ESRF	**Experiment title:** Self-assembly pathways of hepatitis B virus capsids	**Experiment number**: SC-4761
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Report:

Introduction and motivation. Hepatitis B virus (HBV) is one of the most serious human pathogens: 350 million people suffer from chronic HBV and 600,000 die from it annually. HBV is an enveloped virus with an icosahedral nucleocapsid packaging a partially double-stranded DNA. DNA is synthesized by reverse transcription of a RNA pregenome (pgRNA) after its packaging in the immature nucleocapsid. The basic building block of the capsid is a dimer of a 183-residue protein (Cp183). The N-terminal 149 residues form the assembly domain that self-assembles *in vivo* and *in vitro* into icosahedral capsids with ~95% of $T = 4$ (120 dimers) particles and ~5% of $T = 3$ (90 dimers) particles [1]. The 34 remaining arginin-rich amino acids form the C-terminal domain (CTD), which is essential for the packaging of pgRNA but is not required for the formation of empty capsids.

Dimers of Cp149 (devoid of CTD) can then self-assemble *in vitro* into empty capsids at room temperature upon an increase of the salinity from low (<0.1 M) to high (>0.1 M) values. Studies by time-course static light scattering suggested that dimers followed a nucleation-elongation pathway in which the nucleus is a trimer of dimers. This conclusion must be however considered with caution because the technique did not provide structural information and the trimer of dimers was inferred indirectly from a kinetic model fitting time traces. Recently, the assembly of HBV capsids was probed at the single particle level by resistive-pulse sensing in nanofluidic devices [1]. Large long-lived intermediate species comprising around 100 dimers were detected over the course of the assembly (>1 h). Small intermediate species were detected as well but the limited time resolution of resistive-pulse sensing did not allow the investigators to capture the early oligomers such as the putative trimer of dimers.

Our goal was to identify the structure of long-lived intermediate species involved in the formation of HBV empty capsids. We also aimed at devising kinetic models valuable for a better understanding of the HBV assembly mechanisms and that could be translated to *in vivo* situations.

Results and conclusions of the study. Cp149 proteins were expressed in *E. coli* and purified by size-exclusion chromatography. They were initially dispersed in 50 mM HEPES pH 7.5 prior to being rapidly mixed with 50 mM HEPES pH 7.5, 2 M NaCl, via a stopped-flow device. The final protein concentrations varied from 0.3 to 0.9 $g.L^{-1}$ and all the experiments were carried out at room temperature. SAXS patterns were collected with a geometric progression of 5-ms exposures over one hour. The sample-to-detector distance was set to 1.3 m so that the wavenumbers q ranged from 0.03 to 4 nm^{-1}.

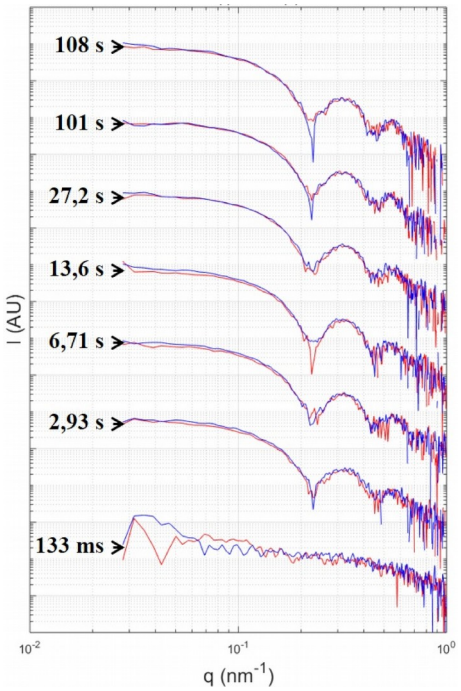

Figure 1 | SAXS patterns of self-assembling Cp149 capsids carried out in duplicate (red and blue lines). Protein concentration was 0.6 g.L^{-1} and salinity 1 M.

Figure 1 shows two kinetics: in the early time steps, only dimers were present in solution. Over the course of the assembly, scattering patterns displayed oscillations characteristic of the presence of spherically-symmetric objects, namely, icosahedral capsids consistent with a $T = 4$ diameter of 38 nm. The onset of the oscillations was within the first two seconds of the assembly process. Notice that we had a good repeatability of the time-resolved data.

