Isoelectric titration curves of viral particles as an evaluation tool for ion exchange chromatography

S. Herzer*, P. Beckett*, T. Wegman†, and P. Moore*
*Amersham Biosciences Corp, Piscataway, NJ, USA; †Mayo Clinic, Rochester, MN, USA

Electrophoretic titration curves (ETC) were used to determine the charge characteristics of viral particles as part of the development of a chromatographic purification strategy. CyDye™ labelling of viral particles and in-gel detection using Typhoon™ scanner contributed to a fast and sensitive tool for determination of chromatographic behavior of intact viruses on ion-exchange columns. ETC results facilitated the choice of ion exchange media and separation conditions. Results are reported for adenovirus and measles virus. The method also worked for adeno-associated virus (AAV), murine leukemia virus, and bacteriophages such as lambda and M13.

Introduction
Electrophoretic titration curves (ETC) are a powerful tool to determine charge characteristics of biomolecules over a preset pH range. ETC of protein mixtures is well documented (1–10) and especially useful for the evaluation of ion exchange chromatography (1, 7).

Gene therapy and the demand for high purity viral particles at large scale have created an increased interest in chromatographic purification (11–14). Complexity and fragility of the particle impair approximations of charge behavior. Agarose gel electrophoresis in a pH gradient offers the advantage of relatively mild conditions. We describe here a fast, reproducible, sensitive method to determine a useful working pH for chromatographic separation of intact viral particles.

Materials
All materials including bacteriophages were obtained from Amersham Biosciences unless otherwise mentioned. Generic chemicals for buffer preparation were obtained from Sigma, Aldrich. SYPRO™ Ruby was obtained from Molecular Probes. All other viruses were obtained from ATCC, except measles virus (kind donation of Professor M. Federspiel, Mayo Clinic, Rochester, MN).

Methods

CyDye™ fluor labelling was achieved at a slightly basic pH compatible with virus stability. To avoid over-labelling and crosslinking, only monoreactive dye was used. Labelling conditions were kept at low temperature (on ice) for short duration to ensure that surface charge characteristics would not be affected by over-labelling.

Manufacturers’ instructions were followed for all other procedures unless otherwise mentioned. 5% glycerol was added to agarose ETC gels to circumvent aggregation, which appears as excessive smearing on the scanned gel.

Control agarose ETC gels were run on PhastSystem according to manufacturer’s instructions and visualized with SYPRO Ruby on a Typhoon scanner.

The HiTrap™ IEX Selection Kit, RESOURCE™ S, and RESOURCE Q columns were used to separate out the viral particles by chromatography. A pH value as determined by ETC was chosen for separation and the desalted or dilute virus (at > 5 mS/cm, appropriate pH) was applied to the pre-equilibrated column. Generally, columns were equilibrated in a low strength buffer (buffer A, e.g. 25–50 mM Tris at appropriate pH) and 1–3 column volumes (CV) of virus was applied.

The column was washed for at least 1–2 CV and virus was eluted with a 10 CV gradient to 100% B where B contains a salt suitable to ensure virus stability, for example 1 M NaCl if compatible, in buffer A. 0.25–0.5 CV fractions were collected throughout.

Fractions were analyzed for virus by either infectivity or by applying virus to an HR 5/10 column packed with Sepharose™ 6 Fast Flow (17-0159-01) or a MicroSpin™ S-400 HR Column (27-5140-01). The collected void volume was analyzed by gel electrophoresis for characteristic viral banding pattern.
Results and discussion

To ensure that CyDye labelling would not have a large effect on viral charge behavior, two of the viral vectors tested were also separated on agarose ETC without prior labelling. Gels were stained (15) and only a slight shift in migration pattern was observed (data not shown).

Agarose gel electrophoresis of adenovirus (AV) labeled with Cy™5 indicated an overall negative surface charge of the AV from pH 5–10 and a positive charge at pH values below 5 (Fig 1). This coincides well with the isoelectric points of hexon, penton and fiber proteins that make up the adenoviral surface (16). To determine a useful pH range for purification, the unlabeled starting material was also separated by electrophoresis and stained with SYPRO Ruby (data not shown).

The virus could be separated from the bulk of the contaminants at pH 8 on Q Sepharose XL (Fig 2). Lower pH values may be used; however, binding capacity will be affected.

At pH 8, up to 300 mM NaCl can be used in buffer A, but this reduces the binding capacity of the column by half compared with separations performed without NaCl in buffer A (data not shown).

The use of capillary isoelectric focusing has been described to analyze AV type 5 lot stability (17). However, this is the first time the use of electrophoretic titration curves to determine charge behavior of intact viral particles has been described.

Measles virus inactivated by UV irradiation at 320 nm for 15 minutes was also analyzed by ETC. The agarose gel electrophoretic curve indicated that the virus was positively charged at a pH below 7 and negatively charged at a pH above 7 (Fig 3). However, severe aggregation problems seemed to persist at a pH above 7. Based on experiments with non-inactivated measles virus above pH 7, aggregation problems seem to be common and not caused by the UV irradiation process. However UV crosslinking is likely to have increased aggregation.

Based on ETC results, cation exchange was chosen for separation of measles virus. This method choice was also confirmed by electrophoretic analysis of contaminants, which indicated that the bulk of contaminants were negatively charged at a pH > 5.5 (Fig 4). This was somewhat surprising because the two envelope proteins of measles virus indicate that the virus would be negatively charged above a pH of 5.5 according to their isoelectric points (18–20). These observations demonstrate the usefulness of determining charge characteristics empirically rather than based on protein sequence or individual protein isoelectric points. The virus was successfully purified using SP Sepharose XL at pH 6.5 (Fig 5).
Ion exchange was also applicable for purification of adeno-associated virus (AAV), murine leukemia virus, and bacteriophages such as lambda and M13 (data not shown). Analysis of AAV indicated an overall negative net charge above pH 7 and an overall positive net charge below neutral pH. Contaminants appeared to be negatively charged above a pH of 5–5.5. Purification of AAV from cell lysate using SP Sepharose High Performance followed by polishing on SOURCE™ 30 Q works well (21).

Moloney murine leukemia virus displayed an overall net negative charge above pH 6 and a positive net charge below pH 6. This corresponds well with isoelectric points described for surface proteins (22–25). The charge transition was very sharp with a rapid increase in migration velocity above and below that pH. Contaminants appeared to be mostly negatively charged, as well above a pH of 5–5.5, however, charge increase was moderate over a pH range of 5.5–8. Anion exchange chromatography on RESOURCE Q was used to separate the MLV from its major contaminants (data not shown).

References


Fig 5. Separation of UV inactivated measles virus on SP Sepharose XL at a pH of 6.5. Virus containing fractions (based on analytical gel filtration data) are indicated by the black line.

Conclusion

The electrophoretic titration curve is a simple, rapid method for prescreening conditions for ion exchange chromatography of viral particles. The method described here relies on CyDye labelling and detection using Typhoon scanner for rapid and sensitive detection of viral particles. As demonstrated, ETC was used to define an appropriate pH range for ion exchange chromatography of the intact virus particle. In some cases, only empirical data generated by ETC can be relied upon to determine the appropriate pH range because published isoelectric points of capsid proteins may not be suitable for determining purification conditions.