Colorful Virus-Like Particles: Fluorescent Protein Packaging by the Qβ Capsid

Jin-Kyu Rhee, Marisa Hovlid, Jason D. Fiedler, Steven D. Brown, Florian Manzenrieder, Hiroaki Kitagishi, Corwin Nycholat, James C. Paulson, and M.G. Finn*

Supporting Information

A. Qβ basics – see Figure S1.

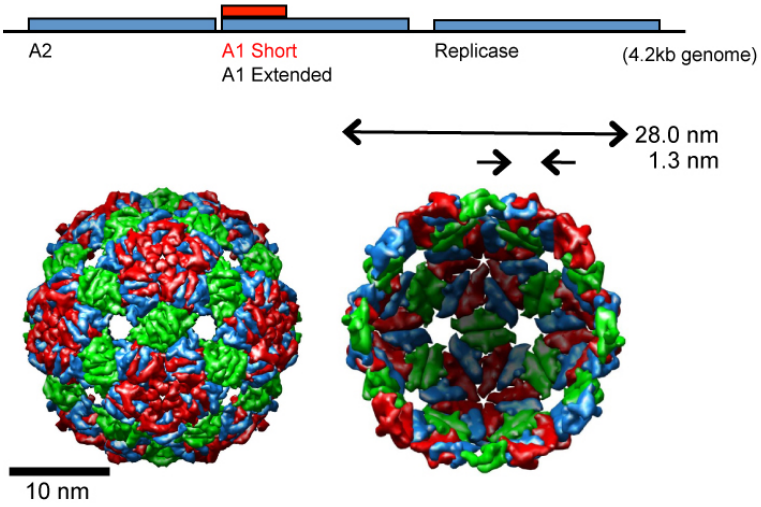

Figure S1. Genomic organization and capsid protein structure of the Qβ particle.

B. Particle Characterization – see Figure S2.

MALDI sample preparation was performed as follows. To a standard sample amount (4 µL of a 1 mg/mL VLP solution) was added dithiothreitol (DTT, 6 µL of 1 M solution), and the mixture was incubated at 37 °C for 1 h. The resulting solution was treated with iodoacetamide (10 µL of 100 mg/mL solution, final conc. approximately 0.5 M) and the tube was stored in the dark at 37 °C for 1 h. More DTT was then added (5 µL of 1 M solution), followed by drying in a Speed-Vac at room temperature. The sample was resuspended in 10 µL of water containing 0.2% trifluoroacetic acid (TFA).

For MALDI analysis (Voyager-DE, PerSeptive Biosystems), the sample was cleaned using ZipTips and the following solutions: wetting solvent = 50% aqueous aceteonitrile; equilibration solution = 0.2% aqueous TFA; wash solution = 95:5 water:methanol, with the water containing 0.1% TFA; elution solution = 35:65 water:acetonitrile, with the water containing 0.1% TFA. The final elution volume was 5 µL. 0.5 µL of the matrix material (sinapinic acid) was spotted on the MALDI plate, followed by 0.5 µL of the sample solution, and the two materials were mixed on the plate.

C. Fluorescence Properties

Non-encapsidated fluorescent proteins were made for comparison by subcloning the cDNA of each to produce an N-terminal His_6 fusion protein. The His_6-tagged proteins were

expressed in *E. coli* and purified on a Ni-NTA agarose column. The very similar excitation and emission properties of free and packaged fluorescent proteins are illustrated in Figure S3. Figure S4 shows similar rates of photochemical bleaching for free and packaged forms as well. In this experiment, the light flux at various wavelengths was not standardized, and so no quantitative conclusions should be drawn from this data concerning the photostabilities of the various fluorescent proteins with respect to each other. However, the results are roughly similar to the relative bleaching times previously reported for mCherry, EBFP, and ECFP/EGFP (3, 85, and 100 minutes, respectively).[1] Lastly, the dependence of the fluorescence emission of sfGFP on pH is shown in Figure S5, provided as justification for examining this material and its Qβ encapsidated version at pH 8. Figure 3 in the main text was obtained with free sfGFP (0.079 mg/mL, 2.9 μM) and Qβ@$(sfGFP)_{11}$ (6.5 mg/mL in total protein, 25 μM in sfGFP).

