Charge, size distribution and hydrophobicity of viruses:  
Effect of propagation and purification methods

Accepted for publication in Journal of Virological Methods on February 9, 2018
Published article DOI: 10.1016/j.jviromet.2018.02.008

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Abstract

Two virus propagation methods (in broth and on double agar overlay) and three purification procedures (PEG precipitation, centrifugal diafiltration and CsCl density gradient centrifugation) were comparatively evaluated using MS2 and P22 bacteriophages as model viruses. The prepared stocks were characterized in terms of electrophoretic mobility as a function of pH, particle size distribution, surface tension components and overall hydrophobicity of the virus, as well as the percentage of infectious and total virus recovered. The obtained data were used to rank the purification methods according to six criteria of likely practical relevance. Regardless of the purification method applied, virus propagation in broth media resulted in higher purity virus stocks as the growth on double agar overlay introduced difficult-to-remove residual agar. CsCl density gradient centrifugation gave the highest quality bacteriophage suspensions, recovered infectious P22 at least as efficiently as the other two purification methods and selected for intact P22 virions over damaged ones. The impurities remaining in the virus suspension after PEG precipitation and centrifugal diafiltration broadened the size distribution and interfered with electrophoretic mobility measurements. The residual impurities had a major impact on the free energy of virus-virus interfacial interaction (the quantitative measure of virus hydrophobicity/hydrophilicity) leading to an incorrect determination of P22 bacteriophage as hydrophilic. The trends in measured physicochemical properties can be rationalized by considering impurity-coated virions as permeable soft particles.

1. Introduction

Viral contamination of the water supply is a common cause of waterborne diseases worldwide (Fong and Lipp, 2005; Gall et al., 2015; Sinclair, Jones, and Gerba, 2009). Combined and sanitary sewer overflow events, illicit discharge to storm water systems and septic system failures can lead to a release of viruses into environment, posing a threat to public health (Arnone and Walling, 2007; USEPA, 2001). Comprehensive studies of viruses’ interactions with various natural and engineered surfaces are essential for understanding the mechanisms that control virus transport and fate in various environmental media (e.g. water, air, soil) and viruses’ ability to pass engineered barriers (e.g. in water treatment plants and air filtration units). The dependence of such interactions on surface properties necessitates a thorough physicochemical characterization of viruses. However, viruses directly harvested from host
bacteria or human cell lines are stored in growth media containing impurities such as host cell
debris, soluble microbial products (e.g. DNA, proteins), and incomplete virions, all of which can
confound physicochemical characterization of viruses. Virus purification methods include
density gradient centrifugation (Cromeans, Kahler, and Hill, 2010; Shi et al., 2016; Torres-
Salgado et al., 2016; Wong et al., 2012), precipitation (e.g. with polyethylene glycol) (Cromeans
et al., 2010; Nguyen et al., 2011; Tanneru, Rimer, and Chellam, 2013; Tong et al., 2012), direct
diafiltration (Armanious et al., 2016b; Gutierrez et al., 2009), dialysis (Langlet et al., 2008a;
Michen et al., 2012) as well as ultracentrifugation (Attinti et al., 2010) and chromatography
(Farkas, Varsani, and Pang, 2015; Monjezi et al., 2010). The first three methods (see
Supplementary material (SM), Figure S1) are broadly used in studies on virus fate and transport
in the environment and were chosen for evaluation in the present work.

Density gradient centrifugation can be of two general types: rate-zonal centrifugation and
isopycnic centrifugation. Sucrose and CsCl are commonly used to prepare density gradient
materials. Rate-zonal centrifugation separates viruses primarily based on differences in size
and mass, which result in different sedimentation rates; in this method, a virus sample is layered
as a narrow band on the top of a continuous density gradient. In contrast, isopycnic
centrifugation separates viruses based solely on differences in the density rather than size and
a virus sample could be either overlaid on or placed under a prepared density gradient. Both
rate-zonal and isopycnic centrifugation methods could effectively separate viruses from
impurities by forming a band that contains only the target virus. Certain viruses (e.g.
herpesvirus (Pertoft, 1970), rotavirus (Chen and Ramig, 1992), human respiratory syncytial
virus (Gias et al., 2008)), however, may lose infectivity during density gradient centrifugation.

Virus precipitation with polyethylene glycol (PEG) relies on PEG acting as an "inert solvent
sponge" that sterically excludes viruses from the solvent and causes them to precipitate from
the growth medium (Atha and Ingham, 1981; Lewis and Metcalf, 1988). Other growth medium
components including metals (Prussin, Marr, and Bibby, 2014), proteins (Prussin et al., 2014)
and DNA (Hammerschmidt, Hobiger, and Jungbauer, 2016) can be co-precipitated. PEG
removal by dialysis is time-consuming due to the low diffusivity of PEG (Cantor and Scopes,
1994) and, possibly, its complexation with other components of the virus stock solution (Kleiner,
Hooper, and Duerkop, 2015). Centrifugal diafiltration uses ultrafiltration membranes for a size-
based separation of virions from other components of the growth medium. A pressure-driven
process, diafiltration is faster than dialysis. In addition, diafiltration concentrates and purifies
viruses in one batch process minimizing virus loss whereas conventional dialysis only purifies
the virus. Two major drawbacks common to dialysis and diafiltration are possible virus aggregation (Dika et al., 2013a) and retention of impurities with the molecular weight larger than the pore size of the dialysis or diafiltration membrane.

