Supporting Information for

Retargeting polyomavirus-like particles to cancer cells by chemical modification of capsid surface

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EXPERIMENTAL PROCEDURES

*Mouse polyomavirus-like particles (*PVLP*)*

Spodoptera frugiperda (Sf9) cells were infected (10 PFU/cell) with recombinant baculovirus carrying the VP1 gene (GenBank accession number: AF442959) for production of mouse polyomavirus VP1 protein.[1] Cells were harvested 72 h post-infection and lysed. PVLPs were purified by cesium chloride gradient and concentrated by sucrose cushion as previously described [1,2] and resuspended in buffer B (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.01 mM $CaCl_2$).

*Rhodamine-labeled PVLP-alkyne (***PVLP****)*

The PVLP were dialyzed against 0.1 M HEPES, pH 7.9, and the protein concentration was adjusted to 2.5 mg/ml (7.5 mg VP1 protein in total). This solution was treated with NHS-rhodamine (ThermoFisher Scientific, final concentration 34 nM, 0.1 equivalents per surface lysine; each PVLP contains 720 surface-exposed lysines) at room temperature overnight with shaking. Two-thirds of the mixture were taken for preparation of **PVLP-Tf*** (see below) and the rest proceeded directly to **PVLP*** production. Excess dye was removed by dialysis against 0.1 M HEPES, pH 7.9 (4 °C, overnight). Finally, the particles were concentrated through a 20% (w/w) sucrose cushion (35,000 x g, SW41 Beckman rotor, 3 h) and dissolved in buffer B, providing the **PVLP*** conjugate (1 mg). Based on spectrophotometric analysis, we found 24 ± 1 molecules of rhodamine per particle.

Preparation of Alexa Fluor 488-labeled Tf-*azide conjugate (***Tf****)*

Holotransferrin (30 mg) was dissolved in 15 ml acetate buffer (0.1 M, pH 5.5). Sodium periodate ($NaIO_4$) was added on ice (final concentration 1 mM) and kept in the dark for 30 min. The resulting Tf-aldehyde was purified with HEPES buffer (0.1 M, pH 7.4) using an ultrafiltration cell and concentrated to 10 ml. 3-Aminooxypropylazide (16.2 mg in 500 ml DMSO) was added to Tf-aldehyde solution, and the mixture was gently stirred for 5 h. The product was purified with HEPES (0.1 M, pH 8) in an ultrafiltration cell and concentrated to 3 ml.[3] Lysine residues of Tf-azide were labeled with Alexa Fluor 488 SDP ester (0.6 mg) by stirring overnight and then purified with HEPES (0.1 M, pH 7.4) in an ultrafiltration cell, providing the **Tf*** conjugate. Concentration of **Tf*** was determined by amino acid analysis (5.0 mg/ml), and the ratio of Alexa Fluor 488 per **Tf*** was determined spectrophotometrically (1.8).

Preparation of **PVLP-Tf*** *conjugate by click reaction*

First, **PVLP*** was modified with heterobifunctional linker containing propargyl and N-hydroxysuccinimidyl ester moieties (Sigma-Aldrich, see Figure 1 for structure). To solution of **PVLP*** in 0.1 M HEPES, pH 7.9 (2.5 mg/ml; total amount 5 mg), 1.74 mg of the linker (35-fold excess per surface lysine) dissolved in 220 µL of DMSO was added (10% final concentration of

DMSO). The reaction mixture was incubated at room temperature overnight with light shaking. Excess reagents were removed by dialysis against 0.1 M HEPES, pH 7.4 (4 °C, overnight, first two buffer changes contained 10% DMSO) and concentrated in an Amicon Ultra centrifugal filter device (Millipore), providing **PVLP***-alkyne conjugate.

PVLP*-alkyne (4.8 mg in a final reaction volume of 6 ml) was reacted with **Tf*** (12 mg) in 0.1 M HEPES buffer, pH 7.4, containing 10 mM copper sulfate, 50 mM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, synthesized according to previously published procedure)[4] and freshly prepared 100 mM sodium ascorbate. Copper sulfate and THPTA were mixed in a separate tube in a 1:5 concentration ratio prior to addition to the reaction mixture. The reaction mixture was well-sealed, mixed, and allowed to stand undisturbed at room temperature for 3 h. The resulting **PVLP-Tf*** conjugate was purified from excess **Tf*** by dialysis (cellulose ester membrane, 300 kDa, Biotech) in 0.1 M HEPES, pH 7.4 (4 °C, overnight), and buffer B (4 °C, overnight). Finally, the **PVLP-Tf*** particles were purified and concentrated by two successive 20% sucrose cushions and dissolved in buffer B. The quality of the preparation was examined by SDS-PAGE, Western blotting, and electron microscopy. The amounts of VP1 were determined by Qubit protein assay kit (ThermoFisher Scientific).

