93 (m, 1H), 6.80 (s, 4H). ^{13}C NMR (125 MHz, MeOD): δ, 24.2, 28.7, 29.2, 32.8, 36.6, 38.3, 51.4, 56.2, 80.7, 135.4, 157.9, 169.9, 172.6, 175.0. HRMS (ESI) calculated for $C_{17}H_{26}N_3O_8S^-$ ($[M]^-$) 432.1446, found 432.1453.

(*S*)-2-(2-amino-6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)ethanesulfonate (4). To a solution of **3** (10 mg, 23.2 μmol) in 1 ml of CH_2Cl_2 cooled to 0 ºC was added 1 mL of TFA. The resulting solution was stirred for 15 min. The resulting solution was concentrated under reduced pressure, and the product was precipitated by triturating with three 1 mL portions of

EtOAc. The product was obtained as a white solid (6.7 mg, 90%). HRMS (ESI) calculated for $C_{12}H_{18}N_3O_6S^-$ ($[M]^-$) 332.0922, found 332.0924.

Taxol maleimide conjugate 6. To a solution of taxol-succinate (10 mg, 0.011 mmol),[4] **4** (4.18 mg, 0.0126 mmol), and DIPEA (7.18 µL, 0.42 mmol) in 1 mL of DMF was added HATU (6 mg, 0.0158 mmol). The resulting solution was stirred for 4 h. After the solvent was removed under reduced pressure, the reaction mixture was first purified on a Phenomenex Strata X-AW SPE column (loaded with MeOH, washed with MeOH and water, and eluted with 2% NH_4OH in MeOH). The yellow solid was then purified by reversed phase HPLC on a C-18 semi-preparative column, using an isocratic 34% ACN/66% H_2O/ 0.1% TFA mobile phase. ^{1}H NMR (500 MHz, MeOD): δ, 1.13 (m, 6H), 1.23-1.35 (m, 15H), 1.54-1.66 (m, 6H), 1.70-1.84 (m, 3H), 1.91 (s, 3H), 2.10-2.20 (m, 4H), 2.39 (s, 3H), 2.44-2.68 (m, 3H), 2.78 (t, 2H, *J* = 6.6 Hz), 2.94 (m, 2H), 3.48 (t, 2H, *J* = 6.9 Hz), 3.52-3.66 (m, 2H), 3.80 (d, 1H, *J* = 7.2), 4.15-4.22 (m, 3H), 4.34 (m, 1H), 5.01 (m, 1H), 5.50 (d, 1H, *J* = 6.8 Hz), 5.62 (d, 1H, *J* = 7.2 Hz), 5.77 (t, 1H, *J* = 7.8 Hz), 6.04 (t, 1H, *J* = 9.1 Hz), 6.45 (s, 1H), 6.79 (s, 2H), 7.25 (t, 1H, *J* = 7.5 Hz), 7.41-7.56 (m, 6H), 7.61 (t, 2H, *J* = 7.7 Hz), 7.69 (t, 1H, *J* = 7 Hz), 7.85 (d, 2H, *J* = 7.3 Hz), 8.11 (d, 2H, *J* = 7.4 Hz). HRMS (ESI) calculated for $C_{63}H_{71}N_4O_{22}S^-$ ($[M]^-$) 1267.4286, found 1267.4304.

Typical MS2 modification with taxol maleimide 6. To a solution of N87C MS2 (5 nmol, 33 µL, 300 µM in phosphate buffer) was added **6** (25 nmol, 25 µL, 2 mM in pH 7.2 10mM phosphate buffer). The reaction mixture was incubated at RT for 1 h, followed by purification by size exclusion chromatography (NAP-5). Longer reaction times and/or higher buffer pH led to detectable degradation of both the starting maleimide and the taxol-MS2 conjugates. The use of higher concentrations of protein, as well as more equivalents (20x) of **6**, were able to increase the yield to almost 75%. However, this was typically avoided to maintain a workable volume of protein solution, and to conserve **6**. A control reaction was also run with wild type MS2, which lacked the N87C mutation, to test the reactivity of the native cysteine residues in the presence of **6**. No appreciable amounts of reaction product were observed using the conditions described above (Figure S1), demonstrating the low reactivity of the native cysteine residues.

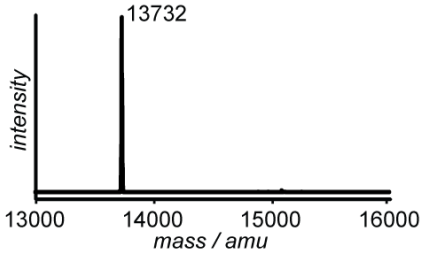

Figure S1. ESI-MS reconstruction of wtMS2 after 1 h reaction with **6**. Expected mass of wtMS2 m/z [M + H^+] = 13730 amu. Expected mass of taxol-MS2 m/z [M + H^+] =14997 amu.

