Supporting information

A dimorphism shift of hepatitis B virus capsids in response to ionic conditions

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Experimental details and calculations (HBV Cp149 expression, capsid assembly and characterizations, protein surface charge calculation and measurement, free energy calculation of capsid formation, and mutation capsid assembly)

1. HBV Cp149 expression

The gene of WT HBV Cp149 was cloned into the expression vector pET24a and the sequence was confirmed via DNA sequencing. The plasmids pET24A-Cp149 was then transformed into BL21(DE3) strain for protein expression. A single colony was used to inoculate 10mL of LB media supplemented with 50 µg/mL Kanamycin (kan). The culture was incubated with shaking overnight and then diluted into 1 L fresh LB/kan media. After OD600 reached 0.8, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 2 mM to induce the expression of Cp149. Cells were incubated for another 5 hours before collected by centrifugation at 7,000 g for 10 minutes. Purification was conducted following procedures as previously described.[1] Briefly, cells were resuspended in a buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM DTT, 0.01 mg/mL RNase, and 2U/mL DNase 1. The cells were lysed by sonication in ice/water bath and centrifuged to collect the supernatant. Solid sucrose was added to the supernatant to a concentration of 0.15 M followed by centrifugation. Solid $(NH_4)_2SO_4$ was added to the supernatant and then incubate the solution with gentle stirring at room temperature for 1 hour. Following the pellet collection and resuspension, HBV Cp149 capsids were analyzed by a Sephadex column. Solid urea and EDTA were added to the capsid solution. After incubated for 3 hours on ice, the sample solution was loaded onto a Sephadex column and dimers fractions with high purity were collected and concentrated. WT dimer solution was dialyzed to $NaHCO_3$ (pH 9.6) buffer for storage.

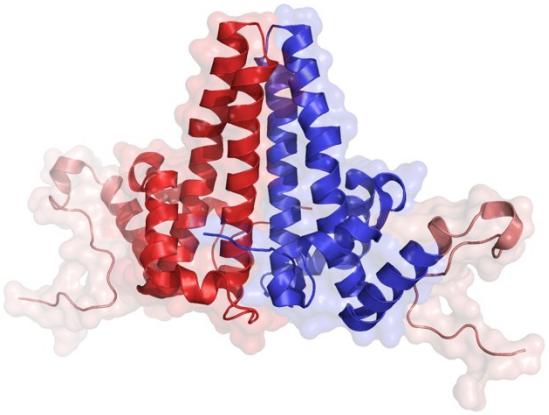

Figure S1. Graphic illustration of HBV Cp149 dimer. A WT Cp149 dimer consists of two identical subunits (red and blue). The edge of the dimer (light pink) is responsible for hydrophobic contact when associated. Charged capsid dimers can be regarded as macroions.

2. Capsid assembly and characterizations

The stock solutions of HBV $Cp149_2$ were first dialyzed to the desired buffer incubated in an ice bucket overnight. Protein concentrations were determined through UV absorbance at 280 nm using an extinction coefficient of 29280 $cm^{-1}M^{-1}$(for protein monomer). A 2.7 M KCl (or 2.7 M NaCl, 270 mM $CaCl_2$) buffered stock solution was titrated into protein solution to achieve the desired salt concentration. A typical assembly reaction was performed at 22 ℃. The buffer used for pH 7.5 and pH 8.5 assembly was Tris buffer (50 mM Tris, 10 mM BME, pH adjusted by HCl); assembly conducted at pH 9.6 was performed in $NaHCO_3$ buffer (50 mM NaHCO3, 10 mM BME, pH adjusted by NaOH).

Sucrose density gradient centrifugation was adopted to differentiate assembled $T = 3$ and $T = 4$ core particles. 0.3 mL of capsid solution was loaded onto a 5%-40% filtered sucrose gradient solution till a total volume of 5 mL. The sucrose solutions were prepared in the same buffer and under the same ionic strength as the assembly conditions. Solutions were spun in a Ti-55 rotor (performed by Beckman ultracentrifuge) at 45,000 rpm, temperature 20 ℃, for 4 h. Separated $T = 3$ and $T = 4$ capsids can be visualized as two bands by shining green light due to Tyndall effect.