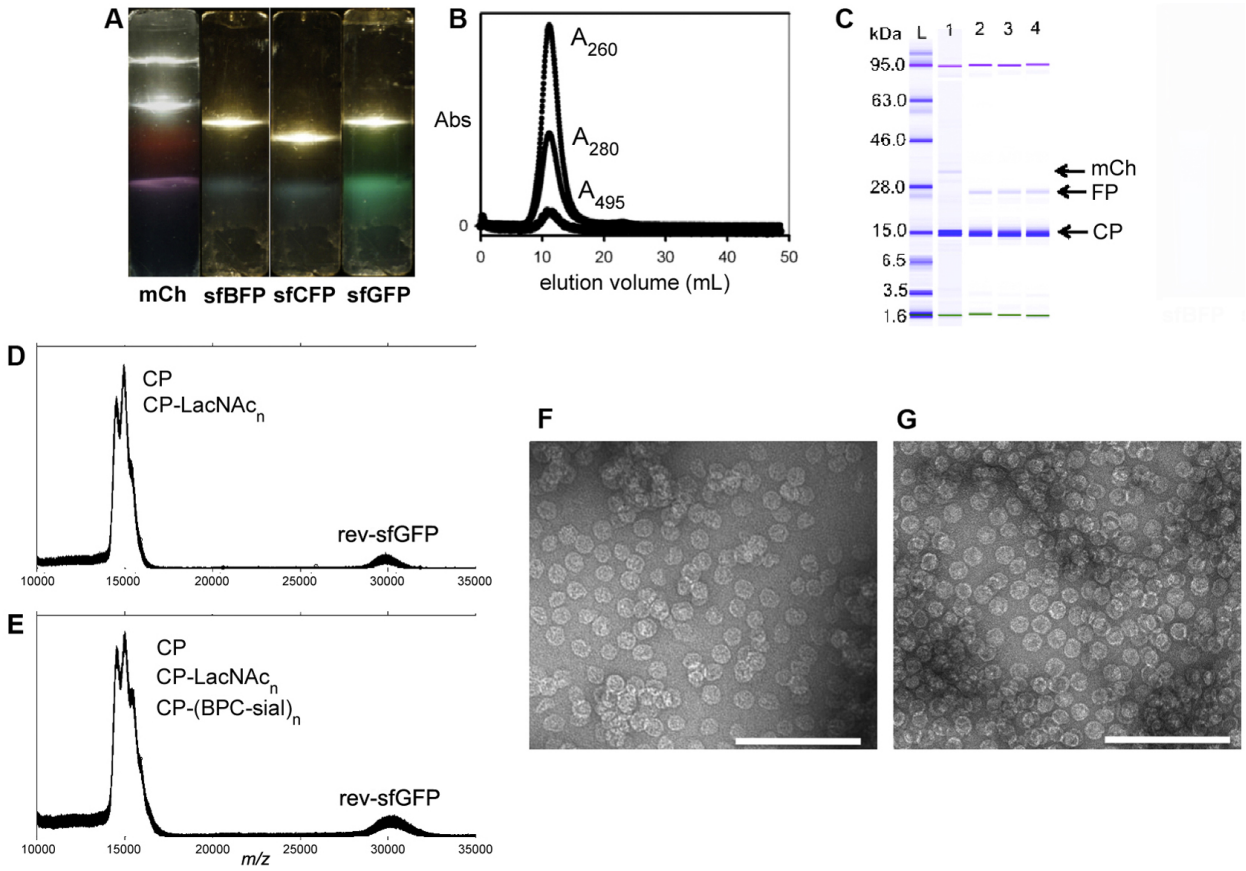

Figure S2. Characterization of Qβ VLPs containing superfolder fluorescent proteins. (A) Purification of Qβ particles containing the indicated fluorescent protein by sucrose density gradient ultracentrifugation (image under visible light). (B) size-exclusion chromatography of Qβ@$(sfGFP)_6$, representative of all of the packaged fluorescent proteins, showing an elution volume of 14 mL, characteristic of intact VLPs. (C) Electrophoresis (Agilent Bioanalyzer) analysis of purified Qβ@(protein) particles showing the band for CP and the following encapsidated proteins: mCherry (lane1), sfBFP (lane 2), sfCFP (lane 3) and sfGFP (lane 4). (D,E) MALDI-TOF of denatured particles **5** and **6**, respectively. (F, G) TEM of the particles used for E and F (scale bar = 200 nm).

