ESRF	**Experiment title:** Effects of antivirals on the assembly pathway of hepatitis B virus capsids	**Experiment number**: LS-3151
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Introduction and motivation. Hepatitis B virus (HBV) is one of the most serious human pathogens with more than 0.8 million people dying yearly from chronic HBV. HBV is an enveloped virus with an icosahedral nucleocapsid packaging a partially double-stranded DNA. The constituent of the capsid is the Core protein. Core is a 183-residue-long protein (Cp183) where the 149 N-terminal residues (Cp149) can self-assemble into capsids. The C-terminal domain (CTD), which is made up of the 34 remaining arginin-rich amono acids, is necessary for the packaging of pregenomic RNA (pgRNA) but not for the formation of empty capsids. CAMs (for Capsid Assembly Modulators), which are new antiviral molecules, bind at the interface between subunits and can disrupt HBV capsid assembly. There are two groups of modulators that have been established according to their mode of action. CAMs A misdirect assembly into non-capsid, aberant species and CAMs E promote assembly into empty capsids.

Our previous TR-SAXS (time-resolved small angle X-ray scattering) study [1] of HBV capsid assembly kinetics formed from Cp149 dimers in the presence of modulators has provided valuable insights that refine our understanding of CAMs. Notably, we found that CAMs E not only accelerate assembly but also influence the assembly process, leading to the formation of elongated capsids.

In this new TR-SAXS study, the main objective was to investigate the assembly pathways of Cp149 capsids in the presence of new antiviral molecules (GLP-26 and molecule B) in order to determine whether these molecules are indeed CAMs. For comparison, the kinetics of capsid formation in the presence of a CAM E, JNJ-632, was also performed. The secondary aim was to acquire the first data concerning the assembly kinetics of capsids formed from the Cp183-EEE protein (a mutant mimicking a phophorylated state of the Cp183 protein). The aim was to determine wheter the formation process was similar to that observed for Cp149, and if not, to identify any differences that might exist.

Results and conclusions of the study. Core protein (Cp149 and Cp183-EEE) were expressed in *E.coli* and purified by size-exclusion chomatography. Cp149 proteins were dispersed in 20 mM Tris HCl pH 7.5 prior to rapid mixing with 20 mM Tris HCl pH 7.5, 750 mM AmAc, in the presence or not of antivirals via a stopped-flow device. The final protein concentration was 1 $g.L^{-1}$, and the final salt concentration was 150 mM. The final concentration for the 3 molecules tested was 90 µM and the experiments were carried out at 37°C. Cp183-EEE proteins were dispersed in 10 mM CHES pH 9, 1.5 M GuHCl before being rapidly mixed with 50 mM Tris-HCl pH 7.5 in the presence or not of the pgRNA, via a stopped-flow device. The final protein concentration was 0.5 $g.L^{-1}$, and the final pgRNA concentration was 0.1 $g.L^{-1}$. The experiments were carried out at 15°C. SAXS patterns were collected with a geometric progression of 5-ms exposures over 15 minutes. The sample-to-detector was set to 2 m so that the wavenumbers q ranged from 0.034 to 3.825 nm^{-1}, and each kinetics was performed at least twice.