Viruses propagated and purified by different methods may exhibit different adsorption (Armanious et al., 2016a), aggregation (Dika et al., 2013a; Nguyen et al., 2011) and electrokinetic (Dika et al., 2013a) behaviors. The differences are likely caused by the residual impurities, either in the dissolved phase or associated with virions (Michen and Graule, 2010). Using quartz crystal microbalance measurements, Armanious et al. showed that bacteriophage MS2 purified by PEG precipitation adsorbs on poly-L-lysine surface to a lesser extent than MS2 purified by diafiltration (Armanious et al., 2016a). Such differences can be understood by elucidating physiochemical properties of viruses grown and purified via different protocols. Using dialysis, PEG precipitation and density gradient centrifugation, Dika et al. showed that size and charge of MS2 and MS2-like particles were affected by the choice of the purification method (Dika et al., 2013a). The effects of growth and purification protocols on other important physicochemical properties such as isoelectric point, surface tension, and hydrophobicity have not been explored and are the focus of the present work.

The goal of this study was to comparatively evaluate several commonly used methods of virus propagation (in broth and on double agar overlay) and purification (CsCl density gradient centrifugation, PEG precipitation, and centrifugal diafiltration) in terms of their impact on key physicochemical properties of viruses. MS2 and P22 bacteriophages were employed as model microorganisms. The two bacteriophages are often used as surrogates for human enteric viruses (Bae and Schwab, 2008; Dawson et al., 2005; Masago, Shibata, and Rose, 2008; Pasco et al., 2014) and differ significantly in terms of size and hydrophobicity. MS2 is small and hydrophilic while P22 is larger and slightly hydrophobic. (Published data on MS2 hydrophilicity are inconsistent (Chattopadhyay and Puls, 1999; Dika et al., 2015; Dika et al., 2013b; Heldt et al., 2017)). Particle size distribution, electrophoretic mobility, isoelectric point, surface tension components (apolar, electron donor and electron acceptor) and the overall virus hydrophobicity were determined for virus stocks prepared using different combinations of growth and purification methods. The characterization data were used to compare and rank the purification methods according to six criteria of potential practical relevance.
2. Material and Methods

2.1 Reagents

All reagents were of analytical grade or higher purity. Sodium chloride, calcium chloride dihydrate and polyethylene glycol (MW 6,000 Da) were purchased from Sigma-Aldrich. Cesium chloride, magnesium chloride (anhydrous) and sucrose were obtained from VWR and Tris base was purchased from Fisher Scientific. Tryptone, agar and yeast extract were purchased from BD biosciences. Luria broth was purchased from DOT Scientific.

2.2 Virus propagation and purification methods

Bacteriophage MS2 was purchased from ATCC (ATCC 15597-B1) and propagated both on double agar overlay (recommended by ATCC) and in liquid broth with Escherichia medium (ATCC medium 271) and Escherichia coli strain C3000 (ATCC 15597) as the host.

Bacteriophage P22 and its host, Salmonella enterica subsp. enterica serovar Typhimurium strain LT2, were provided by Dr. Kristin Parent (Michigan State University). In contrast to MS2, P22 was propagated only in broth (Luria broth (LB)). The detailed description of the two propagation procedures is provided in SM, section S1. Figure 1 schematically outlines the three purification methods evaluated in this work. Each purification method is described in detail in SM, section S2. Table 1 lists nine sample types evaluated in this study as well as the nomenclature used to denote the samples.
Figure 1: Three purification methods evaluated in this study: CsCl density gradient centrifugation, PEG precipitation, and centrifugal diafiltration. See SM for detailed descriptions of each purification method.
Table 1: Nomenclature used to describe MS2 and P22 stocks produced using different combination of propagation and purification methods.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Phage</th>
<th>Propagation medium</th>
<th>Purification method</th>
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</thead>
<tbody>
<tr>
<td>“MS2-agar-CsCl”</td>
<td>MS2</td>
<td>Double layer agar</td>
<td>CsCl ρ-gradient centrifugation</td>
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<tr>
<td>“MS2-agar-PEG”</td>
<td></td>
<td></td>
<td>PEG precipitation</td>
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<tr>
<td>“MS2-agar-DF”</td>
<td></td>
<td>E. coli broth</td>
<td>Centrifugal diafiltration</td>
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<td>“MS2-broth-CsCl”</td>
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<td>CsCl ρ-gradient centrifugation</td>
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<td>PEG precipitation</td>
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<td>“MS2-broth-DF”</td>
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<td>Centrifugal diafiltration</td>
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<tr>
<td>“P22-broth-CsCl”</td>
<td>P22</td>
<td>Luria broth</td>
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<td>“P22-broth-DF”</td>
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<td>Centrifugal diafiltration</td>
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2.3 Hydrodynamic diameter and electrophoretic mobility measurements

Malvern Zetasizer Nano-ZS was used to measure both the hydrodynamic diameter ($d_h$) and the electrophoretic mobility ($\mu$) of virions. Hydrodynamic diameter was measured by dynamic light scattering and $\mu$ was determined by phase analysis light scattering. Prior to virus size and charge characterization, each purified stock was filtered using 0.22 µm filter. The hydrodynamic diameter and electrophoretic mobility were measured as functions of pH, which was adjusted using NaOH and HCl. For all pH except pH 7.6, the background electrolyte was 1 mM NaCl solution. The pH of 1 mM NaCl solution could not be stabilized at pH 7.6 by adding NaOH or HCl only; instead, Tris-MgCl$_2$ buffer (10 mM Tris, 10 mM MgCl$_2$) was used as the background solution at this pH.