1. Patterson, G.; Day, R.N.; Piston, D. Fluorescent protein spectra. *J. Cell Sci.* **2001**, *114*, 837-838.

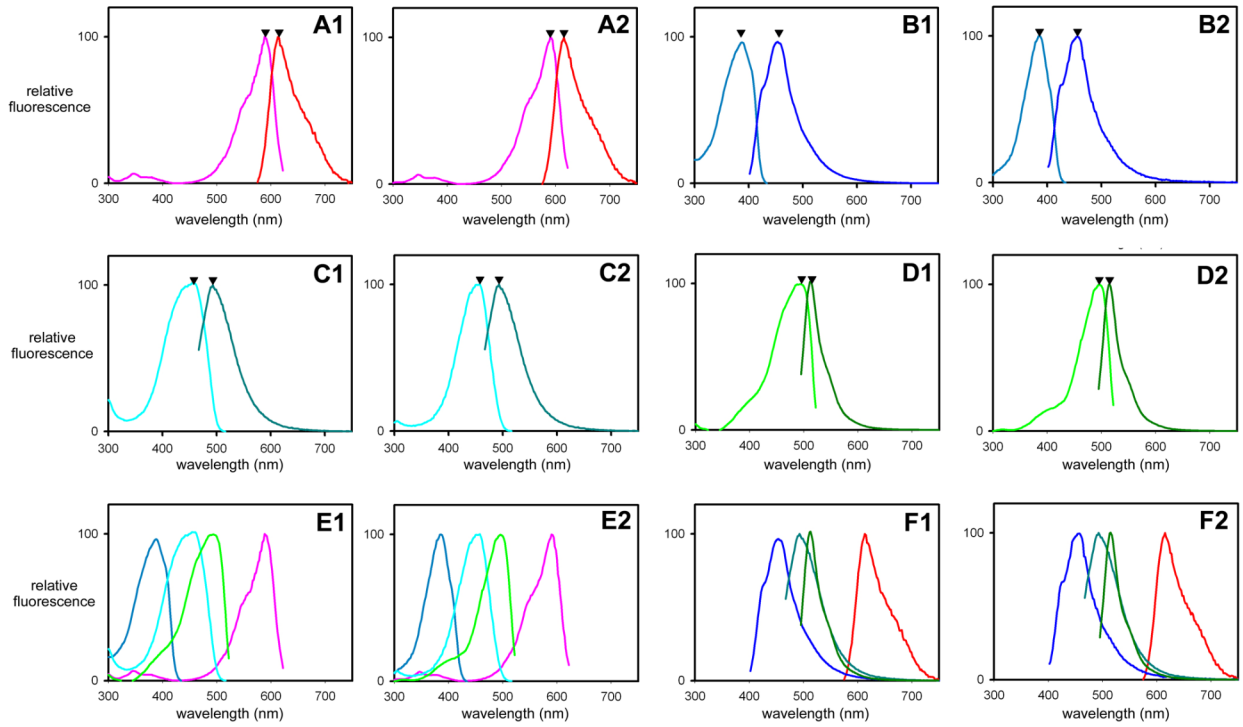

Figure S3. Excitation and emission spectra of the following proteins in TBS buffer at pH 8.0. (A1) mCherry, (A2) Qβ@mCherry, (B1) sfBFP, (B2) Qβ@sfBFP, (C1) sfCFP, (C2) Qβ@sfCFP, (D1) sfGFP. (D2) Qβ@sfGFP, (E and F) overlapped excitation and emission spectra, respectively, for free (E1, F1) and encapsidated (E2, F2) proteins. Excitation spectra were obtained monitoring emission at: mCherry = 613 nm, sfBFP = 454 nm, sfCFP = 489 nm, sfGFP = 513 nm. Emission spectra were obtained with excitation at: mCherry = 590 nm, sfBFP = 386nm, sfCFP = 454 nm, sfGFP = 488 nm. Comparing the "1" spectra to the "2" spectra shows the very similar wavelengths and peak shapes for free and encapsidated proteins.