SDS-PAGE and Western blot analysis

All types of prepared PVLPs were separated on SDS-PAGE (NuPAGE Novex 4-12% Bis-Tris precast polyacrylamide gels, ThermoFisher Scientific).[5] Proteins were stained with colloidal Coomassie dye G-250 according to the manufacturer's protocol (GelCode Blue Stain Reagent, ThermoFisher Scientific). For Western blot analysis and fluorescence scanning, proteins were electro-transferred (1 h, 250 mA) onto a nitrocellulose membrane (GE Healthcare Life Sciences). The membrane was scanned for rhodamine (excitation at 552 nm) and Alexa Fluor 488 (excitation at 488 nm) fluorescences on a PharosFX Molecular Imager (Bio-Rad). Subsequently, the membrane was used for immunodetection of transferred proteins. Nonspecific antibody binding sites were blocked by incubation with PBS containing 5% fat-free milk for 1 h. The membrane was then incubated for 1 h with specific monoclonal antibody against VP1 protein.[1] Unbound antibody was removed by washing in PBS, followed by incubation with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse, Bio-Rad) for 30 min. After washing in PBS, the proteins were exposed to X-ray film using a chemiluminescence detection system (Amersham ECL; GE Healthcare Life Sciences). The signal was quenched by incubation in a 30% solution of H_2O_2 (37 °C, 15 min) according to a previously published protocol[6] and the membrane was re-probed overnight with the primary antibody against Tf (Abcam; ab66952), in 10 µg/ml concentration. The membrane was incubated for 30 min with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit, Bio-Rad).

Immunodot blot analysis

All types of PVLPs were diluted either in buffer B or Laemmli buffer (0.1% 2-mercaptoethanol, 0.0005% bromophenol blue, 10% glycerol, 2% electrophoresis-grade SDS, 63 mM Tris-HCl, pH 6.8) to a concentration of 100 ng/µl. The samples in Laemli buffer were further denatured by boiling at 100 °C for 5 min. All samples (200 ng/one dot) were loaded onto a nitrocellulose membrane (GE Healthcare). Nonspecific antibody binding sites were blocked by incubation with PBS containing 5% fat-free milk for 1 h. The membrane was then incubated for 1 h with specific monoclonal antibody against VP1 protein.[1] Unbound antibody was removed by washing in PBS, followed by incubation with the secondary antibody for 30 min (horseradish peroxidase-conjugated goat anti-mouse, Bio-Rad). After washing in PBS, the proteins were exposed to X-ray film using a chemiluminescence detection system (Amersham ECL; GE Healthcare Life Sciences).

Densitometry analysis

The Coomassie-stained SDS-PAGE gels were scanned on a Molecular Imager GS 800 densitometer (Bio-Rad). The density was quantified with Quantity One image analysis software, version 4.5.0 (Bio-Rad). For calculation of **Tf*** to **PVLP-Tf*** particle ratios, two gels with standards were prepared: **Tf** and VP1 protein were loaded onto SDS-PAGE in various concentrations (0.1, 0.5, 1, 2, and 4 µg of each protein). Subsequently, a standard curve was made for calculation of protein concentrations from measured densities.

Electron microscopy

For morphology analysis, PVLPs were visualized by negative staining. Samples (7 µl) were adsorbed on carbon-coated formvar copper grids (Electron Microscopy Sciences). Grids were washed twice in redistilled H_2O and then contrasted on two drops of 2% solution of phosphotungstic acid (pH 7.3). The grids were visualized with a JEOL JEM-1011 transmission electron microscope.

Cell cultures

Spodoptera frugiperda (Sf9) cells were grown in monolayer in TNM-FH medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS, Gibco) at 27 ˚C as described by Hink.[7] The osteosarcoma cell line U2OS, lymphoblastoid leukemia cell line CCRF-CEM, and human vein endothelial cell line HUVEC were grown at 37 ˚C, 5% CO_2, in media supplemented with 4 mM L-glutamine (Gibco) and 10% FBS. The cultivation medium used for CCRF-CEM was Roswell Park Memorial Institute medium (RPMI-1640; Sigma-Aldrich), for U2OS it was Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich D5796), and for HUVEC it was Endothelial Basal Medium-2 with supplements (EBM-2 SingleQuots™ Kit; Lonza). The cells were subcultured 2 to 3 times per week. To detach adherent cells (U2OS and HUVEC) from the well, we used a solution of 0.25% trypsin and 0.03% EDTA in PBS.