To tell the proportion of small and large capsids, the images of sucrose density analytes were taken and then processed by Image J software. Images were converted to 8-bit and inverted color, with the optical density measurements calibrated. A suitable threshold was adjusted to cover the capsid band. The integrated optical density of the selected upper and lower band reflected the band brightness and width, telling the relative amount of capsids in the corresponding position.

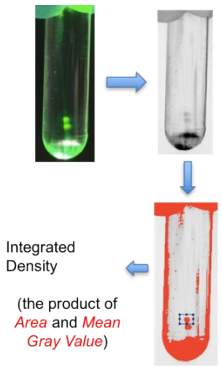

Figure S2. An illustration of optical density calculation.

The morphology of assembled Cp149 capsids was also characterized by a JEOL-1230 electron microscope. Samples for the transmission electron microscopy (TEM) analysis were prepared by a standard negative staining method. A droplet of 8 μL sample solution was placed onto a carbon film coated copper grid (Ted Pella, Inc.), incubated for 90 s and then washed with water. Immediately, the sample was then stained with 1% uranyl acetate for 90 s. The excess staining solution was removed and the copper grid was left dry overnight prior to taking TEM images.

To tell the capsid fractions after assembly, agarose gel electrophoresis was adopted as another characterization method, where 7 μL of assembly mixture solution was injected into a 1.25% gel and moves under 100 V for 60 mins.

3. **Table S1.** WT Cp149 dimer charges under different pH conditions.

Charge	pH 7.5	pH 8	pH 8.5	pH 9.6	pH 10.5
Theoretical calculation	-14.4	-16.4	-18.8	-25.6	-33
Mobility measurement	-14.4653		-17.5748	-28.8072	

The overall net charge on HBV Cp149 dimer is pH dependent and can be estimated using Henderson-Hasselbalch equation. Theoretical results were obtained from protein calculator 3.4 (http://protcalc.sourceforge.net). The mobility of pure dimer was measured by Wyatt Mobius Zeta Potential under a voltage of 1.5 V, and the effective charge was further obtained through:

$$Z * e = 6\pi\eta R_h \mu_E \frac{1 + \kappa R_h}{f(\kappa R_h)} \tag{1}$$

in which η is the solution viscosity; R_h is the measured hydrodynamic radius; μ_E is the dimer mobility under an applied voltage; κ is the inverse Debye length and $f(\kappa R_h)$ is Henry's function.

The Debye length was obtained through:

$$\kappa^{-1} = \sqrt{\frac{\varepsilon_r \varepsilon_0 k_B T}{2 N_A e^2 I}} \tag{2}$$

where ε_0 is the permittivity of free space; ε_r is the dielectric constant; k_B is the Boltzmann constant; T is the absolute temperature in kelvins; N_A is the Avogadro number; e is the elementary charge and I is the ionic strength.

A simple approximation formula[4] for Henry's function $f_o(KR_h)$ was applied in Equation S1:

$$f_o(\kappa R_h) = 1 + \frac{1}{2\left(1 + \frac{2.5}{\kappa R_h (1 + 2\exp(-\kappa R_h))}\right)^3} \tag{3}$$

4. Size Exclusion Chromatography (SEC)[2] was performed using a 0.5 cm width Econo® column packed with Sephacryl® S-300 HR medium, which is desired to separate HBV capsids and Cp149 dimers. The column was pre-equilibrated with 50 mM Tris buffer as used for the Cp149 assembly reaction plus an extra 10 mM $CaCl_2$ to prevent capsids from disassembly during the separation. After more than 24 h equilibrium, 80 μL of the assembly sample was injected onto the column under a mild pressure of 2.5 psi, and peaks of capsids and dimers were determined by UV absorbance at 280 nm via a Hewlett Packard Variable Wavelength Detector. Baseline was corrected and subtracted using Origin, and the integrated peak areas were used to calculate the capsid and dimer concentrations.