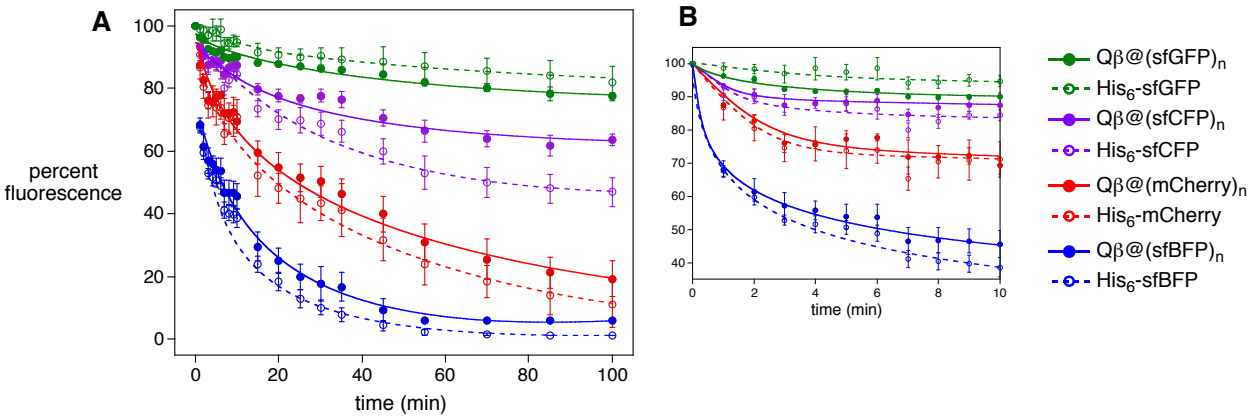

Figure S4. Photobleaching of superfolder fluorescent proteins. Fluorescence emission with continuous irradiation (room temperature) at the following wavelengths: mCherry, 613 nm (excited at 590 nm) sfBFP, 454 nm (excited at 386 nm); sfCFP, 489 nm (excited at 454 nm); and sfGFP, 513 nm (excited at 488 nm). Filled circles mark the fluorescence of the superfolder fluorescent protein encapsulated in the Qβ VLP, and open circles record the values for the His_6-tagged fluorescent protein. Panel B is an expansion for the first 10 minutes of the data in panel A.

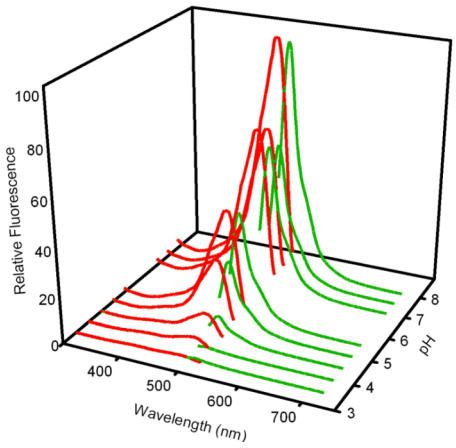

Figure S5. pH-dependence of excitation (red and emission (green) spectra for sfGFP.

Absorbance and Emission Intensity.

The packaged fluorescent proteins appear to have stronger absorbance (excitation) and emission spectra than the free (His_6-tagged) proteins on a per-chromophore basis. For example, Figure S6 shows peak intensities of excitation and emission spectra for free and packaged proteins, normalized to their concentrations. The samples were made in such a way as to provide similar intensities for the free and packaged pairs. However, in our experience the intensity *vs.* concentration values can vary quite a bit, based on the age of the fluorescent protein samples, their conditions of storage, and the presence of impurities. We are therefore not yet prepared to claim that packaged fluorescent proteins show inherently greater absorbance and emission than the non-encapsidated proteins. From a practical perspective, however, the order of apparent "brightness" of the packaged proteins are as indicated in Figure S6: Qβ@sfGFP > Qβ@mCherry >> Qβ@sfBFP ≈ Qβ@sfCFP.

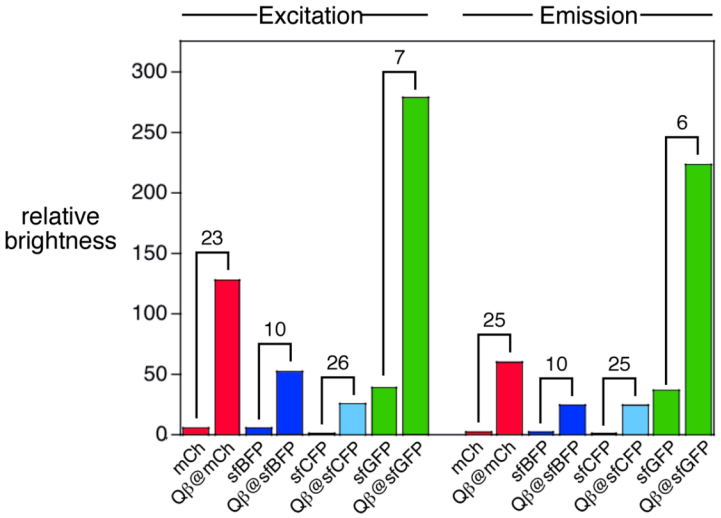

Figure S6. Relative peak intensities of emission and excitation spectra (recorded as noted in Figure S3) for the indicated proteins. The spectra were acquired at the concentrations listed below, and the intensity values were normalized by linear correction for concentration. A relative value of 1.0 was assigned to the lowest value in the absorbance (excitation) and emission series, respectively (in both cases, this was for sfCFP). The numbers above each paired set are the approximate factors by which the packaged proteins were found to be brighter than the free proteins.

