Time-resolved scattering investigations of brome mosaic virus microcrystals appearance

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The behavior of brome mosaic virus in solution as a function of physico-chemical conditions has already been characterized by Small Angle X-ray Scattering studies. The most striking result was that the precipitates induced by the addition of polyethylene glycol were in fact made of microcrystals. This result was reinvestigated on the ID2 beamline at ESRF (Grenoble, France) to determine whether there was an amorphous state before the organized one, and measure the necessary period of latency for the microcrystals to form. The stopped-flow device associated to the high brilliance of the beamline enabled us to characterize the growth of the diffraction peaks as a function of time.

Keywords: brome mosaic virus; icosahedral virus; microcrystals; polyethylene glycol; small angle X-ray scattering; stopped-flow.

1. Introduction

Brome mosaic virus is a small icosahedral plant virus of 268 Å in diameter and of a molecular weight of 4.6×10^6 Da. Preliminary static small angle X-ray scattering studies at the Laboratoire pour l'Utilisation du Rayonnement Electromagnétique (LURE, Orsay, France) had permitted us to characterize the behavior of brome mosaic virus (BMV) in solution as a function of the precipitant nature and/or concentration (pH, salts and polymers) (Casselyn *et al*, 2001). Such a method is very useful in determining attractive conditions between macromolecules, where protein crystallization usually occurs. The addition of polyethylene glycol (PEG), combined or not with salts, could induce a solid-liquid phase separation. The appearance of diffraction peaks revealed the microcrystalline nature of the solid phase.

The presence of PEG in solution induces a non-specific attractive potential called the depletion attraction. This effect is dependent on the size and concentration of the PEG (Asakura & Oosawa, 1958, Poon *et al*., 1996, Ye *et al*., 1996). In our experiments, the nucleation rate was found to be dependent on the PEG molecular weight and concentration, and this result enabled us to establish phase diagrams of BMV concentration as a function of PEG concentration with microbatch experiments. Three phases could be determined, where the virus is respectively soluble, crystalline or precipitated. On the phase diagrams, the boundaries of the different domains are shifted as a function of the PEG molecular weight. This result is consistent with the crystallization of proteins in solution in the presence of PEG (Bonneté *et al*., 2001, Budayova *et al*., 1999, Finet & Tardieu, 2001, Galkin & Vekilov, 2000, Kulkarni *et al*., 2000, Vivares & Bonneté, 2002).

As the period of latency for the microcrystal formation could not be measured at LURE, we used the high brilliance of ID2 beamline at the European Synchrotron Radiation Facility (ESRF), associated to a stopped-flow device and to a fast 2D detector (Narayanan *et al*., 2001). The stopped-flow method permitted the quick mixing and pushing under the beam of precise volumes of virus and precipitant agent. Our purpose was to observe diffraction peaks as soon as they appear, and study their evolution as a function of time. For each sample, exponentially spaced measurements were carried out from 180 milliseconds after the mixing up to 10 or 25 min according to the composition of the sample. PEGs of three sizes (3 000, 8 000, 20 000) were separately studied to characterize the effect of the polymer as a function of its molecular weight.

2. Material and methods

2.1. Sample preparation and stopped-flow device

BMV was purified from infected barley leaves as previously described (Casselyn *et al*, 2001), and concentrated to 40 mg/ml at pH 5.9. The mixing of the viruses with pH 5.9 PEG solutions was performed with the stopped-flow device (Bio-Logic™) available on ID2, that included three tanks. The first was filled with a 20 mM Na acetate (Calbiochem®) buffer adjusted to pH 5.9 with acetic acid, the second contained the PEG (Hampton Research) solutions, filtered by Millipore 0.22 µm filters, and the third tank contained the BMV solution at a concentration of 40 mg/ml. The final concentrations obtained were 2.5, 5, 10 or 20 mg/ml virus, in the presence of 2.5, 5 or 10 % (w/v) PEG. A software (Bio-Logic™) permitted the definition of the desired volumes. The experiments were carried out at a constant temperature of 15°C.

2.2. SAXS experiments

Time-resolved SAXS experiments were performed on the ID2 beamline at ESRF (European Synchrotron Radiation Facility, Grenoble, France). The sample to detector distance was of 3 m. The range of the scattering vector s we recorded (with $s = 2\sin\theta/\lambda$) was from $1.17\ 10^{-3}$ to $3.50\ 10^{-2}$ Å^{-1}.