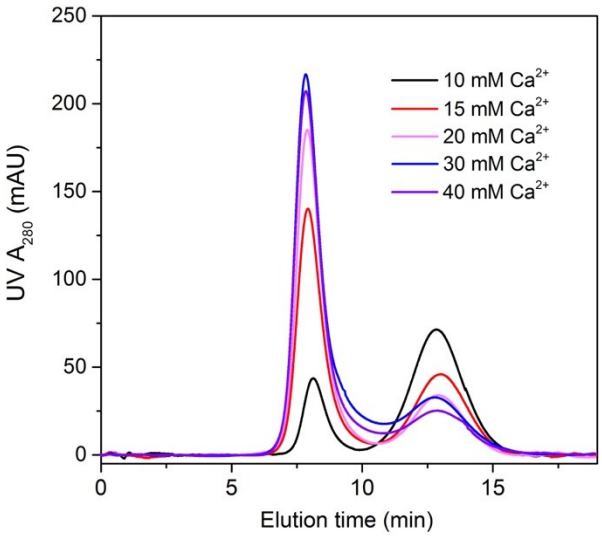

Figure S3. SEC analysis of $Cp149_2$ assemblies. 1.0 mg mL^{-1} protein was quickly assembled by various concentrations of $CaCl_2$ (10 - 40 mM) in pH 7.5 Tris buffer. Capsids elute at 8.0 min, while dimers at 13.0 min.

The theoretical free energy change expression was demonstrated by Adam Zlotnick and his co-workers.[3] Briefly, considering the assembly of 120 dimers associate into a $T = 4$ icosahedral capsid and 90 dimers into a $T = 3$ one, the equilibrium constant K can be expressed by:

$$K_{T=4\ capsid} = [capsid]/[dimer]^{120} \tag{4}$$

$$K_{T=3\ capsid} = [capsid]/[dimer]^{90} \tag{5}$$

Assuming all the contacts in the capsid are identical and then this equilibrium constant can be partitioned into a single contact equilibrium constant $K_{contact}$ by applying a statistical factor:

$$K_{\text{capsid}} = (\frac{j^{N-1}}{N})(K_{\text{contact}})^{cN/2} \tag{6}$$

Here, j-fold degeneracy with N subunits describes the degeneracy of association based on capsid geometry, and the total number of contacts forming a capsid is $cN/2$, with N subunits and c number of contact surfaces per subunit. Thus, the association energy per contact can be calculated as following:

$$\Delta G_{\text{contact}} = -RT\ln(K_{\text{contact}}) \tag{7}$$

The nucleation energy (ΔG_*) of the $T = 3$ and $T = 4$ capsid formation was calculated based on the equation 1 in the manuscript. Take the $T = 4$ capsid assembly with 10 mM $CaCl_2$ as an example:

$$\Delta G_* \approx \frac{\left(\frac{\pi c \Delta g}{k_B T}\right)^2}{4\ln\left(\frac{\rho}{\rho^*}\right)} k_B T = \frac{\left(\frac{\pi * 2 * \left(-3.0252481 * 4\frac{\text{kcal}}{\text{mol}}\right)}{k_B T}\right)^2}{4 * \ln\left(\frac{1\frac{\text{mg}}{\text{ml}}}{0.46747181\frac{\text{mg}}{\text{ml}}}\right)} k_B T = \frac{\left(\frac{\pi * 2 * (-1.2227078 * 4 * k_B T)}{k_B T}\right)^2}{4 * \ln\left(\frac{1\frac{\text{mg}}{\text{ml}}}{0.46747181\frac{\text{mg}}{\text{ml}}}\right)} k_B T$$

Table S2. Raw data and calculation results of the free energy per dimer contact and the nucleation energy.

