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Short communication

A plate-based high-throughput assay for virus stability and vaccine formulation

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A B S T R A C T

Standard methods for assessing the thermal stability of viruses can be time consuming and rather qualitative yet such data is a necessary requisite for vaccine formulation. In this study a novel plate-based thermal scanning assay for virus particle stability has been developed (PaSTRy: Particle Stability Thermal Release Assay). Two environment-sensitive fluorescent dyes, with non-overlapping emission spectra and different affinities, are used to accrue simultaneously independent data for the overall stability of the virus capsid, as judged by the exposure of the genome, and for capsid protein stability according to the exposure of hydrophobic side chains which are normally buried. This offers a fast and efficient high-throughput method to optimise vaccine formulation and to investigate the processes of virus uncoating.

Determination of the stability of virus particles is critical to the development of vaccines which must remain stable for transport and storage. This issue is especially important in the developing world where longer transport times and the absence of an established cold chain can hamper vaccination programmes. Current methods for assaying particle stability use comparative infectivity with respect to standards to determine the number of infective virions per unit volume (WHO, 2006).

Thermal scanning is a well-established method for the assessment of protein stability, in which the protein sample is slowly heated and its state monitored. Fourier-transform infrared spectroscopy (FTIR (Cooper and Knutson, 1995; Niesen et al., 2008)) and circular dichroism (CD (Benjwal et al., 2006)) have both been used to follow the loss of protein secondary structure as the temperature of the sample is increased, however calorimetry (Brandts and Lin, 1990) and light-scattering (Kurganov, 2002), are more popular due to the ready availability of these instruments.

For soluble proteins the so-called Thermofluor (also known as differential scanning fluorimetry, DSF, or thermal shift assay) (Pantoliano et al., 2000) has proven useful for identifying conditions which change the stability of the protein (Niesen et al., 2008) or for finding more stable homologues and mutants (Lavinder et al., 2009). Thermofluor uses an environment-sensitive fluorescent dye to monitor the exposure of the hydrophobic core of the protein to solvent and hence protein unfolding, and has become popular due to the ease of implementation on conventional quantitative PCR instruments (qPCR) combined with fluorescent dyes originally developed as gel stains, especially SYPRO orange. The use of standard format 96-well plates and relatively small amounts of material make this an attractive method, which has been successfully deployed with proteins for several years by ourselves and others (Ericsson et al., 2006; Geerlof et al., 2006; Sainsbury et al., 2008).

Many viruses comprise an effectively sealed protein shell harbouring the viral genome. It might be expected therefore that the loss of capsid integrity would proceed, perhaps via conformational changes, to the point where the protein shell either undergoes complete dissolution releasing the genome, or reconfigures to such an extent that the genome either escapes or is rendered accessible to dye. In either case, although the protein subunits are unlikely to unfold they may well expose hydrophobic regions concealed in the native capsid. Whilst changes in virus capsid protein association might therefore be monitored using a fluorescent dye such as SYPRO orange, a dye sensitive to the presence of nucleic acid would allow the accessibility of nucleic acid to be directly monitored. The use of two dyes simultaneously would provide a picture of the changes in the protein components, via a series of melting temperatures, T_ms, alongside the temperature at which nucleic acid is released or exposed, T_R, (calculated in the same way as T_m). Such a nucleic acid dye must be sensitive over a broad range of temperature and physical and chemical conditions, have excitation and emission spectra compatible with the instrumentation available, and, critically, be sufficiently large so as not to prematurely penetrate the mature viral capsid through pores or "thermal breathing",

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thereby obscuring any change in fluorescence upon capsid opening (Tuthill et al., 2009).

This study sought to devise a thermofluor-type assay to accurately assess the stability of viral capsids. Such an assay could be useful for improving vaccine stability, and as a general tool to examine the dynamics of viral uncoating.

Equine rhinitis A virus (ERAV) was produced and purified as previously described (Tuthill et al., 2009). Briefly, infected Ohio HeLa cells were lysed by freeze–thawing at the peak of virus growth. After centrifugation to remove cell debris virus particles were precipitated with ammonium sulphate and pelleted through a 30% sucrose cushion before separation by ultracentrifugation in a 15–45% sucrose gradient. The virus was located by measuring the absorbance at 260 nm of the fractionated gradient.

The preparation used for the initial proof of principle experiment contained approximately 30% sucrose and contained ERAV at a concentration of approximately 0.04 mg/mL. The investigation of the pH-dependence of particle and capsid protein stability used material from which the sucrose had been removed and which had been concentrated to approximately 0.15 mg/mL in 50 mM HEPES pH 7.3, 50 mM NaCl.

The type 1 Mahoney strain Polio virus (PV) was produced and purified as previously described (Tuthill et al., 2006). Briefly, PV was grown in HeLa cells maintained in suspension culture. The cells were harvested by centrifugation and were subjected to two cycles of freeze–thaw to release the virus before removal of cell debris by low speed centrifugation. The virus was purified by ultracentrifugation in a CsCl density gradient and the fractions containing the virus particles pooled and concentrated.

Bovine enterovirus type 2 (BEV2) was produced and purified as described previously for BEV type 1 (Smyth et al., 1993). Briefly, BHK 21 cell monolayers were infected with BEV2 and the cells and supernatant harvested at maximum cpe. After freeze–thawing the debris was removed by centrifugation and the supernatant made 50% saturated ammonium sulphate to precipitate virus. The resuspended virus was pelleted through a 30% sucrose cushion and finally purified by ultracentrifugation through a 15–45% sucrose gradient.