2.4 Surface tension and hydrophobicity determination

Apolar, electron donor and electron acceptor components of the surface tension as well as the overall hydrophobicity of viruses purified by different methods was determined using the protocol described in our previous work (Pasco et al., 2014; Shi et al., 2016). Briefly, to prepare a virus lawn, purified virus stock was filtered through a 50 kDa ultrafiltration membrane. The membrane coated with a multilayer cake of virions was dried at room temperature until the water contact angle on the membrane stabilized. Contact angles of three probe liquids (water,
glycerol and diiodomethane) on the virus lawn were then measured using sessile drop method.

Surface tension components of each virus ($\gamma_{vlw}^L$, $\gamma_{vlw}^+$, $\gamma_{vlw}^-$) were obtained by substituting measured contact angles and known surface tensions of probe liquids into the Young-Dupré equation (van Oss, 2006):

$$(1 + \cos \theta)\gamma_i^{TOT} = 2 \left( \sqrt{\gamma_{vlw}^L \gamma_i^{LW}} + \sqrt{\gamma_i^+ \gamma_i^-} + \sqrt{\gamma_i^- \gamma_i^+} \right)$$

where $\theta$ is the contact angle of the probe liquid on the virus lawn, $\gamma_i^{TOT}$ is the total surface energy, while $\gamma_{vlw}^L$, $\gamma_i^+$ and $\gamma_i^-$ are Lifshitz-van der Waals (i.e. apolar), electron acceptor, and the electron donor components of surface energy. Subscripts $l$ and $v$ refer to the probe liquid and the virus, respectively. The right hand side of eq. (1) represents the work of adhesion of a probe liquid to a lawn of viruses. The free energy of interfacial interaction between two virions immersed in water ($\Delta G_{vww}$) was calculated using eq. (2) (van Oss, 2006):

$$\Delta G_{vww} = -2 \left( \sqrt{\gamma_{vlw}^L} - \frac{1}{2} \sqrt{\gamma_{vw}^L} \right)^2 - 4 \left( \sqrt{\gamma_{vlw}^L \gamma_{vw}^L} + \sqrt{\gamma_{vlw}^+ \gamma_{vw}^+} - \sqrt{\gamma_{vlw}^- \gamma_{vw}^-} - \sqrt{\gamma_{vlw}^- \gamma_{vw}^+} \right)$$

and used as a quantitative measure of hydrophobicity of the virus in question. A virus is hydrophobic when $\Delta G_{vww} < 0$ (van Oss and Giese, 1995). The absolute value of $\Delta G_{vww}$ indicates the degree of hydrophobicity (or hydrophilicity, when $\Delta G_{vww} > 0$) of the virus.

### 2.5 Virus recovery

To evaluate the recovery in terms of the total virus count (i.e. count of genome copies) and in terms of infectious virus count, 200 mL of the initial bacteriophage stock was purified and concentrated to $\sim 2$ mL with one of the three methods described above. Enumeration of viruses in the original stock and the purified sample was performed using double layer plaque assay (to obtain infectious virus recovery) and quantitative polymerase chain reaction, qPCR (to obtain total virus recovery). The sequences of primers and probe as well as qPCR parameters were described in our previous study (Pasco et al., 2014). The virus recovery, $r$, is given by:

$$r = \frac{C_p V_p}{C_o V_o}$$

where $C_p$ and $C_o$ are virus concentrations (PFU/mL or DNA copies/mL) in the purified sample and the initial sample, respectively, while $V_p$ and $V_o$ are volumes of these samples.
3. Results

3.1 Effects of the virus propagation method on size and electrophoretic mobility measurements for purified virus stocks: Tests with MS2 bacteriophage

3.1.1 Effect of the propagation method on the particle size measured for the purified MS2 stock

MS2 propagated either on double layer agar or in broth was purified by one of the three methods: CsCl density-gradient centrifugation, PEG precipitation, and centrifugal diafiltration (Table 1). To evaluate the effect of the propagation method on virus physicochemical properties, we compared results obtained with MS2 stocks purified by the same method, but propagated differently. Figure 2 shows intensity-based size distribution of MS2 in the storage solution (1 mM NaCl; pH 5.5 to 6.0, unadjusted) for MS2 grown on agar or in broth and purified by one of the three methods. Values and corresponding error estimates of the average hydrodynamic diameter ($d_h$), polydispersity index and half width at half maximum (HWHM) of virus suspensions in the storage solution were determined for each preparation (see SM, Table S1).

In MS2-broth-CsCl, $d_h$ was 26.9 ± 0.1 nm matching the individual virion size obtained by TEM (27 nm (Strauss and Sinsheimer, 1963)). The small HWHM (~ 9 nm) indicates that the virus suspension is close to monodisperse. MS2-agar-CsCl had higher $d_h$ (~ 32 nm) and HWHM (~ 15 nm) and included an additional small peak (3.1% of the intensity) at ~ 2 to 5 μm. Because the purification method was the same (CsCl density gradient centrifugation), the broadening of the size distribution was an effect of propagation on agar.