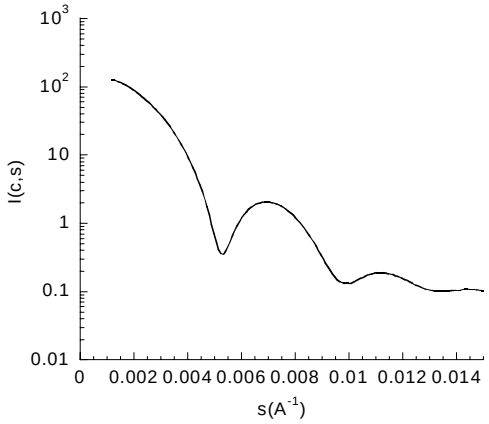

Figure 1
Form factor of the virus. The form factor was obtained by combining the signal at small angles of a sample containing 2.5 mg/ml BMV without PEG, where the interactions are almost negligible, with the signal at higher angles of a 20 mg/ml BMV sample to improve the signal-to-noise ratio.

The signal of the less concentrated sample (2.5 mg/ml) without PEG at low angles (up to s = 0.0050), completed with the signal of a

20 mg/ml BMV sample at higher angles, was taken as the form factor I(0,s) of the virus (figure 1).

The total scattered intensity signal $I(c,s)$ follows the expression: $I(c,s) = I(0,s).S(c,s)$, where $S(c,0)$ is the intensity of the structure factor, which accounts for the interactions between particles in solution.

The acquisition sequence followed the formula : $t_i = t_{i-1} + \Delta t_{meas} + c^{i-1}.t_{int}$, where t is the time after the mixing, t_0 =10 ms, Δt_{meas} the duration of each measurement (50 ms), $c^{i-1}.t_{int}$ is the time interval between two exposures; this time laps is equal to 120 ms (t_{int}) between the two first measurements and then increased by a coefficient c (equal to 1.57 or 1.6 according to the experiments). The first reliable measurement was undertaken 180 ms after the mixing (second frame).

A fast 2D detector, i.e. X-ray image intensifier (XRII) coupled to an ESRF developed FreLoN (Fast Read-out and Low Noise camera) CCD camera (Narayanan *et al*., 2001), connected to the FBS, permitted to record 2D scattering frames.

3. Results and discussion

The precipitation/crystallization of BMV samples of 2.5, 5, 10 and 20 mg/ml was induced by the controlled addition of polyethylene glycol (20 000, 8 000 or 3 000) at different concentrations (2.5, 5 and 10 % w/v). Each of the precipitated samples were microcrystalline and provided diffraction peaks.

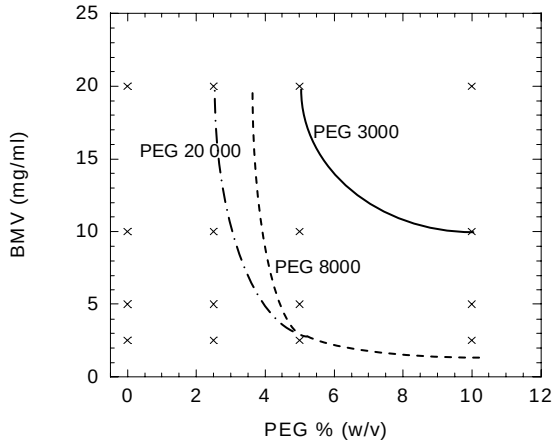

Figure 2
Phase diagram. The crosses represent all the conditions tested for the three PEGs. A curve indicates the boundary between the diffracting conditions (above the curve) and the soluble conditions (below the curve).

For each sample, twenty measurements were carried out with exponentially spaced intervals.

As expected, the use of PEG 20 000 and 8 000 provided more crystallization conditions than PEG 3 000. On figure 2, a curve indicates for each PEG (PEG 3000 (solid line), PEG 8000 (dashed line) and PEG 20000 (dot-dashed line)) the boundary between the diffracting conditions (above the curve) and the soluble conditions (below the curve). This phase diagram is consistent with the one previously established for PEG 20 000 at LURE, at pH5.9 (Casselyn *et al*., 2001). The shift of the present diagram of PEG 8000 with regard to the previous results, for which pH 5 buffers were used, is due to the difference of pH between the two series. Actually, the increase of pH reduces the coulombian repulsion between BMV particles in solution by modifying their net charge, and would thus favor the precipitation in presence of PEG, since the pI is above 7.5 (Casselyn *et al*., 2001).