Transmission Electron Microscopy. TEM grids were prepared by charging carbon-coated, Formvar-supported copper mesh grids with argon plasma (40 mA at 0.1 mbar for 30 s) in a Cressington 108 Auto Sputter Coater. Protein samples were prepared for TEM analysis by pipetting 5 µL samples onto these grids and allowing them to equilibrate for 3 minutes. The samples were then wicked with filter paper and rinsed with ddH_2O. The grids were then exposed to 5 µL of a 1% (w/v) aqueous solution of uranyl acetate for 90 s as a negative stain. After excess stain was removed, the grid was allowed to dry in air.

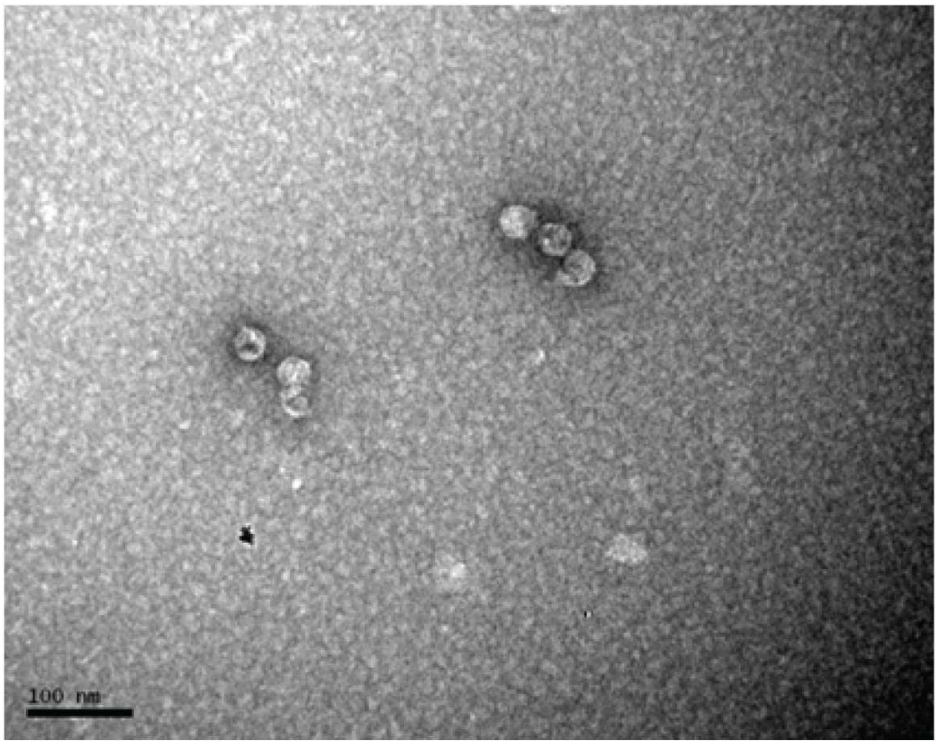

Figure S2. Transmission Electron Micrographs of taxol-modified N87C MS2.

General Procedure for Analysis of Taxol-MS2 by Dot Blot. Taxol-MS2 was prepared using the procedure described above. MS2 samples were denatured by making 1:1 solutions of 5 M aqueous GnHCl and MS2 in 10 mM phosphate buffer. To small rectangles of dry nitrocellulose membrane were applied 2 μl of 50 uM solutions of either wtMS2 or taxol-MS2. The membranes were air-dried for approximately 30 min before being blocked overnight with 2% BSA in phosphate buffered saline (PBS). After the removal of the blocking buffer, the nitrocellulose membranes were incubated with mouse anti-taxol IgG or rabbit anti-MS2 antibody (1:5000 dilution) for 30 min in PBS/0.1% Tween containing 2% BSA. The membranes were then washed four times (30 min each) with PBS/0.1% Tween. The membranes were then incubated with anti-mouse or anti-rabbit-HRP antibodies (1:5000 -1:20,000 dilution) for 30 min in PBS/0.1% Tween containing 2% BSA. The membranes were washed four times (30 min each) with PBS/0.1% Tween before imaging.

