Supporting Information

Potato virus X as a novel platform for potential biomedical applications

Nicole F. Steinmetz,[†∏] *Marianne E. Mertens,*[†Δ] *Rebecca E. Taurog,*[‡] *John E. Johnson,*[‡] *Ulrich Commandeur,*[Δ] *Rainer Fischer,*[Δ¥]* *Marianne Manchester*[†∏]*

[†]Department of Cell Biology, [∏]Center for Integrative Molecular Biosciences, and [‡]Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA; [Δ]Institute for Biology VII (Molecular Biotechnology), RWTH Aachen University, Worringer Weg 1, and [¥]Fraunhofer Institute for Molecular Biology and Applied Ecology, Forckenbeckstrasse 6, 52074 Aachen, Germany

Supporting Materials and Methods:

1. Propagation of PVX

Propagation and purification of PVX was performed by standard procedures (http://www.scribd.com/doc/3288758/Protocols-for-the-Purification-of-Plant-Viruses). The concentration of purified virions was determined photometrically at a wavelength of 260 nm using the molar extinction coefficient of $\varepsilon = 2.97$ mL mg^{-1} cm^{-1}.

2. Biotinylation of PVX particles

2.1 Amine-selective chemistries

Biotinylation at amines derived from Lys side chains in the PVX coat proteins was carried out using biotin-LCLC-NHS esters (Pierce, Waltham, MA). PVX contains 1270 copies of CP subunits per particle. The biotin moiety was dissolved in DMSO and added in a 10-fold excess per coat protein (CP), to PVX (1 mg/ml) in 50 mM potassium phosphate buffer pH 7.0. The final DMSO concentration was 10% by volume. The mixture was allowed to react under shaking at room temperature overnight.

2.2 Carboxylate-selective chemistries

Carboxylates derived from Asp and Glu on PVX were probed using biotin-LC-hydrazide (Pierce, Waltham, MA) in combination with NHS and EDC (Sigma Aldrich, St. Louis, MO). Biotin-hydrazide was dissolved in DMSO and added to PVX (1 mg/ml) in a 10-fold excess per CP. The same excess was used for EDC dissolved in MilliQ water. NHS dissolved in DMSO was used in a 40-fold excess per CP. A final concentration of 10 % v/v DMSO was used. The reaction was allowed to proceed under shaking at room temperature overnight.

2.3 Carbohydrate-selective chemistries

To test whether the glycosylation state could be utilized for selective bioconjugation, carbohydrates were oxidized to give aldehydes followed by reaction with biotin-LC-hydrazide. An equal volume of ice cold sodium meta-periodate (Sigma Aldrich, St. Louis, MO) solution in 0.1 M sodium acetate buffer pH 5.5 and PVX (2 mg/ml) in 50 mM phosphate buffer pH 7.0 were mixed on ice. The reaction was protected from light and incubated for 30 min on ice. Excess of sodium meta-periodate was removed by purifying the mixture using 10 kDa cut-off spin columns (Millipore, Billerica, MA). Biotin-LC-hydrazide dissolved in DMSO was added in a molar excess of 20 moieties per CP. The reaction was allowed to proceed overnight at room temperature with shaking.

2.4 Western Blot analysis

To detect the biotin labels, samples (20 μg) were separated on a 4-12% NuPage Precast gel using MES SDS buffer (Invitrogen, Carlsbad, CA). After electrophoretic separation the proteins were transferred onto nitrocellulose membranes. The membranes were blocked for 60 min at room temperature using Tris-buffered saline (TBS) pH 7.6 containing 0.05% v/v Tween20 and 5% w/v milk, detection was carried out using alkaline phosphatase-conjugated streptavidin (GE Healthcare, Salt Lake City, UT; 1:1000 in TBS+Tween+milk). After 60 min incubation at room temperature with shaking the blot was washed several times with TBS+Tween20. Detection of alkaline phosphatase activity was carried out using BCIP/NBT liquid substrate system (Sigma Aldrich, St. Louis, MO).

