Engineered M13 Bacteriophage Nanocarriers for Intracellular Delivery of Exogenous Proteins to Human Prostate Cancer Cells

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Materials and Instrumentation

PC-3 prostate cancer cells- ATCC
Phosphate buffered saline (PBS) - Hyclone/Thermo Scientific
0.25% Trypsin - Hyclone/Thermo Scientific
Brilliant Blue R-250 - J.T.Baker
Bovin serum albumin - Sigma Aldrich
Fetal bovin serum (FBS) - PAA Laboratories
Triton X-100 - Fisher Scientific
F-12K Nutrient Mixture (Kaighn's Mod.) - Cellgro/Corning
Mammalian cell culture dishes - Fisher Scientific
B-PER Bacterial Protein Extraction Reagent - Thermo Scientific
Imidazole - Sigma Aldrich
Modified Lowry Protein Assay Kit - Pierce/Thermo Scientific
Thiazolyl Blue Tetrazolium Bromide - Sigma Aldrich
PageRuler Prestained Protein Ladder - Thermo Scientific
NeutrAvidin-HRP - Thermo Scientific
TMB One Solution – Promega
15% Tris-HCl ready gel – Biorad
12% Mini-Protean TGX precast gels – Biorad
Anti-GFP antibody – Abcam
Anti-FLAG antibody – Abcam
Goat anti-rabbit IR Dye 800 CW – LI-COR
Odyssey blocking buffer – LI-COR

All water was obtained from a Milli-Q water purification system.

Instrumentation

MTT assay absorbance and TMB assay absorbance was measured on Synergy Mx Microplate Reader from BioTek.
Western blots were imaged on LI-COR odyssey imager.
Experimental Procedures and Data

**Mammalian cell culture.** PC-3 human prostate cancer cells were cultured in F12K with 10% Fetal Bovine Serum (FBS) and incubated at 37 °C with a 5% CO₂ environment.

**Genetic modification of M13 phage.** M13KE filamentous phage vector was purchased from NEB. Ypep(T7A) and the SBP sequences were inserted onto the N-terminus of p3 between the KpnI and EagI restriction sites. The biotin acceptor peptide (BAP) was cloned in using overlapping PCR to separate the C-terminal end of p7 and the N-terminal end and to insert the BAP sequence (GLNDIFEQKIEWHE) at the N-terminus of p9.

**Phage amplification and purification.** *E. coli* ER2738 was inoculated into an overnight culture for phage amplification. The next day, a fresh plaque of Ypep2(p3)/BAP(p9) or SBP(p3)/BAP(p9) phage was grown in LB containing a 1/100 dilution of the ER2738 culture and tetracycline. The phage were amplified overnight at 37 °C. The supernatant was collected and the amplified phage were precipitated with PEG 8000/2.5 M NaCl overnight at 4 °C. After resuspension of the phage pellet in 1 ml PBS, the phage were filtered with a 0.45 µm Nalgene syringe filter to remove any remaining bacterial cells. The concentration of the phage was determined using a plaque forming assay.

**SAV-GFP protein purification.** SAV-GFP-pETduet in BL21-DE3 *E. coli* was inoculated from an overnight culture into 1 L LB and grown at 37 °C and OD₆₀₀= ~0.6. Protein expression was induced with 1 mM IPTG at 30 °C overnight. Cells were harvested by centrifugation, the pellet was resuspended in 10 mL B-PER and stored at -20 °C. The cells were thawed and 10 mL of Tris lysis buffer (25 mM Tris-HCl, 100 mM NaCl, 10 mM Imidazole, pH=8.0) was added to the solution. The cells were sonicated for 2 minutes and the lysate was cleared by centrifugation (150000 rpm, 30 min.). The supernatant was mixed with 1 mL of Ni-NTA agarose resin for 1 hour and the resin was collected by centrifugation (4750 rpm, 10 min.). The resin was washed with Tris wash buffer (25 mM Tris-HCl, 100 mM NaCl, 20 mM Imidazole, pH=8.0) and the protein was eluted with Tris elution buffer (25 mM Tris-HCl, 100 mM NaCl, 400 mM Imidazole, pH=8.0). The protein was dialyzed against 25 mM Tris-HCl, 100 mM NaCl, pH=8.0 and analyzed for purity by SDS-PAGE staining with Coomassie blue. The concentration of the protein was determined by Modified Lowry Protein Assay Kit. The SAV-GFP-FLAG construct was purified in the same manner.