[mCherry] = 4.9 mg/mL (163 μM); [Qβ@$(mCherry)_{16}$] = 3.7 mg/mL (21.7 μM in fluorescent protein)
[sfBFP] = 3.1 mg/mL (103 μM); [Qβ@$(sfBFP)_{7.9}$] = 3.8 mg/mL (10.8 μM in fluorescent protein)
[sfCFP] = 4.1 mg/mL (136 μM); [Qβ@$(sfCFP)_8$] = 2.6 mg/mL (7.4 μM in fluorescent protein)
[sfGFP] = 2.4 mg/mL (81 μM); [Qβ@$(sfGFP)_{8.1}$] = 3.5 mg/mL (10.1 μM in fluorescent protein)

D. Protection from protease digestion

To test the ability of the capsid to protect the encapsidated proteins from enzymatic degradation, Qβ@(sfGFP)$_n$ was treated with proteinase K, and the fluorescence monitored over time (**Figure S7**). These tests were performed with added detergent (SDS) to enhance peptide cleavage,[2,3] since the superfolder GFP variant used here is known to be resistant to cleavage in its absence.[45] Under these conditions, the encapsidated protein was completely stable over a period of 8 hours, during which time a majority of the fluorescence signal from the free protein was lost.

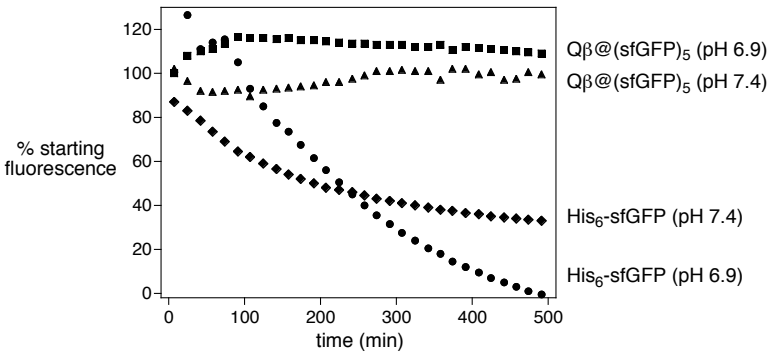

Figure S7. Time-dependent emission spectra for Qβ encapsulated sfGFP upon incubation with proteinase K at 25°C at the indicated pH (25 mM Na phosphate buffer for pH 6.9, 25 mM K phosphate buffer for pH 7.4, both with 0.5% SDS). Concentrations: sfGFP, 6.2 μM (168 μg/mL); Qβ@sfGFP$_{10}$, 1.25 mg/mL; proteinase K, 250 μg/mL. Fluorescence scans were collected at 20 min intervals.

E. Control of sfGFP packaging

sfGFP was used as the test case for an exploration of the factors contributing to packaging in the Qβ VLP. Table S1 shows the results of expression trials with the use or omission of each of the components of the packaging system, in SOB media. Note that the number of packaged sfGFP molecules followed fairly closely the ratio of expressed proteins (sfGFP *vs.* capsid protein), except when the Rev tag was omitted. In that case, expression of sfGFP was found to be much better, but packaging was not improved at all. We believe that the absence of the Rev tag in this case was counterbalanced by the much larger amount of sfGFP available, such that mass action effects compensated for the lack of a directing Rev-RNA interaction. Similar trends but modestly reduced packaging efficiencies were observed in minimal expression media (MEM), with the exception of an increase in packaging efficiency in the absence of the Qβ hairpin, which we cannot explain at present.

2. Hilz, H.; Wiegers, U.; Adamietz, P. Stimulation of proteinase K action by denaturing agents: application to the isolation of nucleic acids and the degradation of 'masked' proteins. *Eur. J. Biochem.* **1975**, *56*, 103-108.

3. Ebeling, W.; Hennrich, N.; Klockow, M.; Metz, H.; Orth, H.D.; Lang, H. Proteinase K from Tritirachium album Limber. *Eur. J. Biochem.* **1974**, *47*, 91-97.