3. Chemical bioconjugation of O488, A647, and PEG to PVX

OregonGreen 488 (O488), AlexaFluor 647 (A647), and PEG molecules were covalently attached to solvent exposed Lys residues on PVX using succinimide carboxylic esters (NHS). O488-NHS (5-isomer) and A647-NHS were purchased from Invitrogen, Carlsbad, CA. PEG succinimidyl ester with a molecular weight of 1000 Da and 2000 Da were purchased from NANOCS, New York, NY. The following formulations were made: PVX-O488, PVX-A647, P1 (PVX-PEG1000), and P2 (PVX-

PEG2000). For bioconjugation each label was dissolved in DMSO and used in a molar excess of 10 to 1 PVX CP. In addition, the following double-labeled formulations were made: P1-O488, P2-O488, and P2-A647. In order to generate double-labeled dye-PEG formulations, the dye and PEG label were each added at a molar excess of 5:1 per CP. All reactions were carried out with shaking overnight at room temperature (in the dark when working with a fluorescent dye). The final DMSO concentration was 10% by volume. Samples were purified by ultrapelleting in 50 mM phosphate buffer pH 7.0 (Beckman 50.2 Ti rotor, 40000 rpm, 3 hrs, 4°C). PVX was re-suspended in 50 mM phosphate buffer pH 7.0 and stored at 4°C.

4. Click chemistry on PVX

Synthesis of PVX-fluorescein was achieved using Cu(I)-catalyzed azide-alkyne cycloaddition (“click” chemistry),[1] where in the first step, an alkyne is installed to serve as a ligation handle for the click reaction with an azide-activated fluorescein conjugate (second step). Amine-selective chemistry was used to conjugate NHS-alkyne (*N*-(4-pentynoyloxy) succinimide) to amines derived from Lys side chains on the PVX CPs. The NHS-alkyne was added in a 10-fold molar excess per CP. The final DMSO concentration was 10% by volume. The reaction mix was incubated overnight at room temperature. The mixture was purified using 10 kDa cut-off spin columns (Millipore, Billerica, MA).

Azide-fluorescein, (5-carboxamido-(3-azidopropyl) fluorescein, was coupled to PVX-alkyne. This was accomplished using an optimized protocol.[1]

4.1 Quantification of alkynes per PVX using a Coumarin Assay

The fluorescence assay used to quantify the number of free alkynes per PVX was adapted from a previously reported method.[2] In brief, non-fluorescent 3-azidocoumarin was added to PVX-alkyne under click conditions as described above. Coupling of the non-fluorescent 3-azidocoumarin to an alkyne leads to the formation of a fluorescent triazolylcoumarin, which can be followed at 475 nm.

Propargyl alcohol at a known concentration was used as an internal standard, allowing quantification of free alkynes. The fluorescence assay was performed using a Varioskan Flash Fluorescence Reader (Thermo Electron Corporation, Waltham, MA).

5. Denaturing gel electrophoresis

Chemically modified PVX CPs were separated on a 4-12 % NuPage Precast gel using MES SDS buffer (Invitrogen, Carlsbad, CA). 20 μg sample were analyzed. After electrophoretic separation the proteins were stained using Safestain Blue (Invitrogen, Carlsbad, CA).

6. Size Exclusion Chromatography (SEC) of PVX particles

PVX-O488, P1-O488, P2-O488, P1, P2, PVX-A647, P2-A647, and PVX-Fluorescein particles were analyzed on a Superose6 column using the ÄKTA Explorer. 500 μl of 0.2 mg mL^{-1} concentrated samples in 50 mM phosphate buffer pH 7.0 were analyzed at a flow rate of 0.5 mg mL^{-1}.

7. Transmission Electron Microscopy (TEM) of PVX particles

PVX formulations (4-5 μl, ~1 mg/ml) were applied to continuous carbon grids (Ted Pella, Redding, CA), which were washed three times with 100 μl of deionized water, then stained with 4 μl of 2% (w/v) uranyl acetate. Images were collected on Kodak film with a CM100 electron microscope at 53,000 magnification and scanned at 605 dpi using a Fuji FineScan 2750xl (Hempel Hempstead, Herts, UK).