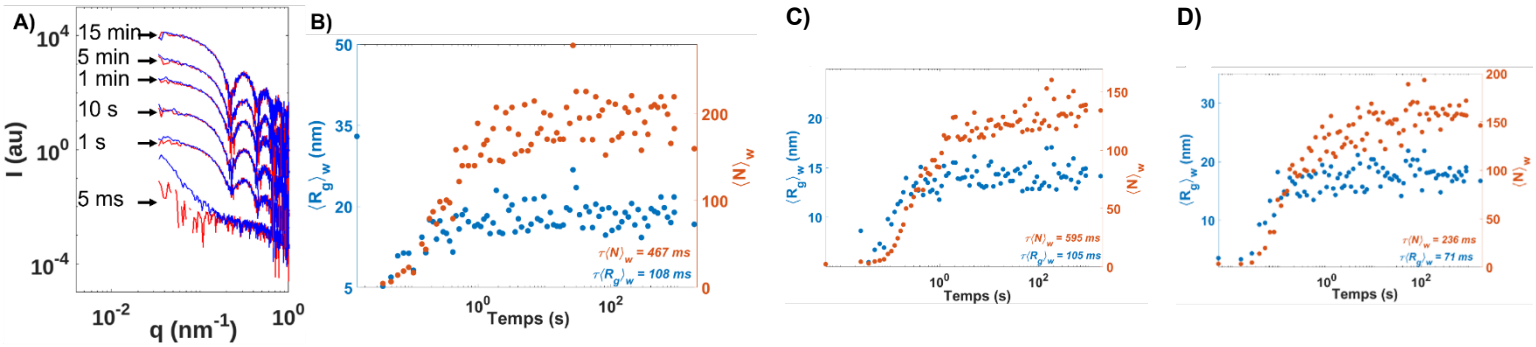

Figure 1: TR-SAXS measurements of Cp149 capsid assembly. (A) TR-SAXS curves of two assembly experiments (blue and red curves) of 30 µM of subunits in the presence of 90 µM GLP-26. **(B)** Evolution of mean aggregation number $\langle N \rangle_w$ (orange discs) and mean radius of gyration $\langle R_g \rangle_w$ (blue discs) as a function of time for capsid assembly from 30 µM subunits in the presence of 90 µM GLP-26. **(C), (D)** Same as **(B)** but for 90 µM of molecule B **(C)** and 90 µM JNJ-632 **(D)**. Characteristic time scales were obtained by fitting an exponential decay function.

SAXS curves of capsid formation kinetics in the presence of GLP-26 are shown above (Figure 1.A). In the presence of this molecule, objects are formed and a first oscillation at q = 0.023 Å^{-1} is observed. The value of the wavenumber q of this first oscillation indicates that the majority of objects formed in this condition are T=4 capsids. Figure 1.B represents the evolution of $\langle R_g \rangle_w$ and $\langle N \rangle_w$ during capsid assembly. These results indicate that some objects formed in the presence of 90 µM GLP-26 are larger than T=4 capsids and that structural differences can be observed during assembly in the presence of this molecule. Furthermore, it is interesting to note that the time scale calculated for the mean aggregation number is shorter in the presence of 90 µM GLP-26. We can therefore conclude that this molecule accelerates assembly kinetics.

The same kind of analysis was carried out to study capsid assembly kinetics in the presence of molecule B. Figure 1.C represents the evolution of $\langle R_g \rangle_w$ and $\langle N \rangle_w$ during capsid assembly. In the presence of 90 µM of molecule B, capsid formation is not accelerated and the objects formed are T=4 capsids. Moreover, the presence of molecule B would lead to the formation of fewer T=4 capsids. Indeed, a decrease in I(0) intensity, translating into a lower $\langle R_g \rangle_w$ value in our analyses was observed. However, the appearance of the first oscillation at q = 0.23 nm^{-1} clearly confirms that the majority of the objects formed are T = 4 capsids and that this decrease in intensity is due to the formation of a smaller number of capsids. To compare these results with another CAM-E modulator, the assembly kinetics of 30 µM of Cp149 subunits in the presence of 90 µM JNJ-632 were performed (Figure 1.D). The values obtained for $\langle R_g \rangle_w$, the associated characteristic time scale, as well as the value of $\langle N \rangle_w^{\infty}$, are similar to those calculated when assembling 30 µM of subunits in the presence of 90 µM GLP-26.

The kinetic and structural data clearly indicate that GLP-26 is a CAM-E. It exhibits the same kinetic effects as JNJ-632, and SAXS curves show that it also has the same structural effects. However, the presence of molecule B during assembly does not appear to accelerate capsid growth, as suggested by TR-SAXS kinetics, and leads to the formation of a smaller number of capsids. One hypothesis is that the molecule B strongly inhibits the formation of HBV capsids when bound to a dimeric subunit of Cp149.

Assembly kinetics of Cp183-EEE in the presence or absence of pgRNA (data not shown here) indicate that the presence of pgRNA influences the size and kinetics of capsid formation. Objects form faster and are smaller in the presence of pgRNA. However, the upward shift in intensity values at small angle did not allow further analysis of these data.

Publications. A publication presenting the results obtained from this TR-SAXS experiment is currently being written.

[1] K. Kra *et al*. "Energetics and kintic assembly pathways of Hepatitis B Virus capsids in the presence of antivirals". *ACS Nano* 2023, 17, 13, 12723–12733