4. Alkaabi, K.M.; Yafea, A.; Ashraf, S.S. Effect of pH on thermal- and chemical-induced denaturation of GFP. *Appl. Biochem. Biotechnol.* **2005**, *126*, 149-156 .

5. Saeed, I.A.; Ashraf, S.S. Denaturation studies reveal significant differences between GFP and blue fluorescent protein. *Int. J. Biol. Macromol.* **2009**, *45*, 236-241.

Table S1. Expression and packaging of sfGFP inside Qβ VLPs using SOB media.

A	B	C	D	E	F
Rev tag	Qβ hairpin	α–Rev aptamer	sfGFP expressed per 180 CP expressed [a]	sfGFP incorporated per VLP	average yield (mg/L) [b]
+	+	+	10.8. ± 2.1	10.5 ± 5.0	97.3 ± 2.5
+	–	+	8.4 ± 1.4	6.2 ± 1.6	29.0 ± 35.6
+	+	–	6.1 ± 2.4	4.0 ± 0.3	88.3 ± 36.8
+	–	–	4.7 ± 1.1	4.5 ± 2.0	197.3 ± 82.7
–	+	+	42.2 ± 4.8	6.6 ± 1.0	93.7 ± 54.3

(a) The capsid is composed of 180 coat proteins; therefore, this value represents the approximate number of sfGFP molecules that would be packaged inside VLPs if the packaging were dependent only on the relative number of proteins. (b) yield of purified particles.

Table S2. Expression and packaging of sfGFP inside Qβ VLPs using MEM media.

A	B	C	D	E	F
Rev tag	Qβ hairpin	α–Rev aptamer	sfGFP expressed per 180 CP expressed [a]	sfGFP incorporated per VLP	average yield (mg/L) [b]
+	+	+	13.0 ± 2.7	7.2 ± 3.6	72.2 ± 18.8
+	–	+	10.0 ± 2.3	9.4 ± 1.8	76.2 ± 18.9
+	+	–	5.5 ± 0.3	4.6 ± 1.1	70 ±17.1
+	–	–	2.2 ± 1.8	1.8 ± 0.5	139.3 ± 20.6
–	+	+	50.8 ± 20	2.3 ± 0.7	42.3 ± 5.7

(a) The capsid is composed of 180 coat proteins; therefore, this value represents the approximate number of sfGFP molecules that would be packaged inside VLPs if the packaging were dependent only on the relative number of proteins. (b) yield of purified particles.

The volume occupied by a sfGFP molecule is approximately 31 nm^3 (0.031 zeptoliters),[6] The inner diameter of the Qβ capsid is 21.4 nm,[7] ignoring the packaged RNA, giving a volume of 5.1 zeptoliters, approximating the capsid as a sphere. While GFP has a cylindrical shape, for the purpose of approximation in a general sense, we will assume that it is a sphere of radius 3.9 nm diameter (corresponding to the same volume of 31 nm^3). The maximum possible number of spheres that can be packed inside a spherical shell is an unsolved mathematical problem,[8] but can be estimated experimentally[9] at approximately 60% density (the fraction of the available space taken up by the packaged spheres). This corresponds to about 3 zeptoliters of space inside the Qβ capsid, or about 100 sfGFP molecules. If one wishes to take into account packaged RNA, the closest quantitative estimate available is that of the genomic RNA of bacteriophage MS2, which

6. Willard, L., Ranjan, A., Zhang, H., Monzavi, H., Boyko, R. F., Sykes, B. D. & Wishart, D. S. VADAR: a web server for quantitative evaluation of protein structure quality. *Nucleic Acids Res.* **2003**, *31*, 3316-3319.
7. Reddy, V.; Natarajan, P.; Okerberg, B.; Li, K.; Damodaran, K.; Morton, R.,; Brooks, C.I.; Johnson, J. VIrus Particle ExploreR (VIPER), a Website for Virus Capsid Structures and Their Computational Analyses. *J. Virol.* **2001**, *75*, 11943-11947 (http://viperdb.scripps.edu).
8. See, for example, Hifi, M. & M'Hallah, R. A Literature Review on Circle and Sphere Packing Problems: Models and Methodologies. *Adv. Operations Res.*, doi: 10.1155/2009/150624 (2009).
9. Scott, G.D. Packing of equal spheres. *Nature* **1960**, *188*, 908-909.

has been reported to occupy approximately 25-30% of the interior volume of that particle.[10] If the same volume of RNA is found inside Qβ VLPs, this would reduce the maximum number of sfGFP's that could be packaged to a value of 70-75 per particle. Considering the capsid as a nano-container, the concentration of 10 sfGFP molecules in the particle is approximately 3.2 mM.