8. Quantification of fluorescent dye and PEG labels per PVX particle

Dyes were quantified using UV/visible spectroscopy and the specific extinction coefficient for O488 ($\varepsilon_{496\ nm}$ = 70,000 M^{-1} cm^{-1}) and A647 ($\varepsilon_{650\ nm}$ = 239,000 M^{-1} cm^{-1}). PEG labels per subunit were quantified by comparing the protein band intensity in denaturing protein gels after Coomassie staining using FluorChemSP software, as previously described.[3]

9. CPMV-O488 formulation

For bioconjugation of O488 to CPMV, O488-NHS was used. CPMV particles display 300 solvent-exposed addressable Lys side chains per particle.[4] Bioconjugation on CPMV has been extensively studied and a range of protocols has been developed. NHS-O488 was dissolved in DMSO, the labels were used in a molar excess of 3000 to CPMV (2-3 mg mL^{-1}), the reaction was carried out overnight at room temperature and in the dark. The DMSO concentration was adjusted to 10 % by volume. Samples were purified using gradient ultracentrifugation in 10-40 % sucrose gradients in 0.1 M phosphate buffer pH 7.0 (Beckman SW 28 Ti rotor, 28700 rpm, 3 hrs, 4 °C) followed by ultrapelleting (Beckman 50.2 Ti rotor, 42000 rpm, 3 hrs, 4 °C). CPMV was re-suspended in PBS and stored at 4 °C. Particles remained intact as confirmed by SEC using Superose6 (not shown). UV/visible spectroscopy (see also Section 8) was used to determine the number of dyes per particle. The molar extinction coefficient of CPMV is 8.1 $cm^{-1}mg^{-1}mL$. CPMV-O488 displaying 260 fluorescent labels per particle were used.

10. *In vitro* studies using confocal microscopy

Confocal microscopy studies were carried out using HeLa and BalbCl7 cells. Cells were seeded at $1x10^5$ cells/ml on glass bottom petri dishes (35 mm) and grown overnight in DMEM and MEM medium (Invitrogen, Carlsbad, CA), respectively, added 7 % fetal bovine serum and 1 % glutamine and 1 % penstrep.

Each virus formulation was added using 1 μg of the particles per petri dish (equates to 10E5 particles per cell) and incubated at 37 °C for 3 hrs. Cells were washed three times with DPBS to remove any excess PVX particles prior to fixation with fixation buffer containing 3% paraformaldehyde, 0.3% glutaraldehyde and 1 mM $MgCl_2$ in DBPS for 10 min at room temperature. Cell nuclei were stained by adding 4',6-diamidino-2-phenylindole (DAPI). In between each step the slides were rinsed three times with DPBS. Slides were mounted using Vecta Shield mounting medium (Vector Laboratories,

Burlingame, CA). Sections were imaged using a Biorad 2100 confocal microscope with a 60x oil objective. Data were analyzed and images were created using ImageJ.

Time course studies were also conducted: 1 μg of PVX-A647 was added to HeLa cells. Samples were collected at time point 0, 10 min, 60 min, 180 min, and 360 min post incubation. Cells were washed, fixed, cell nuclei stained, mounted, and analyzed by confocal microscopy as described above.

To gain further insights into whether the PVX particles are surface-bound or internalized, cell membranes were stained with A555-labeled wheat germ agglutinin (WGA-A555) and Z-dimensional data were recorded. Z-section were imaged at a step size of 0.3 μm. PVX (negative control), PVX-A647, or PVX-A647 and CPMV-O488 (positive control) were added at a concentration of 10E5 particles/cell and incubated with HeLa cells for 3 hrs. Cells were washed and fixed as described above. Cells were blocked using 5 % (v/v) goat serum in DPBS for 60 min at room temperature. Staining of the cell membranes was achieved by adding WGA-A555 (Invitrogen, Carlsbad, CA, USA) at a dilution of 1:200 in DPBS containing 1 % (v/v) goat serum and incubation for 60 min at room temperature. Cells were washed and nuclei stained with DAPI (as described above). Imaging was conducted using a Biorad 2100 confocal microscope with a 60x oil objective. Data were anayzed using ImageJ or Imaris software.