Conditions	[dimer] (M)	[capsid] (M)		Capsid percentage	[capsid] (M)	Free energy $\Delta G_{contact}$ (kcal/mol)	Nucleation energy ΔG_* (J/k_BT)
10 mM $CaCl_2$	1.394E-5	4.260E-8	$T = 4$	93.80%	3.995E-8	-3.0252	310.1511
			$T = 3$	6.20%	3.518E-9	-3.0071	306.4456
15 mM $CaCl_2$	7.858E-6	1.478E-7	$T = 4$	72.33%	1.061E-7	-3.1944	197.2186
			$T = 3$	27.67%	5.413E-8	-3.1828	195.7821
20 mM $CaCl_2$	5.654E-6	1.964E-7	$T = 4$	53.59%	1.052E-7	-3.2902	167.7969
			$T = 3$	46.41%	1.215E-7	-3.2812	166.8783
30 mM $CaCl_2$	3.827E-6	2.209E-7	$T = 4$	47.92%	1.097E-7	-3.4040	145.4508
			$T = 3$	52.08%	1.590E-7	-3.3957	144.7467
40 mM $CaCl_2$	3.193E-6	2.206E-7	$T = 4$	42.33%	8.675E-8	-3.4561	137.7855
			$T = 3$	57.67%	1.576E-7	-3.4484	137.1715

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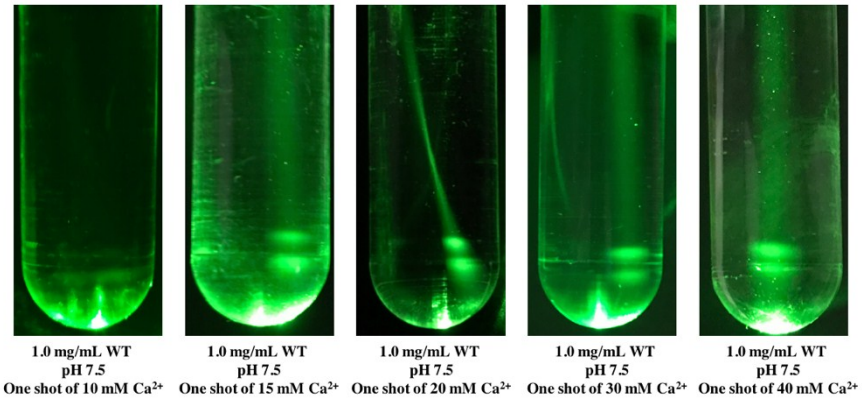

Figure S4. Optical images of $T = 3$ and $T = 4$ capsid distributions through quick nucleation via various concentrations of $CaCl_2$ (10 - 40 mM).

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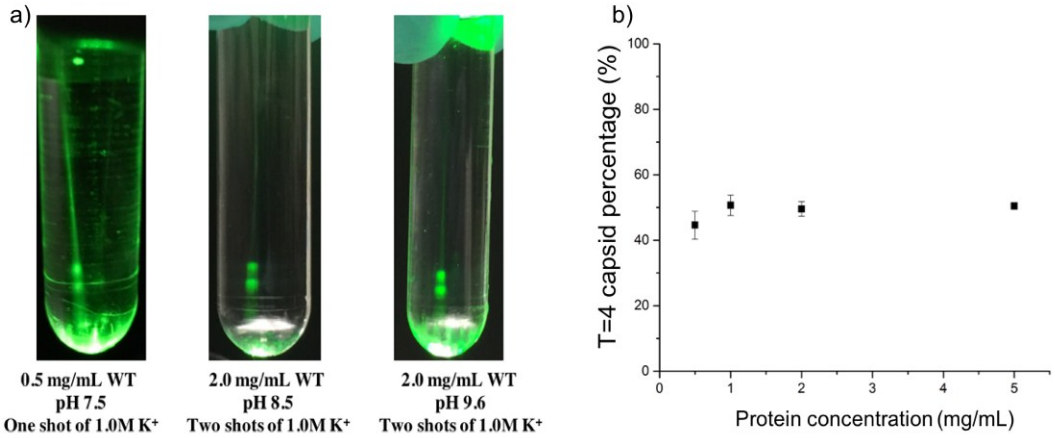

Figure S5. Optical images (a) and results (b) of $T = 3$ and $T = 4$ capsid distributions through quick nucleation via 1.0 M of K^+ under different protein concentrations (0.5 - 5.0 mg/mL protein solutions). Capsid population at 5.0 mg/mL was a result of particle counting from TEM images since smear bands were observed in sucrose gradient due to abundant capsids and possible aggregation.