The same trend was observed for PEG-purified samples. In MS2-agar-PEG, $d_h$ was 32 nm with HWHM of ~ 15 nm, both larger than those for MS2-broth-PEG ($d_h$ 28 nm; HWHM ~ 11 nm). For the same purification method, $d_h$, polydispersity index and HWHM values for agar-propagated MS2 were all higher than the corresponding values for broth-propagated MS2 (see SM, Table S1). We tentatively attribute the broadening of the main peak to the presence of impurities introduced during MS2 propagation on the double agar overlay.
Figure 2: Particle size distribution of purified MS2 stock produced by two different propagation methods - in broth and on double layer agar overlay - and purified by a) CsCl density gradient centrifugation, b) PEG precipitation and c) centrifugal diafiltration. Background solution: 1 mM NaCl; pH 5.5 to 6.0.
Regardless of the purification method, all MS2 suspensions obtained by propagation in broth were characterized by a single peak in the particle size distribution. In contrast, bimodal size distributions were measured for some MS2-agar-CsCl and MS2-agar-PEG preparations and for all MS2-agar-DF preparations. If a bimodal size distribution was obtained, the same virus stock was filtered once or multiple times through a 0.1 μm pore size membrane and additional size measurements were conducted. An extra filtration step eliminated the peak corresponding to large particles in the MS2-agar-CsCl preparation. Surprisingly, in MS2-agar-DF and MS2-agar-PEG, the second peak (~ 0.2 to 1 μm) persisted through multiple filtrations through the 0.1 μm filter. We attribute the peak to the components of the agar - the only additional substance used in the double agar overlay procedure in comparison with the broth-based growth.

Although agar gel had been removed from the virus harvest by centrifugation and filtration, agar molecules such as agarose (> 150 kDa) and agaropectin (< 20 kDa) (Armisén and Gaiatas, 2009) or their aggregates could remain in the stock and re-aggregate. Indeed, gel-like aggregates were observed by naked eye in the UF concentrate during MS2-agar-DF. The aggregates likely formed from smaller agar molecules that passed the 0.1 μm filter and were concentrated along with the virus during the purification step. Such re-aggregation of agar molecules has been reported previously (Armisén and Gaiatas, 2009). In the course of each size distribution measurement with post-ultrafiltration samples we observed a continual increase in the intensity of the peak within 0.2 to 1 μm range.

Particle size distributions of virus-free control samples were also measured. Controls were prepared by following all the steps in the agar propagation method except for the inoculation by MS2. After the harvest, a virus-free solution was purified by either PEG precipitation or diafiltration to obtain control-agar-PEG and control-agar-DF samples, respectively. Broad size distributions ranging from 10 nm to 5 μm were measured in both controls (see SM, Figure S2); this observation confirmed the presence of agar-derived molecules and their aggregates in the virus-agar-PEG and virus-agar-DF samples.
Figure 3: Electrophoretic mobility as a function of pH for MS2 propagated by two different methods (in broth or on double layer agar overlay) and purified by a) CsCl density gradient centrifugation, b) PEG precipitation or c) centrifugal diafiltration. Empty and filled symbols correspond to measurements in 1 mM NaCl background electrolyte and in Tris-MgCl₂ buffer, respectively.
3.1.2 Effect of the propagation method on the electrophoretic mobility measured for the purified MS2 stock

In a separate set of tests, the electrophoretic mobility, $\mu$, of MS2 prepared by six different protocols (Table 1) was measured as a function of pH (Figure 3). The mobility measured for MS2-agar-CsCl in the storage solution (1 mM NaCl; pH 5.5 to 6.0, unadjusted) was $-3.0 \pm 0.1 \mu m \text{cm}^{-1} \cdot \text{V}^{-1}$, the same as for MS2-broth-CsCl samples. Negative $\mu$ for MS2-agar-PEG and MS2-agar-DF were only slightly more negative than corresponding mobilities measured for MS2-broth-PEG and MS2-broth-DF in the storage solution, but the difference increased significantly at pH < 4. The IEP of MS2 was ~ 3.6 in MS2-broth-CsCl and MS2-broth-DF and ~ 2.9 in MS2-broth-PEG. In MS2-agar-PEG and MS2-agar-DF, the electrophoretic mobility was measured to be negative over the entire pH range from 2 to 10 so that the IEP was not observed.

As in the case with size data, the trends in the dependence of electrophoretic mobility with pH could be affected by agar-based impurities. We hypothesize two mechanisms for such interference. First, agaropeptin, which is a non-gelling fraction of agar, could contribute a spurious signal. Agaropeptin is a highly charged anionic polysaccharide with acidic side-groups such as ester sulfate, D-glucuronic acid, and pyruvic acid in the repeating unit (agarobiose, which is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose) (Nussinovitch, 1997). Indeed, high negative charge was measured in control-agar-PEG and control-agar-DF samples, which contained no viruses, over the entire pH range from 2 to 10 (see SM, Figure S3). Second, agaropeptin could potentially coat the virus to impart a strong negative charge to its surface. This explanation is consistent with the divergence of electrophoretic mobility values measured for agar-propagated and broth-propagated samples at pH < 4 where the viral capsid is protonated (Michen and Graule, 2010; Penrod, Olson, and Grant, 1995).