11. *In Vitro* Binding Studies using flow cytometry

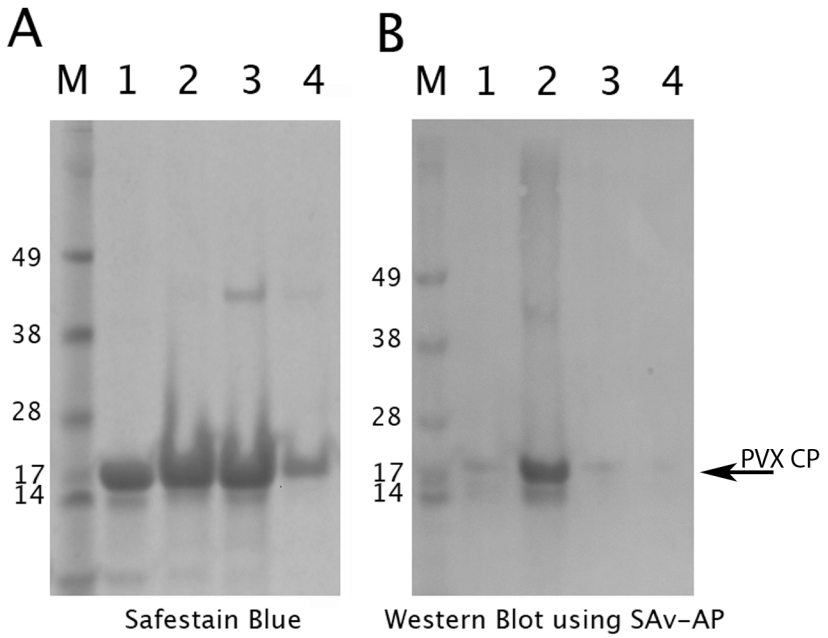
HeLa and BalbCl7 cells were grown in medium (see above). Cells were collected using Enzyme-free Hank's based Cell Dissociation Buffer (Gibco, Carlsbad, CA) and distributed in 200 μl portions at a concentration of 5x 10^6 cells/ml in 96-well V-bottom shaped plates. Different virus formulations were added using 1x 10^5 virus particles per cell and incubated at 37 °C for 3 hrs. To determine the threshold of how many viruses are required to render a positive signal studies were also conducted with 1x 10^3, 1x 10^4, and 1x 10^5 virus particles per cell. After incubation cells were washed two times with DPBS buffer containing 1 mM EDTA pH 8.0, 25 mM HEPES pH 7.5 and 1 % fetal bovine serum and fixed with 2 %

(v/v) formaldehyde in DPBS for 10 min at room temperature. Cells were re-suspended and analyzed using a FACS Calibur instrument (BD Biosciences, Franklin Lakes, NJ). At least 10,000 events were collected. Experiments were repeated at least twice and triplicates of each sample were measured and data were analyzed using FlowJo 8.6.3 software (Tree Star, Inc, Ashland, OR).

Supporting Data:

1. Probing PVX with different chemistries to test for addressable amino acid side chains

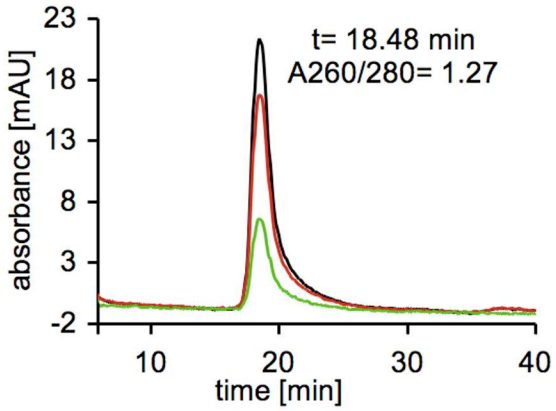
We employed three different chemistries in order to test the addressability of the particles: amine-selective, carboxylate-selective, and carbohydrate-selective reactions were applied. Biotin was used as a probe. Samples were analyzed by denaturing gel electrophoresis and Western blotting probed with streptavidin-alkaline phosphatase conjugates. Successful and efficient biotinylation was confirmed when applying amine-selective chemistries. Biotin-attachment was not indicated when using carboxylate-selective chemistries or carbohydrate-selective conjugation methods (Supporting Figure 1).