7. The capsids assembled through stepwise or single-shot addition of KCl into $Cp149_2$ mutant solutions, D2N/D4N, were analyzed by sucrose gradient density centrifugation. The images and results of $T = 4$ capsid fractions were listed in Figure S6. D2N/D4N self-assembly was performed in pH 8.4 Tirs buffer. In this case, its net protein charge was kept the same as WT $Cp149_2$ at pH 7.5, while displaying different surface charge distribution.

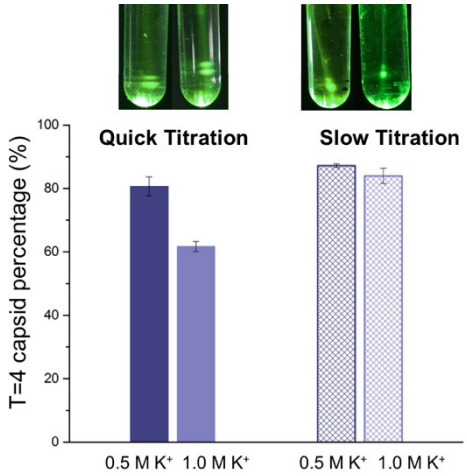

Figure S6. Optical images of sucrose density gradient solutions (above) and the calculated $T = 4$ capsid percentages of the mutant D2N/D4N assemblies (below) through stepwise or single-shot titration methods, respectively.

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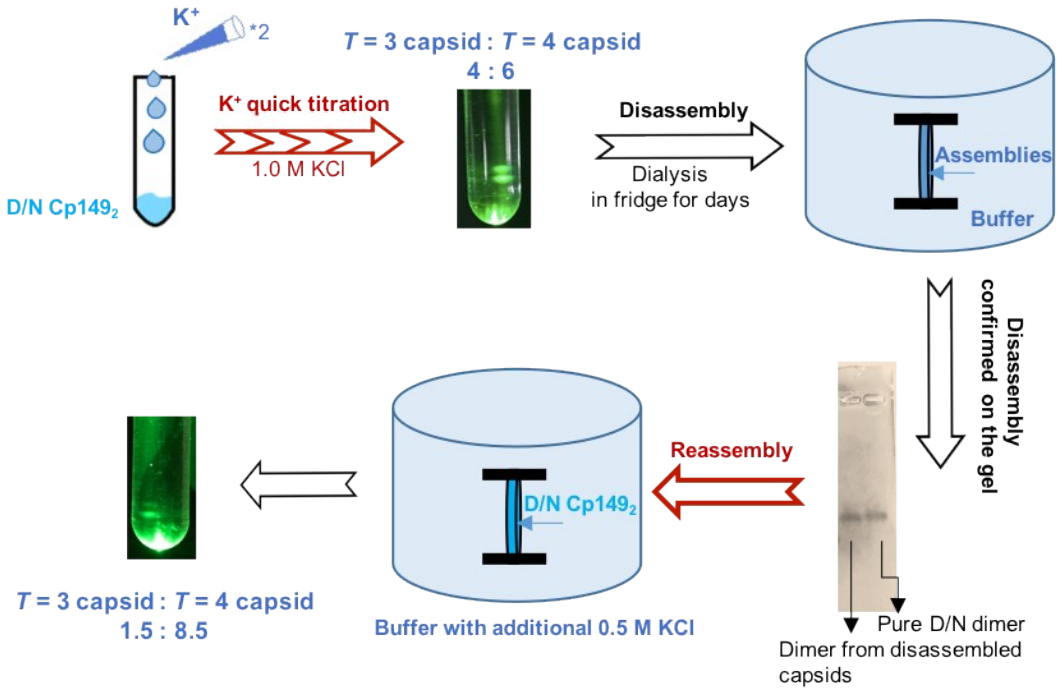

Figure S7. Illustration of disassembly and re-assembly of quickly associated mutant HBV D2N/D4N capsids. A single aliquot of 1.0 M of KCl (final concentration) was quickly introduced into a 1.0 mg/mL D2N/D4N protein solution to trigger capsid assembly. Sucrose gradient centrifugation confirmed a close proportion of $T = 3$ and $T = 4$ capsids. Then the assembled mixture solution was dialyzed against buffer to remove the KCl. The capsids were totally disassociated into dimers as verified on an agarose gel. After that, dimers were re-assembled into capsids by incubating with 0.5 M KCl, and they formed into capsids with the $T = 4$ particles as the dominant one.