For MS2-agar-CsCl, the IEP could be determined (~ 2.9) but was lower than the IEP (~ 3.6) measured for MS2-broth-CsCl (Figure 3a). It appears that CsCl density gradient centrifugation is capable of separating MS2 from agar impurities but the separation is incomplete. The presented results show that in comparison with double layer agar overlay, broth-based propagation introduces less contamination that interferes with size and charge measurements of bacteriophages.
Figure 4: Particle size distribution (a, c) and electrophoretic mobility (b, d) of broth-propagated MS2 (a, b) and P22 (c, d) stocks purified by CsCl density gradient centrifugation, PEG precipitation and centrifugal diafiltration. Data for MS2 partly overlaps with data shown in Figures 2 and 3 and is included here to facilitate data analysis. Empty and filled symbols in (b) and (d) correspond to measurements in 1 mM NaCl background electrolyte and in Tris-MgCl₂ buffer, respectively. Vertical lines mark sizes of MS2 (a) and P22 (c) virions as determined by TEM.
3.2 Effects of the virus purification method on size and electrophoretic mobility measurements for broth-propagated viruses: Tests with MS2 and P22 bacteriophages

3.2.1 Effect of the purification method on the particle size measured for broth-propagated MS2 and P22 stocks

When evaluating the effects of virus purification procedures, P22 and MS2 bacteriophages were propagated in liquid broth to avoid the interference of impurities from agar. Figure 4 shows the size distribution and electrophoretic mobility of broth-propagated MS2 and P22 purified by three different methods. As mentioned in section 3.1, MS2 size in MS2-broth-CsCl samples in the storage solution was measured to be ~27 nm with low HWHM (~9 nm) indicating a stable suspension of monodisperse MS2 virions. MS2-broth-DF had a similar $d_h$ (~30 nm), but a significantly ($p < 0.01$) higher HWHM (~16 nm). The broadening of the main peak and appearance of the additional peak could be due to the presence of cell debris and microbial products retained by diafiltration membrane.

In the MS2-broth-PEG samples at the same pH, $d_h$ was similar (~28 nm) with a slightly higher HWHM (~11 nm) than in MS2-broth-CsCl. The $d_h$ value is close to the size determined by TEM (27 nm) and smaller than MS2 average diameters reported in two other studies that also employed PEG precipitation to purify this phage (40 nm (Nguyen et al., 2011) and 40 to 60 nm (Dika et al., 2013a)). The higher accuracy of our measurement is likely due to the removal of virus-associated PEG by chloroform extraction and dialysis (see SM, section S2.2); this additional step in the purification protocol should reduce the amount of PEG adsorbed on the viral capsid.

Figure 4c illustrates the effects of the three purification methods on the hydrodynamic diameter of bacteriophage P22. In the storage solution, the P22-broth-CsCl preparation had $d_h$ of ~68 nm and with a HWHM of ~23 nm. The individual P22 virion size determined by TEM imaging was ~54 nm (Pasco et al., 2014). The 14 nm difference between the two values of $d_h$ (54 nm and 68 nm) could stem from the tail structure of P22, which impacts its diffusivity (Baltus et al., 2016; Pasco et al., 2014). A higher $d_h$ (~78 nm) and HWHM (~46 nm) were measured for P22 in P22-broth-PEG corroborating the hypothesis of PEG adsorption onto the capsid. Indeed, the
10 nm difference between the two values of $d_h$ (78 nm and 68 nm) could be due to a layer of PEG, a 6,000 Da molecule with ~ 3.1 nm radius of gyration (Holyst et al., 2009). The size distribution for P22-broth-DF ($d_h \approx 91$ nm; HWHM at 75 nm) was bimodal although the secondary peak (3 μm to 6 μm) was very minor.

For both MS2 and P22 in the storage solution, monodispersed virus suspensions with very narrow size distributions were always obtained after purification by the CsCl density gradient centrifugation. Values of $d_h$ as a function of pH for both bacteriophages with each purification method are shown in SM (Figure S5). As pH decreased, severe aggregation of MS2 was observed in all sample preparations (SM, Figure S5a). Larger aggregates (up to ~ 20 μm) were observed after PEG precipitation, while CsCl density gradient centrifugation and centrifugal diafiltration resulted in relatively small aggregates (4 to 6 μm). In comparison with MS2, the largest P22 aggregates detected as pH decreased were ~ 4 μm with CsCl density gradient purification (SM, Figure S5b). For measurements at pH 7.6, Tris-MgCl$_2$ buffer was used as the background solution. At this pH, the size distribution of P22 had a single narrow peak at ~ 70 nm, close to the size measured in 1 mM NaCl at pH 5.5. In contrast, $d_h$ for MS2 in Tris-MgCl$_2$ buffer, pH 7.6 depended on the choice of the purification method. MS2-broth-CsCl preparations had $d_h$ of 29 nm, which was very close to the size obtained by TEM. MS2-broth-DF and MS2-broth-PEG preparations, however, had $d_h$ values of ~ 45 nm and ~ 59 nm, respectively. These larger averages could be explained by impurities that may aggregate in the higher ionic strength (~ 25 mM) of the Tris-MgCl$_2$ buffer. The difference further underscores the advantage for CsCl density gradient centrifugation as the purification method.

3.2.2 Effect of the purification method on the electrophoretic mobility measured for broth-propagated MS2 and P22 stocks

**MS2 bacteriophage: electrophoretic mobility and IEP**

Figure 4b presents electrophoretic mobility, $\mu$, of MS2 as a function of pH. In the storage solution, MS2 has electrophoretic mobility of -2.6 ± 0.1 μm·s$^{-1}$·V$^{-1}$·cm when purified by centrifugal diafiltration. Dika et al. (Dika et al., 2011; Dika et al., 2013b) and Langlet et al. (Langlet et al., 2008a) reported similar MS2 electrophoretic mobilities in NaNO$_3$ and at the same pH of 5.6 for MS2 purified by dialysis, a method closely related to diafiltration. Regardless of
the purification method, a less negative electrophoretic mobility was recorded in the Tris-MgCl₂ buffer (pH 7.6); the difference can be attributed to electric double layer compression at the higher ionic strength of the buffer. The IEP of MS2 was reported to range from 2.2 to 4.0 depending on the solution chemistry (Michen and Graule, 2010). In our study, IEP of MS2 was ~ 3.6 for both MS2-broth-DF and MS2-broth-CsCl preparations in 1 mM NaCl; this value is in agreement with the results reported in other studies where MS2 was purified by similar methods (CsCl density gradient centrifugation (Penrod et al., 1995), diafiltration (Gutierrez et al., 2009), and dialysis (Dika et al., 2011)). No significant differences in \( \mu \) were observed for pH > 5.6 among the three purification methods. At lower pH values, MS2-broth-PEG had a lower value of \( \mu \) than MS2 purified by the other two methods and, therefore, had a significantly lower IEP (~ 2.9).