Supporting Figure 1. Characterization of PVX CPs after biotinylation reactions. A. Denaturing protein gel, 20 μg denatured PVX were separated on a 4-12% NuPage gel, proteins were stained using Safestain Blue. B. Western Blot probed with alkaline phosphatase-conjugated streptavidin (SAv-AP) to probe for biotin functionality. M = protein marker SeeBlue Plus2 (Invitrogen), the molecular weight of the marker bands are indicated in kDa. 1 = PVX, 2 = PVX + biotin NHS (amine-selective), 3 = PVX + EDC + NHS + biotin-hydrazide (carboxylate-selective), 4 = PVX + $NaIO_4$ + biotin hydrazide

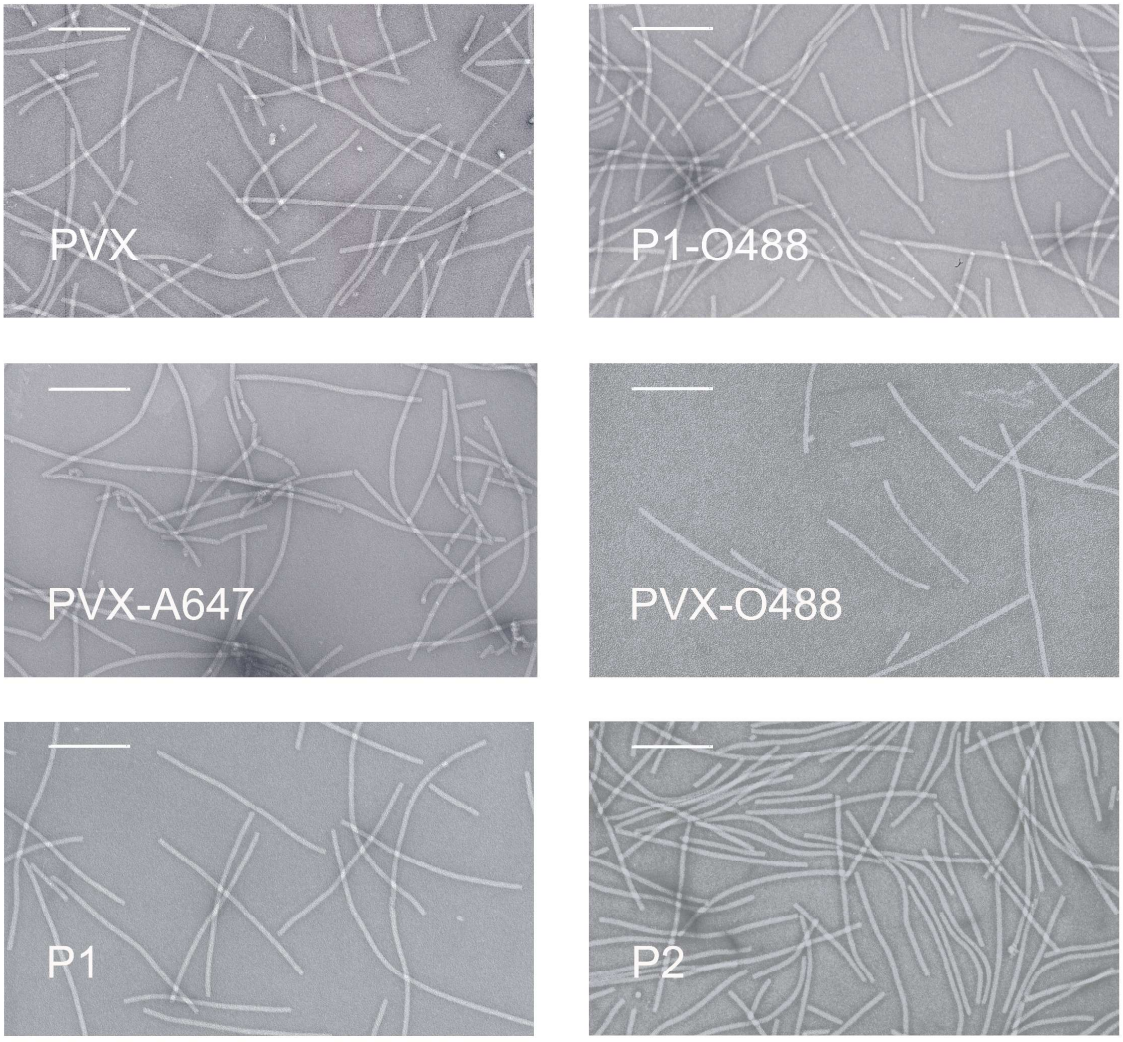
(carbohydrate-selective).