Evaluation of Cytotoxicity. Cell culture was conducted using standard sterile technique. MCF-7 cells were grown in Dulbecco's Modified Eagle Media supplemented with 10% (v/v) fetal bovine serum (FBS, HyClone), 1% non-essential amino acids, and 1% penicillin/streptomycin (P/S, Sigma). Antiproliferative activities of N87C MS2, taxol-MS2, and unconjugated taxol were determined using the Alamar Blue cytotoxicity assay.[5] Samples of 2.5 x 10^3 cells were added to individual wells of a 96-well microtiter plate and incubated at 37 °C for 24 h in 160 μL of media. Following this, 40 μL of sterile-filtered MS2-taxol (in PBS) was added to the cells. Concentrations ranged from 1 x 10^{-10} to 1 x 10^{-6} M, measured in terms of taxol (or in the equivalent concentration of unmodified MS2 monomer for the N87C control sample). The stock solutions were prepared in equal volumes of PBS, and in the case of pure taxol, with <1% DMSO as co-solvent. After 72 or 120 h of incubation in media at 37 °C, the media was removed via aspiration, and a 5% Alamar Blue solution in 10% FBS in PBS was added. The plates were then incubated for 4 h, and the inhibition of cell proliferation was quantified by determining the fluorescence at 530ex/590em with a microplate reader (Molecular Devices, San Diego, CA). For each sample, cell survival was calculated compared to untreated cells. Average cell survival values and standard deviations were calculated from six simultaneous replicate samples. Data after 3 d are shown in Figure S2, and data after 5 d are shown in Figure 4 of the main article.

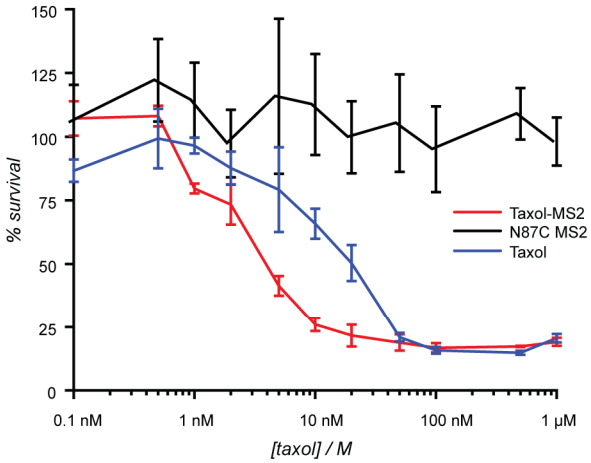

Figure S3. Cell viability assay using an MCF-7 cell line. Three days after the initial treatment, the cell survival was calculated relative to an untreated control by measuring the fluorescence resulting from treatment with Alamar Blue. Taxol-MS2 conjugates were delivered as a solution in 10 mM phosphate buffer, and free taxol was applied as a <1% solution of dimethyl sulfoxide in phosphate buffer. Error bars represent the standard deviation resulting from six replicate experiments.

Determination of Capsid Thermal Stability After Taxol Incorporation. A 2 µM sample of MS2 capsids that were 60% labeled with taxol-maleimide **6** was prepared in 10 mM phosphate buffer, pH 7.4. The particle volume distributions were determined using Dynamic Light Scattering (DLS) at 2 °C temperature increments, with 2 min sample equilibration at each time point. Capsid instability (which resulted in aggregation) was observed at temperatures above 64 °C, Figure S4.

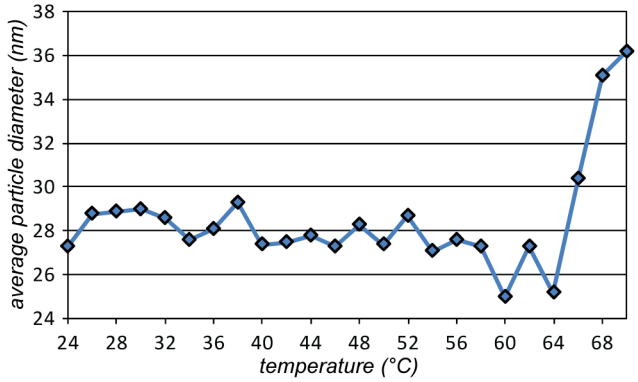

Figure S4. Thermal stability of MS2-taxol conjugates, as measured using DLS. Capsid instability (which results in aggregation) occurs at temperatures above 64 °C.

Determination of Capsid Stability Under Cell Culture Conditions. To verify that intact capsids were the predominant form of MS2 that was present during the cell culture experiments, N87C MS2 was labeled with Oregon Green maleimide dyes (Invitrogen) to allow facile detection in media samples. These samples were incubated for 6 days at 37 ºC in PBS, 10% FBS in PBS, and in media (all samples at pH 7.4). The amount of intact capsid was then measured using SEC (PolySep 5k column, with assembled capsids eluting at 8 min using a 1 mL/min flow rate), with tracking of the Oregon Green absorbance at 488 nm. For all samples, more than 80% of the labeled MS2 capsids were recovered in the assembled state, Figure S5a.

