Assembly of Hybrid Bacteriophage Qβ Virus-Like Particles

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SUPPORTING INFORMATION

Materials and Methods

Chemically competent BL21(DE3) *E. coli* and cloning vectors pET-28b and pCDF-1b were purchased from Novagen, Inc. For cloning, DH5α (Biopioneer, Inc.), NEB (New England Biolabs, Inc.) or TOP10 (Invitrogen, Inc.) *E. coli* were used. Vector pET-11d was a gift from Mr. Isaac Yonemoto and Prof. Claire Waterman-Storer of The Scripps Research Institute. Virulent bacteriophage Qβ was a gift from Prof. John Huelsenbeck of the University of California, San Diego. Oligonucleotides were purchased from IDT, Inc. and used without further purification. Restriction enzymes were purchased from New England Biolabs, Inc. Carbenicillin (100 μg/mL, 50 μg/mL for very low osmolarity media (VLOM)), kanamycin (100 μg/mL, 50 μg/mL for plates and VLOM), and spectinomycin (100 μg/mL, 50 μg/mL for plates and VLOM) were used as required. Protein analysis was performed on an Agilent Bioanalyzer 2100 according to manufacturer protocols. Anti-transferrin IgG and anti-chicken IgY HRP conjugate were purchased from Abcam, Inc.; TMB HRP substrate was purchased from Promega, Inc.. Anti-Qβ IgY was prepared by intramuscular immunization of hens with wild-type Qβ VLPs. After two boosts, a dozen eggs were collected from each hen, pooled, and total IgY was isolated by GenWay Biotechnology, Inc. The resulting polyclonal IgY sample was enriched in Qβ-binding fraction by affinity chromatography on Carboxy-Link resin (Promega, Inc.) derivatized with Qβ VLPs according to the manufacturer's protocol.

Media

Media used for initial experiments (defined minimal expression media, MEM) was based on the MDG formulation of Studier (*1*) with two modifications: methionine was added to 100 μg/mL and glucose omitted in favor of glycerol to 0.5% by weight. MEMS (MEM plus 5 w/v % sucrose), MEMSB (MEM plus 5 w/v % sucrose and 1 mM glycine betaine), and MEMC (MEM plus 0.2M NaCl) were prepared as MEM with a portion of the volume of water replaced by a concentrated solution of the additive. RMEM was prepared as MEM with two modifications: aspartate was omitted and 50xL salts was used in place of 50xM salts (*1*). VLOM media was prepared as described by Capp *et al.* (*2*). Protein concentrations were assayed with Thermo Coomassie Plus reagent. All protein concentration and ELISA assays were measured on a Thermo VarioSkan Flash microtiter plate reader.

Cloning Qβ CP and CP-Z fusions

The short coat protein gene was amplified from live phage by reverse transcription PCR using primers CP-F1 (catgCCATGGcaaaattagagactgttactt) and QBCP-R (gctagcGCTCAGCtcagtacgccggattcaag). The product was agarose gel purified before digesting with restriction endonucleases NcoI and BlpI. Cloning vector pET-28b(+) was digested with the same enzymes and the two fragments were ligated to give the vector designated pET28CP. The native Qβ genome packaging hairpin was inserted immediately downstream of the native TGA stop codon by whole-plasmid PCR using primers HairpinBluntF1 (taagacagcatctgcagaataactagcataaccccttggg) and HairpinBluntR1 (gacatgcatttcatccttatcaatacgctgggttcagctg). The product was circularized with T4 PNK

and T4 DNA ligase, giving plasmid pET28CPH. The Qβ coat protein gene was also cloned into the NcoI and BamHI sites of pET11d in the same manner as for pET28CP. The same primers were used to insert the Qβ genomic hairpin into this vector to create pET11CPH.

A vector for cloning of C-terminal fusions to CP was created in pCDF1b. A vector originally containing a Qβ-mCherry fusion generated by overlap-extesion PCR and cloned into the NcoI and XhoI site was modified to relocate the unique NcoI site at the N-terminus of the fused sequence. This vector was designated pCCP8-mCherry. An oligonucleotide encoding a single Z domain between NcoI and XhoI sites was purchased. This DNA was amplified with primers OptZ-FP (CATGCCatgGTTGACAACAAATTCAACA) and Z-R1 (CCGCTCGAG TTATTTCGGAGCCTGAGCGTCGT), and digested with NcoI and XhoI. This fragment was ligated into similarly digested pCCP8-mCherry to give vector pCCP8-Z.