2. Size-exclusion chromatography of P1-O488



Supporting Figure 2. SEC of P1-O488 using a Sepharose6 column and AKTA Explorer FPLC instrument. The sample was analyzed at a flow rate of 0.5 mL min^{-1}. The elution profile per time (min) is given on the X-axis. Laser settings: black line = 260 nm (RNA), red = 280 nm (protein), green = 496 nm (O488). The inset shows the time at elution peak and the ratio of A 260 nm:280 nm which is 1.22 for native particles.

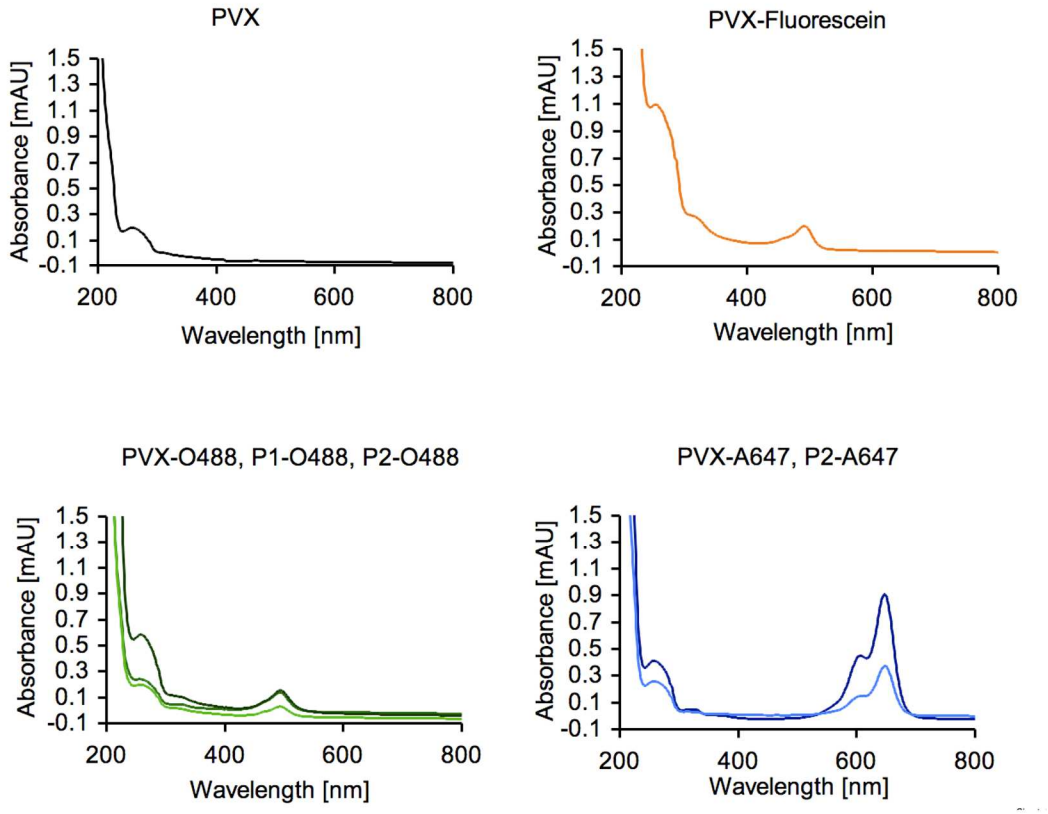
3. TEM data



Supporting Figure 3. TEM micrographs of chemically modified and negatively stained (UAc) PVX particle formulations. The scale bar is 200 nm. The magnification was 53,000x for all samples.

4. UV/visible spectroscopy data

To quantify the number of fluorescent labels per PVX UV/visible spectroscopy was used. The number of labels per PVX particle can be calculated based on the absorbance at λ_{max} and the dye-specific extinction coefficient (see Materials and Methods). The spectra are given in Supporting Figure 4.



Supporting Figure 4. UV/visible spectra of fluorescent-labeled and PEGylated PVX formulations. Black line = PVX, orange = PVX-Fluorescein (via click chemistry), green = PVX-O488, dark green = P2-O488, light green = P1-O488, dark blue = PVX-A647, light blue = P2-A647.