F. Negative controls in confocal microscopy

Figures S8 and S9 show negative control experiments to complement Figure 4. Experiments published earlier with the same targeting ligand on Qβ VLPs bearing attached Alexa dyes in place of the encapsidated GFP showed that binding was abolished in the presence of added BPC-sialoside.[11]

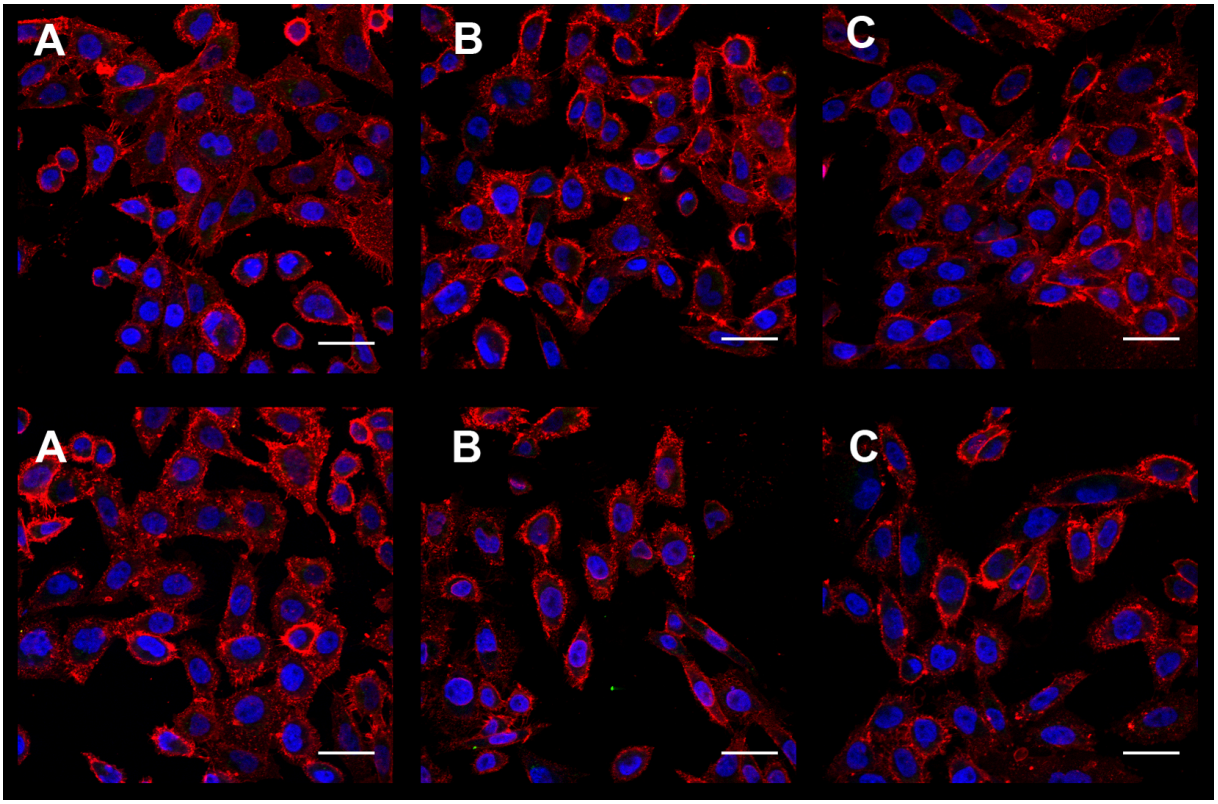

Figure S8. Representative confocal laser microscopy images of the following cells and VLPs (one hour incubation at 37°C, followed by washing): (A) CD22-CHO cells + **5**, (B) WT-CHO cells + **6**, (C) WT-CHO cells + **5**. Blue = DAPI stained nuclei, red = cell membrane (wheat germ agglutinin AlexaFluor® 555 conjugate), green = encapsidated GFP, scale bar = 30 μm in all panels.

10. Toropova, K.; Basnak, G.; Twarock, R.; Stockley, P.G.; Ranson, N.A. The three-dimensional structure of genomic RNA in bacteriophage MS2: Implications for assembly. *J. Mol. Biol.* **2008**, *375*, 824-836. The structures of MS822 and Qß are closely related.
11. Kaltgrad, E.; O'Reilly, M. K.; Liao, L.; Han, S.; Paulson, J. C.; Finn, M. G. *J. Am. Chem. Soc.* **2008**, *130*, 4578-9.