**P22 bacteriophage: electrophoretic mobility and IEP**

Figure 4d presents electrophoretic mobility, \( \mu \), of P22 as a function of pH. As was the case with MS2, regardless of the purification method, \( \mu \), measured in Tris-MgCl₂ (pH 7.6) was lower in magnitude than that in 1 mM NaCl (pH 5.5). For P22-broth-DF and P22-broth-CsCl in 1 mM NaCl, the IEP of P22 was ~ 3.4, consistent with the IEP (3.3 to 3.4) measured by Fidalgo de Cortalezzi et al. for diafiltration-purified P22 in 15 mM NaCl (Fidalgo de Cortalezzi et al., 2014). In contrast to what was observed for MS2, the IEP of the P22-broth-PEG preparation was not lower but slightly higher (~ 3.7) than IEP values measured for P22-broth-CsCl and P22-broth-DF. The theoretical IEP of P22 calculated by ProtParam, an analysis software that calculates various physicochemical parameters for proteins was higher at ~ 5.0 (Parent et al., 2012). For adenovirus, the theoretical IEP was also reported to be higher (5.2 to 5.6 (Mayer et al., 2015)) than the experimentally measured values (4.0 to 4.3 (Shi et al., 2016), 3.5 to 4.0 (Wong et al., 2012), 4.3 (Lu, Li, and Nguyen, 2016)). The discrepancy may be due to a role of protein structure and viral genome, or to an interaction between the virus and other dissolved species in the virus stock. Both of these factors may affect IEP of viruses and yet are unaccounted for in the theoretical determination of IEP.

**IEP shifts for PEG-purified bacteriophages: Possible reasons**

IEP shifts observed for PEG-purified bacteriophages may stem from PEG interactions with the virion. A virus could be regarded as a permeable soft particle (Langlet et al., 2008b), with the
Electrophoretic mobility partly dependent on the particle’s inner structure (i.e., genome and inner proteins). Having measured electrophoretic mobilities of intact MS2 and RNA-free MS2 at different ionic strengths, Dika et al. interpreted their results as stemming from the formation of a PEG coating on the MS2 capsid, which masked the inner structure of the bacteriophage and possibly reduced its permeability (Dika et al., 2013a). Such masking should make MS2 appear less negatively charged because of the low IEP value (~2.9) of the MS2 RNA (Dika et al., 2011; Langlet et al., 2008b)). Consistent with this interpretation, a higher IEP was measured in our study for P22-broth-PEG than for P22-broth-DF and P22-broth-CsCl. The particle sizing data (10 nm increase in $d_h$ measured for P22-broth-PEG sample; see section 3.2) pointed to existence of an adsorbed layer on the P22 surface in P22-broth-PEG corroborating the above interpretation.

As mentioned above, the decrease in permeability of PEG-purified MS2 should lead to an increase in its IEP. Yet, in our study a lower IEP value was measured for MS2-broth-PEG than for MS2-broth-DF or MS2-broth-CsCl. The result can be understood based on the particle size data, which presented no evidence of PEG adsorption to MS2 in MS2-broth-PEG samples. Post-treatment with chloroform likely removed the virus-associated PEG (see SM, section S2.2) and the internal structure of MS2 was not masked by the PEG coating and a increase in MS2 IEP should not be expected.

The observed decrease in MS2 IEP requires an explanation. The observation can still be rationalized in terms of changes in the internal permeability. A neural polymer, PEG induces virion aggregation at lower pH (see SM, Figure S5a) while the aggregate structure affects hydrodynamic permeability and, therefore, electrokinetic properties of the aggregate (Langlet et al., 2008b). Homoaggregation of PEG-purified MS2 or its heteroaggregation with co-precipitated impurities should lead to a higher overall hydrodynamic permeability. Indeed, much larger (~25 μm) aggregates were detected at pH<4 in MS2-broth-PEG preparation than in MS2-broth-CsCl or MS2-broth-DF (~6 μm) (see SM, Figure S5a). Higher phage permeability increased the role of RNA structure of MS2 in defining its electrokinetic behavior. The aggregation-induced increase in permeability can be a contributing factor in the IEP increase detected for PEG-purified P22. Indeed, the size of P22 aggregates in P22-broth-PEG (up to 120 nm) was significantly smaller than in P22-broth-CsCl (~4 μm) and P22-broth-DF (~2.5 μm) samples. Smaller aggregate size should translate into smaller permeability and, therefore, smaller effect.
of the internal virus structure on electrophoretic mobility. Because no published data could be
found on the IEP on P22 DNA, in the P22 case the argument is purely speculative.