**In vitro phage biotinylation.** Phage (1×10¹² pfu/ml) were precipitated with PEG/NaCl and resuspended in 633 µl PBS. The in vitro biotinylation was carried out with a commercially available kit from Avidity. Briefly, 80 µL of Biomix A and Biomix B were added to the phage solution and mixed. Then 7 µl of 3 mg/mL BirA biotin ligase was added to the solution. The mixture was incubated overnight at 30 °C. The phage were purified by precipitation with PEG/NaCl two times and resuspended in PBS. Phage titer was determined using a plaque forming assay.

**Complexation of streptavidin-fusion proteins.** After biotinylation, the phage were incubated with either 20 µM SAV-GFP or 0.05 mg/ml NeutrAvidin-HRP in 500 µL PBS. The solutions were incubated for 3 hours at room temperature with rotation. The phage were purified by precipitation twice with PEG/NaCl at 4 °C and resuspended in 1 mL PBS. Phage titer was determined using a plaque forming assay.
Western blot of phage conjugated to SAV-GFP. Ypep2(p3)/SAV-GFP(p9) phage, SAV-GFP, and phage wash samples were run on 12% TGX precast gels (BioRad), and transferred to PVDF membrane with iBlot transfer stack (invitrogen). The blot was incubated with anti-GFP primary antibody overnight at 4 °C, then incubated with goat-anti-rabbit IR Dye 800 CW secondary antibody for 1.5 hours at room temperature. The western blot was imaged on the LI-COR Odyssey Imager.

Determination of complexation stoichiometry. FLAG(p3)/BAP(p9) phage were purified and isolated as described above. Complexation of SAV-GFP-FLAG to FLAG(p3)/BAP(p9) phage was carried out as previously stated. Samples were run on 10% TGX precast gels (BioRad), and transferred to PVDF membrane with iBlot transfer stack. The blot was blocked with milk for 45 minutes at room temperature, then incubated with anti-FLAG primary antibody for 15 minutes at 4 °C, then incubated with goat-anti-rabbit IR Dye 800 CW secondary antibody for 15 minutes at room temperature. The western blot was imaged on the LI-COR Odyssey Imager. Stoichiometry was determined by comparing band intensity of the FLAG(p3) and the SAV-GFP-FLAG bands.

TMB assay of HRP activity. Ypep2(p3)/SAV-HRP(p9) phage were precipitated twice from 1 mL of PBS, resuspended in 1 mL of PBS, and the final 1 mL wash was collected for the experiment. PBS, wash from phage precipitation, and Ypep2(p3)/SAV-HRP(p9) phage were added (20 µL) to a 96-well black sides, clear bottom plate. To each well, 20 µL of TMB One solution was added and incubated for 5 minutes at room temperature. TMB absorbance was read at 450 nm (yellow) on the Synergy Mx microplate reader.

TMB assay of internalized HRP phage. Ypep2(p3)/SAV-HRP(p9) and SBP(p3)/SAV-HRP(p9) phage were diluted to 50×10^9, 10×10^9, 5×10^9, and 1×10^9 pfu/mL in F12K/10% FBS media prewarmed to 37 °C. The solutions (0.4 mL to each well) were incubated with PC-3 prostate cancer cells in a 24-well plate for 3 hours at 37 °C, 5% CO_2. Cells were then put on ice for 5 minutes before beginning washing with cold reagents. Cells were washed twice with PBS, 3 times with TBS/0.1% tween-20, and 3 times with PBS/heparin sulfate. TMB One solution (250 µL) was added to each well and incubated at room temperature for 2 hours in the dark. Absorbance readings (655 nm) were taken on the Synergy Mx microplate reader.