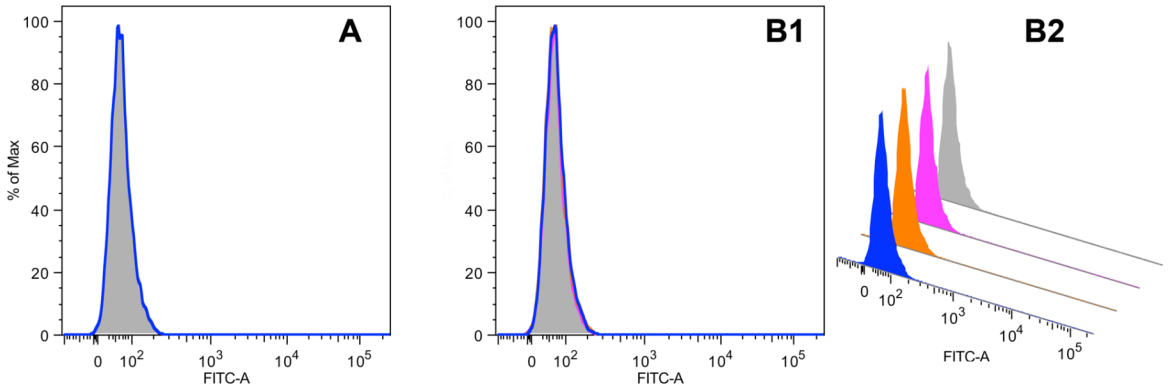

Figure S9. Flow cytometry analysis showing lack of binding to WT-CHO cells, treated with the following reagents. (A) grey = buffer, blue = fluorescently labeled anti-CD22 antibody. (B) grey = buffer, pink = particles **5** (25 µg/mL, 10 nM in particles), orange = **6** (2.5 µg/mL, 1 nM), blue = **6** (25 µg/mL, 10 nM).

Table S3. Primers used for production of a fused coat protein construct as well as encapsidated proteins. Overlap sequences are noted in italics, His tag is bold, and *Nco*I/*Xho*I sites are underlined.

primer name	primer sequence
*Nco*I/xFP-F	**CAT GCC ATG GTT TCT AAA GGT GA**
*Nco*I/xFP-F-His	CAT GCC ATG GCA CAT CAC CAC CAC CAT CAC ATG GTT TCT AAA GGT GAA GAA CTG
sfxFP-R	CCG CTC GAG TTA TTT GTA CAG TT
sfGFP / S30R (top)	GGGCACAAATTTTCTGTCCGTGGAGAGGGTGAAGGTGAT
sfGFP / S30R (bottom)	CCCGTGTTTCCCCGACAGGCACCTCTCCCACTTCCACTA
sfGFP / Y39N (top)	GGTGAAGGTGATGCTACAAACGGAAAACTCACCCTTAAA
sfGFP / Y39N (bottom)	CCACTTCCACTACGATGTTTGCCTTTTGAGTGGGAATTT
sfGFP / N105T (top)	TCTTTCAAAGATGACGGGACCTACAAGACGCGTGCTGAA
sfGFP / N105T (bottom)	AGAAAGTTTCTACTGCCCTGGATGTTCTGCGCACGACTT
sfGFP / Y145F (top)	CACAAACTCGAGTACAACTTTAACTCACACAATGTATAC
sfGFP / Y145F (bottom)	GTGTTTGAGCTCATGTTGAAATTGAGTGTGTTACATATG
sfGFP / I171V (top)	TTCAAAATTCGCCACAACGTTGAAGATGGTTCCGTTCAA
sfGFP / I171V (bottom)	AAGTTTTAAGCGGTGTTGCAACTTCTACCAAGGCAAGTT
sfGFP / A206V (top)	TACCTGTCGACACAATCTGTCCTTTCGAAAGATCCCAAC
sfGFP / A206V (bottom)	ATGGACAGCTGTGTTAGACAGGAAAGCTTTCTAGGGTTG
sfBFP-F (Y66H)	CAC CCT GAC CCA CGG TGT TCA GT
sfBFP-R (Y667H)	ACT GAA CAC CGT GGG TCA GGG TG
sfCFP-F (Y66W)	CAC CCT GAC CTG GGG TGT TCA GT
sfCFP-R (Y66W)	ACT GAA CAC CCC AGG TCA GGG TG