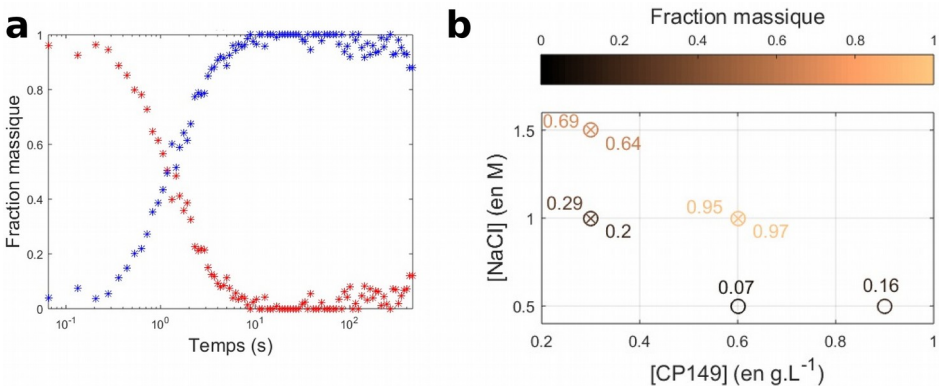

Figure 2 | a, Mass fraction of capsids (blue) and dimers (red) versus time for an experiment at a protein concentration of 0.6 g.L^{-1} and salinity of 1 M. **b**, Final mass fraction of capsids as a function of protein concentration and salinity. Crosses indicate experiments performed in duplicate.

Singular value decomposition applied to all the TR-SAXS data revealed that the scattering patterns of each experiment could be represented with solely two components within the uncertainties. As a consequence, we limited our analyses to the presence of dimers and capsids in solution at each time step. Figure 2a illustrates the evolution of the mass fraction in capsids and dimers. Interestingly, all the capsids were assembled within ten seconds and the remaining fraction of dimers was very small. Figure 2b depicts the final mass fraction of capsids in various experimental conditions: the higher the protein concentration and the salinity, the better the assembly efficiency. The effect of salinity can be understood in terms of screening of the electrostatic repulsion between dimers, promoting subsequently their association into capsids.

The binding and structural time scales of the assembly were estimated by fitting the forward scattering intensity I_0 and the radius of gyration R_g – or the form factor at a given wavenumber – with a decaying exponential function. Quite unexpectedly, we observed three phases in the self-assembly pathway (Fig. 3). In the first one, I_0 and R_g increased simultaneously, which can be ascribed to the growth of capsids by sequential addition of dimers. This phase could last a few seconds at most. In the second phase, R_g plateaued but I_0 continued to increase: the capsids were almost complete in the sense that their final size was nearly reached but they missed several dimers to close up. Again, this phase could last a few seconds. In the third phase, the longest one, if I_0 and R_g did no longer evolve significantly, we observed that the form factor, and more specifically the minima associated to the oscillations characteristic of the icosahedral symmetry, were still changing over five to ten minutes. At the end of the second stage, the capsids were most likely not well-ordered, and the third phase corresponds to their slow self-organization into proper icosahedral shells. At high salinity (1 M), however, we did not observe the self-organization phase, and we hypothesized that the strong effective attraction between dimers led to trap kinetically the objects into misassembled shells comprising defects.

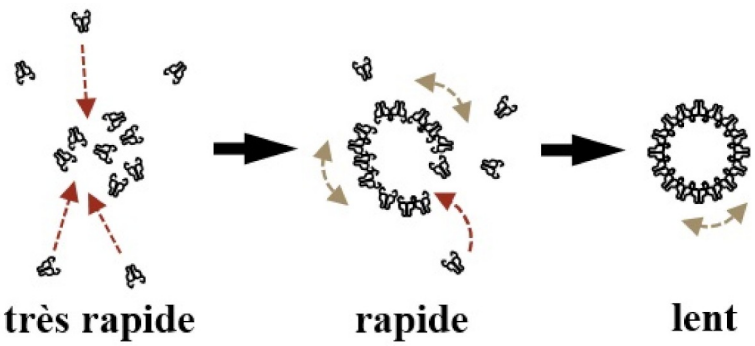

Figure 3 | Schematic illustration of the self-assembly dynamics of Cp149 dimers into empty capsids.

[1] P. Kondylis *et al.* (2017). "Nanofluidic devices with 8 pores in series for real-time, resistive-pulse analysis of hepatitis B virus capsid assembly". *Anal. Chem.* 89 4855.