5. Calculation of the surface grafting area of the PEG chains on PVX

The flexible particle helix has a pitch of 3.4-3.6 nm and 8.875 subunits are organized per turn.[5] On average 1 PEG1000 is attached per turn for P1-O488, and 2-3 PEG2000 chains are found per turn on P2-O488 and P2-A647. Assuming that the PEGs are evenly distributed over the surface area, the average distance of two adjacent PEGs is around 200 Å for P1-O488 and 70-100 Å for P2-O488. The Flory dimension is given by $R_F = aN^{3/5}$, where a is the persistence length of the PEG monomer (a = 3.5 Å)[6] and N the number of PEG monomers (N = 23 for PEG1000 and N = 45 for PEG2000).[7] The Flory dimension of the PEG polymers was calculated and is R_F PEG1000 = 22.8 Å and R_F PEG2000 = 34.5 Å, respectively. Therefore for both particle formulations $D > R_F$ is given, hence the PEG chains are likely to be presented in a mushroom conformation. The theoretical surface area (Γ) occupied by a single PEG molecule can be calculated based on the molecular weight (MW), its density (ρ = 1.1 g cm^{-3}) and height (h = Flory Dimension R_F) using the following equation: $\Gamma = MW/(\rho * h * N_A)$ with N_A = Avogadro's constant[8]. The area covered by the PEG chains is Γ PEG1000 = 66.4 $Å^2$ and Γ PEG2000 = 87.4 $Å^2$. The total coverage area can be estimated taking the total number of labels into account and the dimensions of the PVX particle. We estimated that 0.5% of the surface area of P1-O488 is covered with PEG, for P2-O647 and P2-A647 coverage was 1.3% and 1.6% respectively. Of course, one must take into account that the flexible PEG chains potentially rotate around the attachment site on the virion and thus could occupy and block the surface area around that radius.

6. PVX-cell interaction monitored by flow cytometry

Supporting Table 1: Evaluating PVX-cell interactions using flow cytometry. HeLa cells and BalbCl7 cells were probed with various fluorescent dye-labeled and PEGylated PVX formulations. Mean fluorescence intensities (MFI) are given for each formulation tested. Samples were analyzed in triplicate; average values and the standard deviation (S.D.) are shown. Data were analyzed using FlowJo software. See also Figure 5 in the manuscript.

	Sample	MFI #1	MFI #2	MFI #3	Average	S.D.
HeLa	cells only	269.0	285.0	286.0	280.0	9.5
	PVX-O488	660.0	768.0	757.0	728.3	59.4
	P1-O488	492.0	415.0	662.0	523.0	126.4
	P2-O488	326.0	313.0	298.0	312.3	14.0
BalbC17	cells only	91.1	94.8	93.5	93.1	1.9
	PVX-O488	1065.0	1189.0	1246.0	1166.7	92.5
	P1-O488	458.0	358.0	292.0	369.3	83.6
	P2-O488	196.0	244.0	264.0	234.7	34.9
HeLa	cells only	1403.0	651.0	536.0	863.3	470.9
	PVX-A647	15298.0	24242.0	15415.0	18318.3	5130.4
	P2-A647	60329.0	45458.0	58244.0	54677.0	8051.7
BalbC17	cells only	245.0	213.0	206.0	221.3	20.8
	PVX-A647	18967.0	12791.0	11847.0	14535.0	3867.1
	P2-A647	48494.0	57962.0	71463.0	59306.3	11543.4

	Sample	MFI #1	MFI #2	MFI #3	Average	S.D.
HeLa	cells only	1403.0	651.0	536.0	863.3	470.9
10E3	PVX-A647	662.0	442.0	513.0	539.0	112.3
	P2-A647	692.0	583.0	484.0	586.3	104.0
10E4	PVX-A647	3733.0	1271.0	1165.0	2056.3	1453.0
	P2-A647	4036.0	4037.0	3969.0	4003.0	39.0
10E5	PVX-A647	15298.0	24242.0	15415.0	18318.3	5130.4
	P2-A647	60329.0	45458.0	58244.0	54677.0	8051.7

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