Flow cytometry

The complete medium of adherent cells (U2OS and HUVEC) growing on 12-well dishes was replaced with 800 µl of serum-free medium (SFM). The suspended CCRF-CEM cells were counted, centrifuged (300xg, 5 min, 4˚C), dissolved in 800 µl of SFM, and transferred into a 12-well dish. When all cells were placed in SFM, one part of each cell type was treated with Tf (Sigma-Aldrich, 100µg/ml) for 30 min, 37 ˚C, 5% CO_2. Subsequently, the cells were incubated with **PVLP*** or **PVLP-Tf*** ($1 \cdot 10^6$ PVLP per cell; 0.54 nM) for 1 h at 37 ˚C, 5% CO_2. This incubation was performed in duplicates and with negative control (SFM only). After the treatment, cells were harvested either by scraping (CCRF-CEM) or trypsinization (U2OS, HUVEC). In case of incubation with trypsin solution, the activity of this enzyme was subsequently blocked by soybean trypsin inhibitor (ThermoFisher Scientific, 0.5 mg/ml). The cells were washed twice in 1 ml cold DPBS, resuspended in 300 µl DPBS, and filtered through 35-µm nylon mesh. The samples were analyzed with a BD LSRFortessa flow cytometry analyzer (Becton, Dickinson and Company) with collection of 10,000 events per sample. The data were analyzed with BD FACSDiva Software, version 6.0. Statistical analysis was performed in ANOVA R version 3.2.2. (2015-08-14).

Confocal microscopy

U2OS cells growing in a 24-well dish on cover slips were incubated for 30 min with Tf (Sigma-Aldrich, 100µg/ml) in SFM (DMEM, Sigma-Aldrich) or in SFM only. Subsequently, U2OS were treated with **PVLP*** or **PVLP-Tf*** ($1 \cdot 10^6$ PVLP per cell; 0.54 nM) and incubated for 1h. For experiments with fixed cells, fixation with 4% paraformaldehyde in PBS for 15 min was used. Cells were further permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 5 min, followed by extensive washing with PBS. The cover slips were mounted into ProLong Gold Antifade Mountant with DAPI (Invitrogen) and visualized with a Zeiss LSM 880 confocal microscope.

Fluorescence images were acquired with ZEN 2011 software. The images were further processed in Figi Is Just ImageJ software[8] with the Coloc 2 plug-in. Colocalization analysis was performed ex post in Coloc 2 plug-in from a representative section. After background subtraction, the images were merged with automatically computed threshold levels by Coste's method, and Manders' correlation coefficients were calculated.

UV-vis spectrometry

The spectra were recorded using an Analytic Jena Specord 250 plus spectrophotometer. The particle concentration was 150 µg/ml.

Dynamic light scattering

The particles (0.1 mg/ml) were measured using a Zetasizer Nano ZS system (Malvern Instruments) at 25 ˚C.