3.3 Effects of the virus purification method on surface tension parameters and
hydrophobicity of broth-propagated viruses: Tests with P22 bacteriophage

We also evaluated the effect of the purification method on the values of surface tension
components and the interfacial free energy (i.e. hydrophobicity) of P22. For P22-broth-CsCl,
these values were reported in our previous work and are used here for comparison (Pasco et
al., 2014). The apolar surface energy component ($\gamma_{LW}$) was similar for all P22 preparations and
was within the 41 to 43 mJ/m$^2$ range (Table 2) typical for biological materials (van Oss, 2006).
The purification procedure did have an effect on the polar surface energy components. For P22-
broth-CsCl, $\gamma^-$ and $\gamma^+$ were 25.8 mJ/m$^2$ and 0.06 mJ/m$^2$, respectively, translating into the overall
polar component, $\gamma_{AB}$ of 2.4 mJ/m$^2$. For P22-broth-PEG and P22-broth-DF, slightly negative
values for $\sqrt{\gamma^+}$ were obtained (~ -0.9 and ~ -0.5, respectively). This may be explained by the
presence of impurities that PEG precipitation and diafiltration failed to remove.

Table 2: Measured contact angles, calculated surface energy parameters and free energy of
interfacial interaction when immersed in water for broth-propagated P22 bacteriophage as a
function of the purification method.

<table>
<thead>
<tr>
<th>Virus properties</th>
<th>CsCl density gradient centrifugation</th>
<th>PEG precipitation</th>
<th>Centrifugal diafiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact angle with probe liquid ($^\circ$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$O</td>
<td>55 ± 2</td>
<td>44 ± 5</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>Glycerol</td>
<td>56 ± 6</td>
<td>65± 4</td>
<td>61 ± 4</td>
</tr>
<tr>
<td>DIM$^f$</td>
<td>34 ± 3</td>
<td>34 ± 6</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>Surface energy parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\gamma_{LW}$</td>
<td>42.5</td>
<td>42.5</td>
<td>41.1</td>
</tr>
<tr>
<td>$\gamma^+$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\gamma^-$</td>
<td>25.8</td>
<td>52.9</td>
<td>46.6</td>
</tr>
<tr>
<td>$\gamma_{AB}$</td>
<td>2.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\gamma^{TOT}$</td>
<td>44.9</td>
<td>42.5</td>
<td>41.1</td>
</tr>
<tr>
<td>Free energy of interfacial interaction in water, $\Delta\gamma_{LW}$ (mJ/m$^2$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-6.3</td>
<td>38.1</td>
<td>29.8</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5: Recovery of broth-propagated (a) MS2 and (b) P22 bacteriophages after purification by three different methods.
Concentrated concomitantly with the virus these impurities would be present within the virus lawn leading to errors in contact angle measurements. This interpretation is consistent with the lower average water contact angle and the higher glycerol contact angle on P22 lawn formed from P22-broth-PEG and P22-broth-DF than from P22-broth-CsCl. Indeed, some of the potential impurities (e.g. PEG, DNA) are hydrophilic substances with a negligible $\gamma^+$ (van Oss, 2006).

Based on the surface tension data reported in our previous work (Shi et al., 2016) and other studies (van Oss, 2006), a very small value of $\gamma^+$ is also a common characteristic of non-enveloped viruses so that even a small error in contact angle measurements could lead to $\sqrt{\gamma^+} < 0$. Accordingly, when calculating the interfacial free energy, $\Delta G_{v\omega v}$, (eq. (2)), for P22-broth-PEG and P22-broth-DF, $\gamma^+$ was assumed to be negligible. For P22-broth-CsCl, P22 was found to be hydrophobic ($\Delta G_{v\omega v} < 0$) whereas for the other two methods $\Delta G_{v\omega v}$ was calculated to be positive indicating that P22 is hydrophilic. The results indicate that purification methods could have a dramatic impact on the hydrophobicity of viruses in the purified stock.

3.4 Effects of virus purification on virus recovery: Tests with MS2 and P22 bacteriophages

The optimal growth-purification sequence should yield a virus stock that is pure, high titer and with virus infectivity preserved. High recovery of intact viruses is especially important in the biopharmaceutical industry (e.g. in vaccine manufacturing). To evaluate virus loss during different purification procedures, we determined infectious virus recovery (for both MS2 and P22) as well as the total virus recovery (for P22 only). As shown in Figure 5, no significant differences ($p > 0.5$) were observed between recoveries of infectious MS2 and infectious P22 with either CsCl density gradient centrifugation ($62 \pm 6\% \text{ vs } 59 \pm 4\%$) or centrifugal diafiltration ($63 \pm 6\% \text{ vs } 60 \pm 8\%$). Thus, at least for MS2 and P22, virus recovery during density-based or size based purification is not sensitive to the virus type; this may not hold for viruses that lose infectivity at high ionic strengths typical in density gradient separations (~ 2.8 to 4.9 M in this study). At the same time, a significant difference ($p < 0.01$) was observed for the PEG precipitation method where infectious MS2 recovery was $69 \pm 7\%$ while infectious P22 recovery
was only 36 ± 9%. The result is expected for a solubility-based purification process that depends on the interactions of the precipitating agent (in this case – PEG) with the virus.