MTT assay of IAA-HRP phage cytotoxicity. Solutions of indole-3-acetic acid (IAA), Ypep2(p3)/SAV-HRP(p9) phage and SAV-HRP were prepared in F12K/10% FBS media prewarmed to 37 °C. The various solutions were added to 60% confluent PC-3 prostate cancer cells in a 24-well plate and incubated for 24 hours 37 °C, 5% CO_2. After incubation the cells were washed twice with PBS, and then MTT reagent diluted in prewarmed F12K/10% FBS was added to the cells. The cells were incubated for 3.5 hours with the MTT reagent at 37 °C, 5% CO_2. MTT detergent reagent (4 mM HCl, 0.1% Triton X-100 in isopropanol) was then added to the cells and the cells were incubated for an additional 30 minutes. Absorbance at 570 nm was then recorded with the Synergy Mx microplate reader.
Figure S1. TMB assay of Ypep2(p3)/SAV-HRP(p9) complexation. PBS, Ypep2(p3)/SAV-HRP(p9) phage pellet wash and resuspended Ypep2(p3)/SAV-HRP(p9) phage was incubated with TMB One Solution for 20 minutes at room temperature. Absorbance was measured at 450 nm on the platereader.

It should be noted that at high concentrations of HRP, the TMB assay becomes oversaturated and turns yellow (as in Fig. S1,C).

Figure S2. Sequence information for all proteins used in this work.

**sfGFP**

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MGASKGEEELFTGVVIPILVELGDVNGHKFSVRGEGEGERDATNGKLTLLKFICTTGKLVPWPVLTV
TTLTYGVQCFSRYPDMHKQHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGETDLVNRIELK
GIDFKEDGNILGHKLEYNFNSHNYVITADKQKNGIKANFKIRHNVEDGSVLADHYQQNTPIG
DGPVLLPDNYHLSTQSLSDNPNEKRHDMLVLLEFVTAAIGITHMDELYKHHHHHH
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**SAV-GFP**

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MASMTGGQQMGRDQAGITGTWYNQLGSTFIVTAGADGALTGYESAVGNAESRYVLTGITYD
SAPATDGSQGTLGWVWAVKNNYRNASATTWSSGQYVGGAEARINTQWLLTSTGTTEANAWKS
TLVGHDTFTKVPSAASIDAAKAGVNNPPLDAVQQGGSGGSSGSSGGSGASKGEELFT
GVVIPILVELGDVNGHKFSVRGEGEGERDATNGKLTLLKFICTTGKLVPWPVLVTLLTYGVQCFSR
YPDHEMKQHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGETDLVNRIELKGDIFKEDGNIL
GHKLEYNFNSHNHYVITADKQKNGIKANFKIRHNVEDGSVLADHYQQNTPIGDPVLLPDMINYL
STQSLSDNDPNEKRHDMLVLLEFVTAAIGITHMDELYKHHHHHH
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**p9 BAP**

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MGLNDIFEQKIEWHGGSMSVLVYSSAFVLGWCLRSQITYFTRLMETS
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**SAV-GFP-FLAG**

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MASMTGGQQMGRDQAGITGTWYNQLGSTFIVTAGADGALTGYESAVGNAESRYVLTGITYD
SAPATDGSQGTLGWVWAVKNNYRNASATTWSSGQYVGGAEARINTQWLLTSTGTTEANAWKS
TLVGHDTFTKVPSAASIDAAKAGVNNPPLDAVQQGGSGGSSGSSGGSGASKGEELFT
GVVIPILVELGDVNGHKFSVRGEGEGERDATNGKLTLLKFICTTGKLVPWPVLVTLLTYGVQCFSR
YPDHEMKQHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGETDLVNRIELKGDIFKEDGNIL
GHKLEYNFNSHNHYVITADKQKNGIKANFKIRHNVEDGSVLADHYQQNTPIGDPVLLPDMINYL
STQSLSDNDPNEKRHDMLVLLEFVTAAIGITHMDELYKHHHHHH
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**DYKDDDDK**