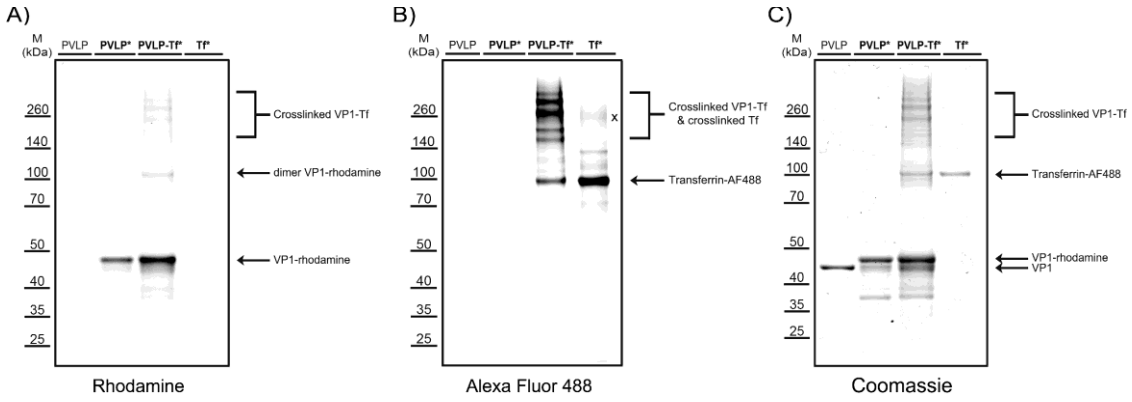

Figure S1. Characterization of **PVLP-Tf*** and **PVLP*** conjugates. PVLP, **PVLP***, **PVLP-Tf***, and **Tf*** were separated on SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was scanned for rhodamine fluorescence (A) or Alexa Fluor 488 fluorescence (B) or stained with Coomassie brilliant blue (C). The molecular weights are indicated on the left. The cross mark (x) represents crosslinked **Tf*** dimer. The other bands present in **Tf*** correspond to side products of **Tf*** modification and impurities contained in the starting Tf protein.

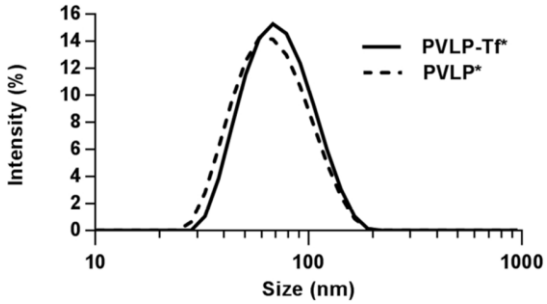

Figure S2. Characterization of **PVLP-Tf*** and **PVLP*** by dynamic light scattering. Obtained maxima were at 59 nm (**PVLP***) and 68 nm (**PVLP-Tf***), polydispersity indexes 0.15 and 0.12, respectively.

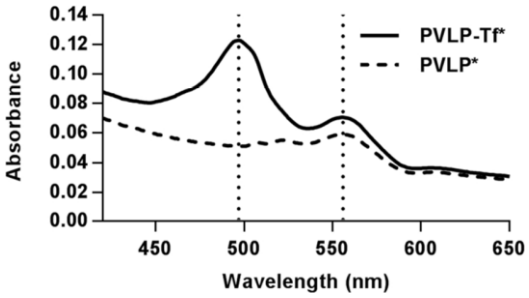

Figure S3. Extinction UV-vis spectra of **PVLP-Tf*** and **PVLP***.