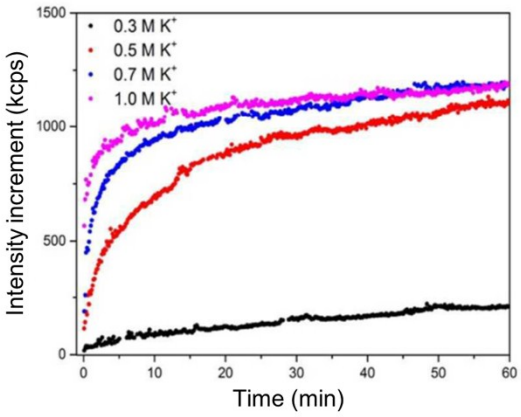

Figure S8. Assembly process monitored by static light scattering. The increment of intensity indicated the formation of capsids. As the concentration of potassium chloride increased, the electrostatic repulsion between dimers is lowered, which facilitates the capsid formation and presents a steeper growth in scattered light intensity. Assembly was triggered by adding various concentrations of K^+ ions into 1 $mgml^{-1}$ of buffered protein solution at 22℃.

9. **Table S3.** WT Capsid dimorphism (represented by the percentage of $T = 4$ capsids) under different assembly conditions.

Conditions	**Analysis methods**	**0.3 M KCl** (quick assembly)	**0.5 M KCl** (quick assembly)	**0.7 M KCl** (quick assembly)	**1.0 M KCl** (quick assembly)	**0.5 M KCl** (slow assembly)	**1.0 M KCl** (slow assembly)
pH 7.5 1.0 mg/mL	sucrose	79.7±0.9%	78.8±0.8%	69.8±1.7%	53.4±4.7%	83.9±0.9%	80.9±0.8%
	TEM	79.2%	66.0%		51%		
	electrophoresis		86%		64%	90%	88%
pH 8.5 1.0 mg/mL	sucrose		79.1±3.6%		51.9±2.9%		
	TEM		71.1%		48.1%		
	electrophoresis				57%		
pH 9.6 1.0 mg/mL	sucrose		84.3±3.5%		50.9±1.5%		
	TEM		74.4%		51.7%		
pH 7.5 0.5 mg/mL	sucrose				44.6±4.3%		
pH 7.5 2.0 mg/mL	sucrose				49.6±2.2%		
pH 7.5 5.0 mg/mL	TEM				50.5%		
			0.5 M NaCl (quick assembly)	**0.7 M NaCl** (quick assembly)	**1.0 M NaCl** (quick assembly)		
pH 7.5 1.0 mg/mL	sucrose		81.8±2.0%	65.8±3.9%	42.1±3.6%		
		10 mM $CaCl_2$ (quick assembly)	**15 mM $CaCl_2$** (quick assembly)	**20 mM $CaCl_2$** (quick assembly)	**30 mM $CaCl_2$** (quick assembly)	**40 mM $CaCl_2$** (quick assembly)	
pH 7.5 1.0 mg/mL	sucrose	93.8±1.1%	72.3±2.6%	53.6±5.5%	47.9±4.0%	42.3±3.4%	

References:

1. A. Zlotnick, N. Cheng, J. Conway, F. Booy, A. Steven, S. Stahl and P. Wingfield, *Biochemistry*, 1996, **35**, 7412-7421.
2. P. Mialane, A. Dolbecq, L. Lisnard, A. Mallard, J. Marrot and F. Secheresse, *Angew. Chem., Int. Ed.*, 2002, **41**, 2398.
3. S. Katen and A. Zlotnick, *Methods in enzymology*, 2009, **455**, 395-417.
4. Ohshima, H., A simple expression for Henry's function for the retardation effect in electrophoresis of spherical colloidal particles. *Journal of Colloid and Interface Science* **1994,** *168* (1), 269-271.