Table 1 Positions of the diffraction peaks. The s values were calculated on the basis of the unit cell side a = 388.7 Å, determined with the first reflection.

Face-centered cubic system	s calculated	s observed
(111) √3	0.0044557	0.004456
(200) √4	0.005145	0.005061
(220) √8	0.007276	0.007224
(311) √11	0.008532	0.008522
(222) √12	0.008911	0.008868
(400) √16	0.010289	0.010100
(331) √19	0.011213	0.011204
(420) √20	0.011504	0.011377
(422) √24	0.012603	0.012502

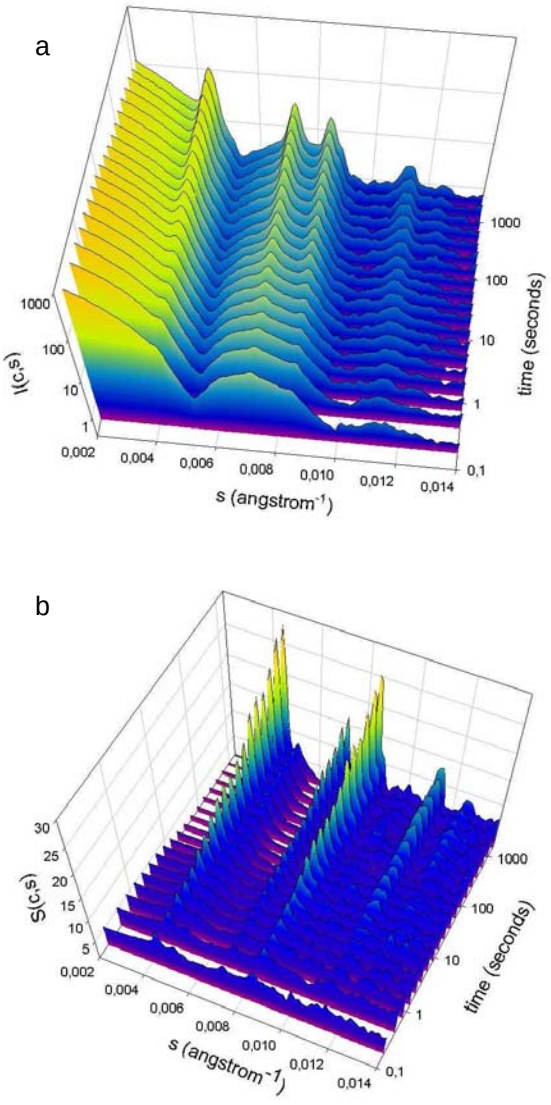

Figure 3
The diffraction peaks on (a) were emphasized by suppressing the contribution of the soluble virus to the signal (b).

The diffraction peaks were more numerous and better differentiated with PEG 20 000 than with PEGs 8 000 and 3 000. To characterize the diffraction peaks, all the data were divided by the form factor of the virus, in order to obtain the structure factor of the diffracting samples and emphasize the peaks (figure 3, a and b). The peaks appeared from one to ten seconds after the mixing, according to the precipitation conditions, and kept on growing continuously throughout the measurements, except when there were no more soluble BMV in solution.

In our previous studies, five diffraction peaks were distinguishable (Casselyn *et al*., 2001), but did not allow us to determine a precise unit cell. With the present experiments, the high brilliance of the beamline permitted to observe up to eight peaks, and their spacing is consistent with a face-centered cubic system (see table 1), with a unit cell side of 390 Å. This system is different from the rhombohedric system (R3) of the crystals that recently permitted the structural resolution of BMV at 3.4 Å, for which a =b = c =263.5 Å and α =61.5° (Lucas *et al*., 2002), but the crystallization conditions were there PEG 550 mono-methyl ether combined with magnesium acetate.

4. Conclusions

The analysis of the results obtained at ESRF by coupling SAXS experiments with a stopped-flow device provided us with informations about the appearance and evolution of BMV microcrystals induced by the addition of PEG.

In all crystalline conditions, the growth of the diffraction peaks was regular as a function of time.

This indicated a continuous formation of microcrystals, until reaching the solubility of the virus. The nucleation rate was related to the PEG size and concentration, as well as to the virus concentration.

All the microcrystals belong to the same crystalline system, as the positions of the peaks are conserved in all conditions. The positions are consistent with a face centered cubic system.

The period of latency for the peaks to appear was from one to ten seconds after the mixing, and no evidence of an intermediary amorphous step before the crystallization of the samples was found during that period.

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