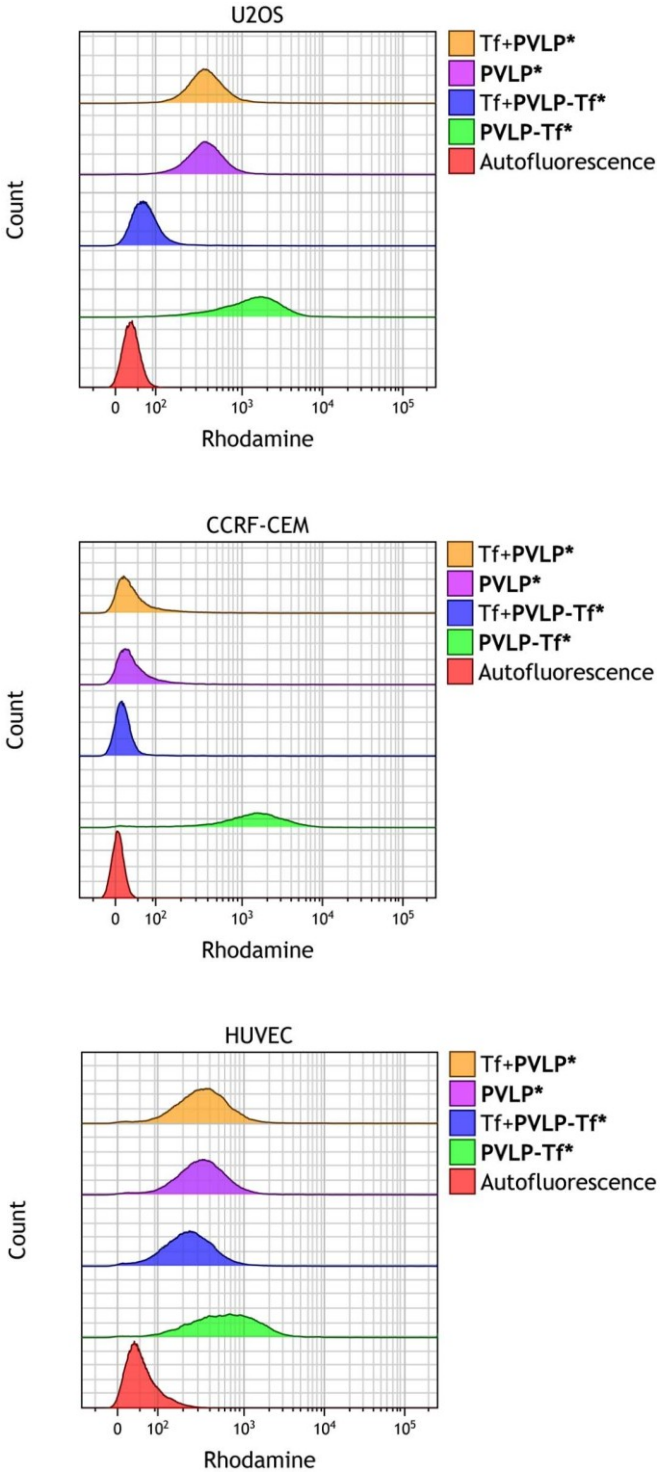

Figure S4. An overlay of representative histograms from flow cytometry measurements of U2OS, CCRF-CEM and HUVEC cells. Data were analyzed by Kaluza Analysis Software (v1.5a).

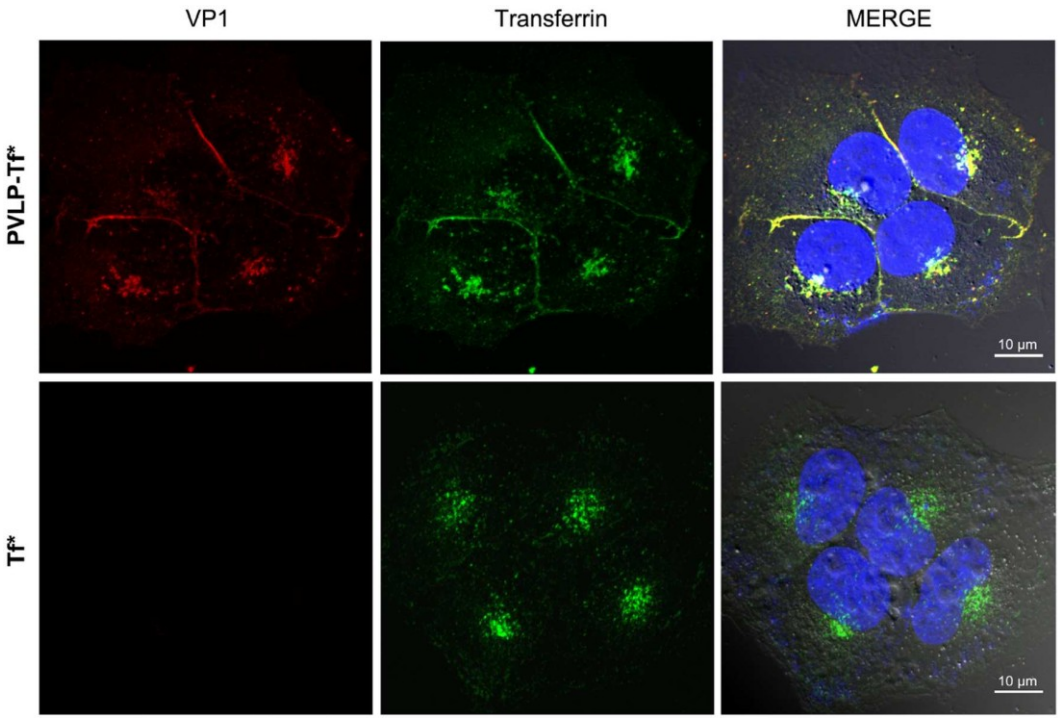

Figure S5. Comparison of intracellular localization of **PVLP-Tf*** and **Tf*** in U2OS cells. The cells were treated for 1 h with particles with **PVLP-Tf*** or **Tf***, fixed and processed for confocal microscopy. Confocal sections of representative cells with corresponding signal in green (Tf conjugated with Alexa Fluor 488 in **PVLP-Tf*** and **Tf***) or red (VP1 conjugated with rhodamine in **PVLP-Tf***) channels are shown. Nuclei are shown in blue (DAPI). Merge is composed of all three channels and bright field image. The images are shown as a maximum intensity Z-projection.