Determination of Taxol Release Kinetics. The quantitative determination of liberated taxol and/or remaining taxol-MS2 conjugates was made difficult by (1) the presence of numerous serum proteins in the FBS culture media and (2) the lack of a distinguishing UV signal for taxol itself. To circumvent these issues, taxol release was measured by first purifying intact capsids from the culture medium, and then by determining the percentage of capsid monomers still bearing taxol groups using RP-HPLC (as exemplified in Figure 2b of the main article). Taxol-MS2 samples were incubated at 37 ºC for 5 days in 10% FBS and aliquots were removed and analyzed every 24 h. Each sample was mixed with an equal volume of saturated ammonium sulfate. This served to isolate the capsids from most other serum proteins via precipitation of the capsids. The resulting suspension was centrifuged and the isolated pellet was resuspended in PBS (10 mM, pH 7.4). The resulting solution was next subjected to SEC using a PolySep 5k column. Intact capsids eluted at approximately 8 minutes using a 1 mL/min flow rate. The capsid-containing fractions were subjected to centrifugal ultrafiltration against a 100 kDa cutoff filter to concentrate the samples. Finally, the isolated capsids were analyzed by reversed-phase HPLC to separate MS2 monomers bearing and lacking taxol groups. The percentages of protein monomers still bearing taxol were calculated from the chromatograms, based on tryptophan fluorescence at 330 nm (280 nm excitation), Figure S5b.

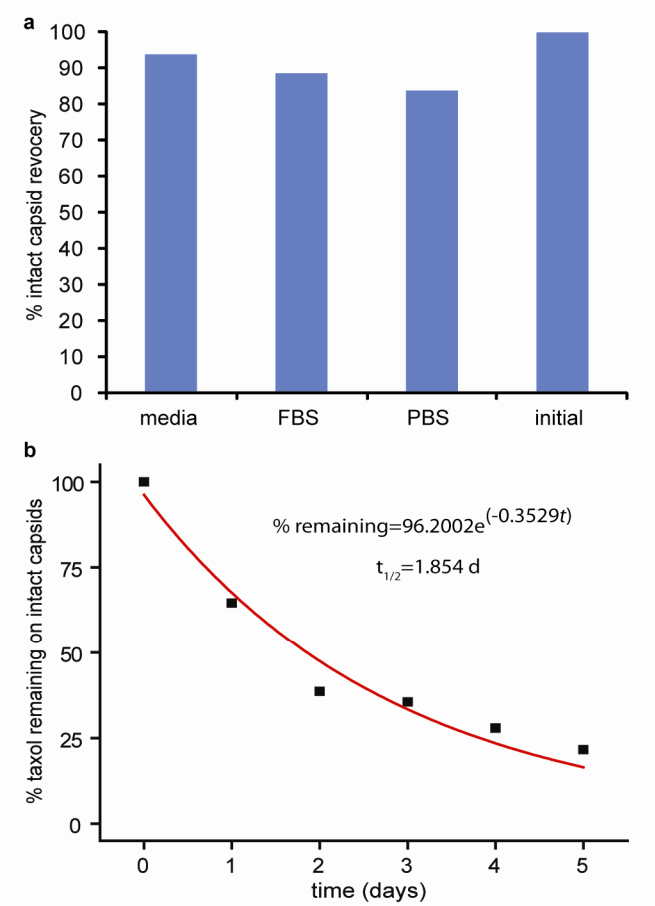

Figure S5. Stability of MS2 conjugates under cell culture conditions. **(a)** Samples of MS2 capsids labeled with Oregon Green maleimide at C87 were incubated at 37 °C for six days in the indicated solutions. Following this, the samples were analyzed using SEC. The amounts of the intact capsids remaining were quantified using the UV absorption at 488 nm. All samples were initially 10 μM, based on MS2 capsid monomer. **(b)** A drug release profile was determined for MS2 capsids containing taxol conjugate **6** at 37 °C. The initial capsid concentration was 10 μM in 10% FBS/PBS, pH 7.4. At the indicated time, the remaining intact capsids were isolated via ammonium sulfate precipitation and SEC. Subsequent RP-HPLC analysis of the capsid samples allowed separation of the protein with and without bound taxol (see trace in Figure 2b as an example). The taxol remaining in the capsid fraction was quantified using the tryptophan fluorescence (λ_{ex}=280 nm, λ_{em}=330 nm) of the associated protein.

References:

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