For the PaSTRy assay, 50 μL volume reactions were set up per well. Typically a small volume of concentrated sample provided 1–2 μg virus per experiment although if the virus concentration was low then a larger volume of sample was required in order to provide a minimum of 1 μg.

Fluorescent dyes were obtained from Molecular Probes (Life Techologies, Paisley, UK); 5000× stocks of both SYPRO orange (product S-6650) and SYPRO red (S-6653), 5 mM stock of SYTO9 (S-34854) and 10,000× of SYBR green II (S-7564), all supplied in dimethylsulphoxide (DMSO). Working stocks of 50× SYPRO orange and SYPRO red, 100× SYBR green II, and 50 μM SYTO9 were produced freshly for each experiment by dilution in milliQ-grade water. The dyes were used at final concentrations of 3× SYPRO orange and SYPRO red, 10× SYBR green II and 5 μM, SYTO9, by diluting the working stocks into the final reaction, or in the case of multiple parallel experiments, by preparation of mastermix solutions containing dyes, buffer and virus sample.

The remainder of the 50 μL reaction volume was made up with buffer, 10 mM HEPES pH 8.0, 200 mM NaCl. By using a large volume of well-buffered screening solution with a small volume of sample it is possible to readily screen a wide range of conditions while diluting the effect of any constituent of the sample buffer.

In order to screen the pH-dependence of ERAV the SPG buffer mixture (Molecular Dimensions, Newmarket, UK) consisting of succinate, phosphate and glycine, (Newman, 2004)) was used at 0.1 M across a pH range of 4.0–9.5 in 0.5 pH unit steps with 0.1 M NaCl in all wells. This buffer mixture contains all ion species at all points in the pH range and so any stabilisation effect of specific ions could be excluded from the analysis.

Typically a dual-dye reaction would contain the following:

Virus sample (1 mg/mL)	1	*Total 1* μg
SYPRO red (50×)	3	*3× final*
SYTO9 (50 μM)	5	*5* μM *final*
Buffer	41	
Total	50 μL	

For the experiments described below we used two different machines, the Opticon2 (Bio-rad, Hemel Hempstead, UK) and the MX3005p (Agilent, Edinburgh, UK). Reactions were set up in 96-well PCR plates (low profile skirted plates AB-1000/w, Thermo, for the BioRad instrument; semi-skirted plates, product 401334, Agilent, for the Agilent instrument), mixed by pipetting, and then centrifuged at 2000 × *g* to remove any bubbles. The plates were then sealed using optically clear foils (BioRad Microseal B, MSB-1001).

The BioRad Opticon2 qPCR machine uses an array of LEDs for excitation in the 470–505 nm range. Emission is detected on two channels: 523–543 nm and 540–700 nm. Both SYPRO orange and SYTO9 (or SYBR green) produced a stronger signal in the second, broader, channel so this was used for detection of all dyes. This instrument therefore can only be used for experiments with one dye per well. Thermal control is provided by the Peltier unit of a MJ Research DNA Engine which can achieve temperatures below ambient.

The experiments were ramped from 4 to 99 °C, taking a fluorescence reading every 0.5 °C after holding for 10 s, each such run taking approximately one hour to complete. Particular attention was paid to ensure that heating of the sample was not excessive while the lid of the instrument was pre-heated.

The temperatures (T_R, T_m) of the transitions for melting curves were calculated from the inflection point (dI/dT) of the fluorescence intensity (I) as a function of temperature (T). When necessary, noisy data were smoothed to aid the identification of the dI/dT peak and hence assign T_R or T_m.

The MX3005 qPCR machine uses a quartz tungsten halogen lamp in combination with one of five selectable bandpass filters for excitation; and a single scanning photomultiplier tube with one of five bandpass filters for fluorescence detection. Both the excitation and emission filters, bandwidth ~10 nm, can be specified as required and are independently selectable in order to allow mismatching. The excitation (ex) and emission (em) bandpass filters used for the dyes were as follows: SYPRO orange, ex: 492 nm, em: 585 nm (~20% stronger than ex: 492 nm, em: 610 nm); SYPRO red, ex: 585 nm, em: 665 nm (~30% stronger than ex: 535 nm, em: 665 nm); SYTO9, ex: 492 nm, em: 517 nm. Thermal control for this instrument is also provided by a Peltier unit which in this case can only operate from 25 to 100 °C limiting the low temperature data that can be gathered.

The experiments were ramped from 25 to 99 °C recording in triplicate fluorescence readings for each of the filter combinations specified every 1 °C, taking approximately one hour to complete.

Initial investigations indicated that the RNA-sensitive dyes SYBR green II and SYTO9 were both suitable (data not shown). Proof-of-principle experiments with these dyes used two picornaviruses which show different capsid alterations during the process of cell entry and uncoating; equine rhinitis A virus (ERAV), an aphthovirus often used as a surrogate for foot-and-mouth disease virus, dissociates into pentameric units after release of the RNA (Tuthill et al., 2009), and the enterovirus poliovirus type I, which undergoes a conformational transition to release the RNA genome from an expanded particle (Hogle, 2002). Parallel experiments containing one dye each were set up to monitor both the protein unfolding, via T_m, and the RNA release, via T_R, from the virus capsid (Fig. 1a).