Protein Expression and Purification

Competent BL21(DE3) *E. coli* cells were simultaneously transformed with either pET11CPH/pCCP8Z or pET28CPH/pCCP8Z and plated on SOB agar media containing appropriate antibiotics. Colonies were used no more than one week after transformation. Well-isolated colonies were picked from plates into 2 mL of freshly prepared selective liquid media as required by the experiment and shaken overnight at 37 °C. The following morning, cultures were pelleted and resuspended in 25 mL of freshly prepared media of the same type. Culture growth was monitored by optical density of 10:1 culture dilutions at 600 nm. When the OD_{600} reached approximately 2.0, 25 µL of culture was withdrawn and frozen for analysis. Protein expression was induced by addition of IPTG to 1 mM and shaking was continued at 37 °C for 4 hours, when another 25 µL sample was withdrawn and frozen. The remaining culture was pelleted in a JA-17 rotor at 15,000 RPM for 5 minutes. The supernatant was decanted and the pellet was resuspended in 10 mL of 1x TBS containing 10 µg/mL each of RNase I and DNase A and frozen overnight in 15 mL conical centrifuge tubes. Cultures were thawed in a 37 °C incubator, then chilled on ice. Cooled samples were sonicated in an ice bath (21W, 5 minutes total sonication time, in cycles of 5 s on, 5 s off). The cellular debris was pelleted in a JA-17 rotor at 15,000 RPM for 10 minutes. The supernatant was transferred to 15 mL conical centrifuge tubes and VLPs were precipitated with 0.265 g/mL $(NH_4)_2SO_4$. Samples were mixed on a rotisserie at 4 °C for 15 minutes and the precipitate was collected in a JA-17 rotor (15,000 RPM, 5 minutes). The pellet was redissolved in 0.9 mL 1x TBS containing 2 mM EDTA. Virus-like particles were then purified by 10-40% sucrose velocity gradients in an SW41 ultracentrifuge rotor (38,000 RPM for 3 hours). For each sample, a 3 mL band of the sucrose gradient containing the particles was removed. This solution was analyzed by FPLC size exclusion chromatography with Superose 6 gel and monitored by eluent absorbance at 260 and 280 nm. VLPs were precipitated with 10 w/v % PEG8000 and incubating on a rotisserie at 4 °C for 30 minutes. The protein content of the purified particles was determined by microfluidic gel electrophoresis on an Agilent Bioanalyzer 2100 using Series II Protein 80 chips. Peak integrations were taken directly from the instrument software's automatic analysis after verifying baseline calculations were appropriate. Each sample was independently prepared and purified at least two times by sucrose density gradient ultracentrifugation. The results were analyzed in the statistical analysis software R. A transmission electron micrograph of a representative sample of purified particles containing approximately 24 Z-domain appendages per particle is shown in Figure S1. These images are indistinguishable from those of wild-type Qβ VLPs. The same result was observed in cryo electron microscopy analysis (thanks to Prof. Bridget Carragher, Prof. Clint Potter, and Mr. Neil Voss of the Automated Molecular Imaging group at The Scripps Research Institute).

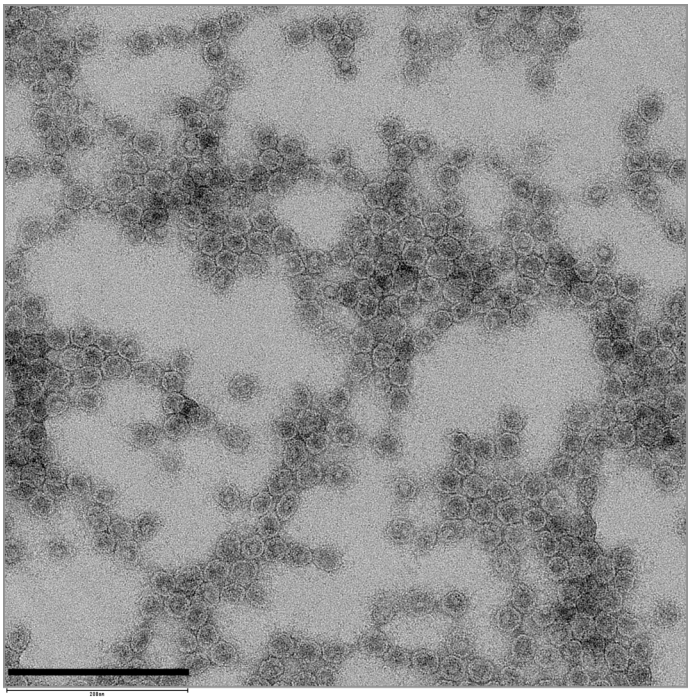

Figure S1. Transmission electron micrograph of a standard Qβ-Z domain hybrid particle containing an average of 24 Z-domain insertions per VLP. The particles were stained with uranyl acetate; the scale bar at the bottom left is 200 nm.

One case proved to be problematic: doubling times for cells incorporating the pET28CPH vector in VLOM media were found to be between 140 and 280 minutes, far longer than all other cultures (60-70 minutes). After four hours of induction, the resulting cell pellet appeared to be primarily cell debris and was accompanied by a decrease in OD_{600}.