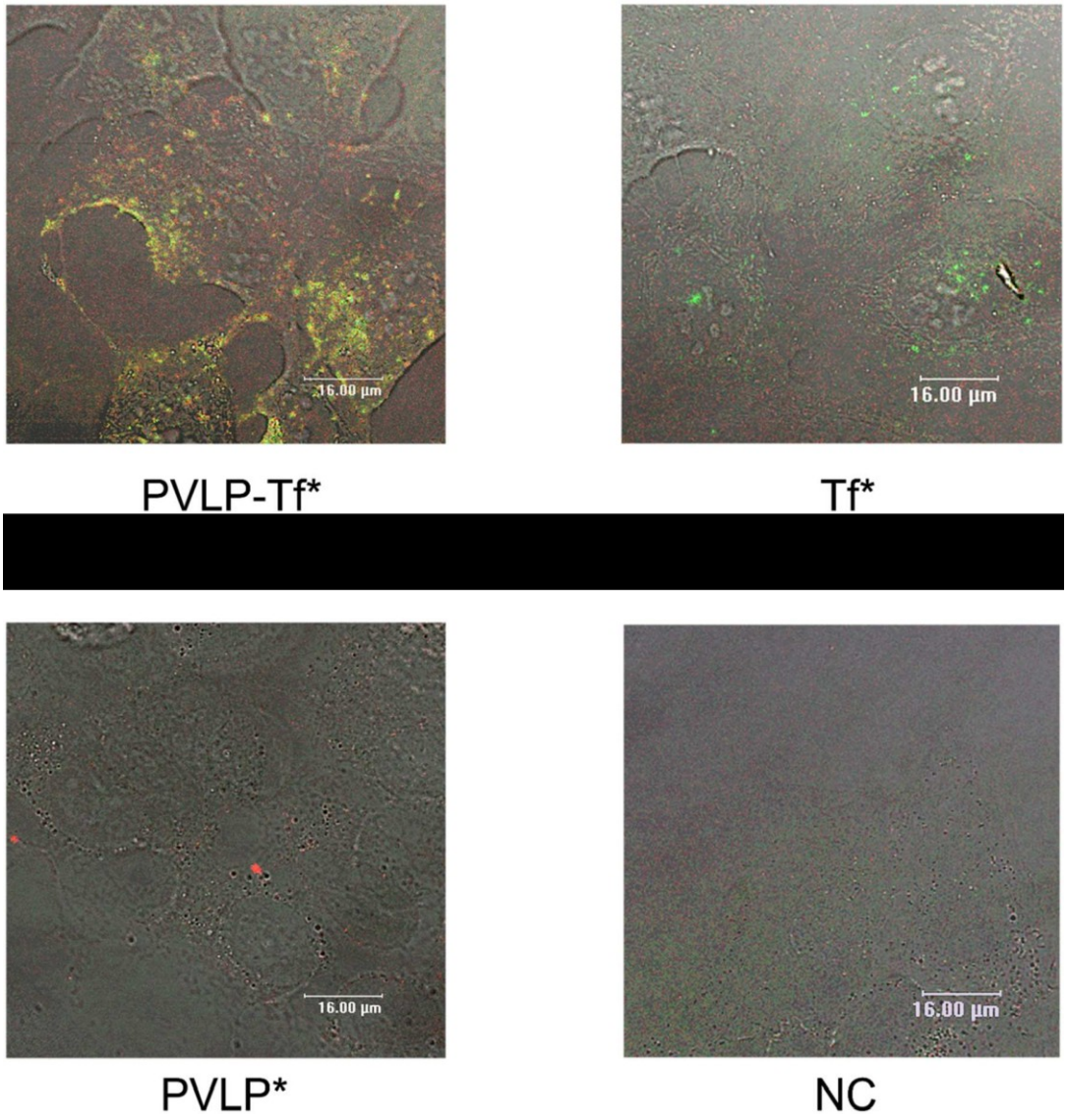

Figure S6. Live cell confocal microscopy of **PVLP-Tf*, Tf*** and **PVLP*** uptake by U2OS cells. The cells were treated for 1 h with particles or **Tf*** and imaged live with Leica TCS SP2 confocal microscope. Merged image of representative cells with corresponding signal in green (Tf conjugated with Alexa Fluor 488 in **PVLP-Tf*** and **Tf***) and red (VP1 conjugated with rhodamine in **PVLP*** and **PVLP-Tf***) channels with bright field channel are shown. U2OS cells incubated in serum-free media are shown as a negative control (NC).

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