Measurement of IgG binding by ELISA (Figure S2)

Anti-transferrin IgG was diluted 1000:1 with 1x TBS containing 0.02 w/v% NaN_3 (TBS-azide). 100 µL of this solution was placed in the wells of an ELISA plate and incubated at 37 °C for 1 hour. Wells were washed once with 200 µL of 1x TBS. Wells were then blocked with 200 µL of 5 w/v% dry milk in 1x TBS-azide at 37 °C for 1 hour followed by 3 washes of 200 µL of 1x TBS. Test particle samples (100 µL, wild type Qβ, Qβ-Z fusions, or buffer-only) at concentrations ranging from 0.01 to 1000 µg per mL was added to each well and incubated at 37 °C for 1.5 hours. Wells were washed 3 times with 200 mL of 1x TBS. Previously prepared anti-Qβ chicken IgY was diluted 100:1 with 1x TBS-azide and 100 µL of this solution was added to all wells. After overnight incubation at 4 °C wells were washed 3 times with 200 µL of 1x TBS. Goat anti-chicken IgY HRP conjugate was diluted 1000:1 with 1x TBS and 100 µL was added to each well. After incubation at 37 °C for 2 hours, wells were washed 4 times with 200 µL 1x TBS. 100 µL of TMB HRP substrate was added to each well and the plate was incubated at room temperature for 1 hour. 100 µL of glacial acetic acid was added to the wells and the absorbance at 450 nm was read in a plate reader.

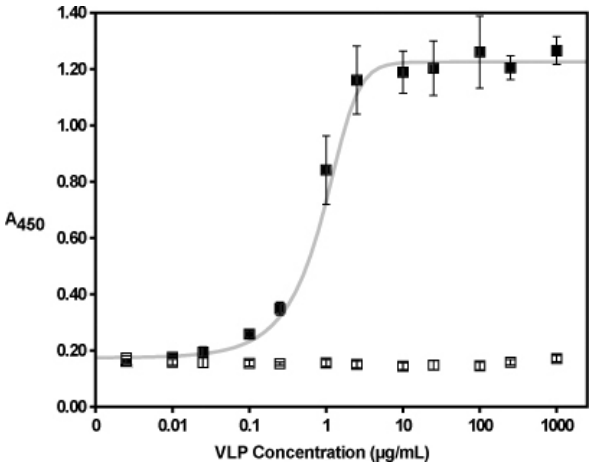

Figure S2. Enzyme-linked immunosorbent assay for binding to immobilized IgG. Empty squares represent wild-type Qβ VLPs; filled squares represent the hybrid Qβ VLP displaying an average of 20 Z domains per VLPs. The calculated ED_{50} of the Z-bearing VLPs was 1.2 µg per mL (0.47 nM in particle, 9.3 nM in Z domains).

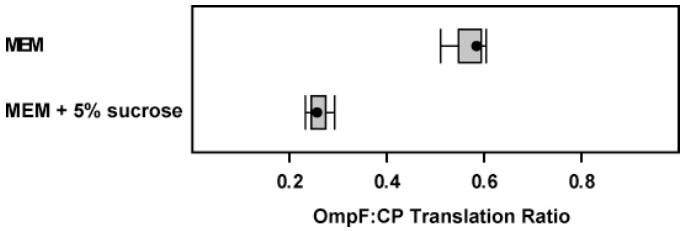

Figure S3. Box plot comparing OmpF to CP expression levels, calculated from normalized integration of peaks corresponding to the appropriate proteins obtained by microfluidic electrophoresis at 4 hours post-induction.

Discussion of plasmid ratio, copy number, and expression levels

The ratio of plasmids used to produce hybrid particles has been reported to be important for adeno-associated virus (AAV).(*3, 4*) We did not explore this parameter because of a fundamental difference between the Qβ/*E. coli* and AAV. In mammalian transfection methods, the amount of input DNA directly correlates to the gene copy number and thus protein expression levels. In *E. coli*, however, the origins of replication of the plasmids actively maintain a constant plasmid copy number, and we chose origins that do not interact or interfere with each other. We also controlled for differences caused by changes in growth phase by always inducing cultures at the same density, and therefore at the same growth stage. Most importantly, the number of Z domains incorporated into VLPs was found to be independent of the expression level of the subunits. In experiments with a different fusion protein, not described here, we tested this correlation in an extreme manner by placing the wild-type coat protein gene under the control of the weak Plac promoter while keeping the fusion protein under PT7 control. The resulting VLPs were obtained in very low yield since not much wild-type coat protein was produced, but the amount of fused protein per VLP was the same as in the standard system that has the coat protein under the control of the PT7 element.

References

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