In addition to the recovery of infectious virus, the total virus recovery (i.e. recovery of genome copies) was also evaluated for P22. For P22-broth-PEG and P22-broth-DF preparations, total P22 recoveries were 33 ± 3% and 59 ± 8%, respectively, the values that were not statistically different ($p > 0.6$ and $p > 0.8$, respectively) from infectious P22 recoveries by the same methods. This suggests that PEG precipitation and centrifugal diafiltration recover infectious P22 simultaneously with inactivated P22. In the CsCl density gradient centrifugation method, however, one can expect a difference in density between intact (infectious) virions and damaged (non-infectious) ones. Indeed, our results with this method showed significantly ($p < 0.01$) higher recovery (59 ± 4%) of the infectious P22 than of P22 genome copies (38 ± 3%).

4. Discussion

Experiments with MS2 (see section 3.1) showed that double agar overlay growth introduces difficult-to-remove impurities that confound results of physicochemical characterization of the virus. Therefore, the selected purification methods were assessed with MS2 and P22 propagated only in broth. We compared the purification methods in terms of several criteria including: the narrowness of the particle size distribution in the virus stock; accuracy electrophoretic mobility, IEP and hydrophobicity determinations, total and infectious virus recoveries, as well as the approximate time and cost of each purification procedure (Table 3). TEM images of MS2 (Hooker, Kovacs, and Francis, 2004) and P22 (Pasco et al., 2014) provided guidance for evaluating size measurement results: the proximity of the average hydrodynamic diameter to the size expected based on TEM and the narrowness of the size distribution were considered when ranking the purification methods. Because there is no “standard” reference data on the electrophoretic mobility, IEP, surface energies or hydrophobicity of viruses, the removal of impurities that interfere with measurements of these virus characteristics was assumed to correlate with the quality of the virus size data. The reproducibility of IEP measurements was a secondary consideration responsible for differences between size- and charge-based rankings. Time and cost demands of each purification method were qualitatively evaluated based on the laboratory-based purification protocols employed in this study.
**Table 3.** Ranking of the three purification methods applied to broth-propagated viruses. A higher mark corresponds to better performance.

a The lower ranking is based on the shift in IEP for viruses purified the PEG precipitation.

b The higher ranking is based on the match of the IEP values measured for viruses purified by centrifugal diafiltration and CsCl density gradient centrifugation.

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Method</th>
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<tbody>
<tr>
<td></td>
<td>CsCl ρ gradient centrifugation</td>
</tr>
<tr>
<td>Narrow, monodisperse particle size distribution</td>
<td>+++</td>
</tr>
<tr>
<td>Accurate determination of electrophoretic mobility, isoelectric point, and hydrophobicity</td>
<td>+++</td>
</tr>
<tr>
<td>Recovery: total virus (genome copies)</td>
<td>+</td>
</tr>
<tr>
<td>Recovery: infectious virus</td>
<td>++</td>
</tr>
<tr>
<td>Rapid</td>
<td>+</td>
</tr>
<tr>
<td>Inexpensive</td>
<td>+</td>
</tr>
</tbody>
</table>

A survey of the presented data shows that in contrast to CsCl density gradient centrifugation and centrifugal diafiltration (density- and size- based purification methods, respectively), PEG precipitation (solubility based purification) affected size distribution, IEP and virus recovery differently for MS2 and P22. Specifically, size distribution measured for MS2-broth-PEG was similar to that in MS2-broth-CsCl while P22-broth-PEG had higher $d_h$ and much broader distribution than P22-broth-CsCl. At pH < 4, large (up to ~ 25 μm) aggregates were observed in MS2-broth-PEG but very limited aggregation (~ 120 nm) was detected in P22-broth-PEG. For MS2 a lower IEP (~ 2.9) was observed after PEG precipitation than in MS2-broth-CsCl and MS2-broth-DF (~ 3.6) while slightly higher IEP (~ 3.7) was measured for P22-broth-PEG in comparison with P22-broth-CsCl and P22-broth-DF (~ 3.4). Finally, PEG precipitation gave significantly different ($p < 0.01$) recoveries for infectious P22 (69 ± 7%) and MS2 (36 ± 9%). We hypothesize that solubility-based purification is generally virus-specific because of its dependence on interactions between the precipitating agent and the virus.
In summary, the highest purity virus stocks were produced by CsCl density gradient centrifugation, which consistently gave MS2 and P22 suspensions with single-band narrow size distribution. In addition to high purity, CsCl density gradient centrifugation recovers infectious MS2 and P22 at least as efficiently as the other two purification methods and appears to select for intact virions (~ 59 ± 4% recovery of infectious P22) over damaged ones (~ 38 ± 3% recovery of total P22 genome copies). PEG precipitation and diafiltration may be appropriate choices when some amount of residual impurities is acceptable. In comparison to the other two purification methods, PEG precipitation reduced the IEP for MS2 and slightly increased the IEP for P22; the shifts were tentatively attributed to a finite permeability of the virus/residual PEG aggregates. PEG precipitation is, however, inexpensive and very simple to implement; as such, it may be the best method for applications where the remnant PEG is not a concern (e.g. due to its biological inertness). Centrifugal diafiltration gave size and electrophoretic mobility data of quality close to that obtained with CsCl density gradient centrifugation, but with a much broader size distribution. Compared with the other two methods, the main advantages of diafiltration are speed and high recovery of both infectious and total virus.

5. Conclusions

The study evaluated two virus propagation methods (in broth and on double agar overlay) and three purification procedures (CsCl density gradient centrifugation, PEG precipitation, and centrifugal diafiltration) in terms of their impact on the properties of resulting virus stocks. The results of the physicochemical characterization of MS2 and P22 bacteriophages were found to depend on the choice of propagation and purification methods. Particle sizing and electrophoretic mobility data for MS2 indicated that double agar overlay growth introduces difficult-to-remove impurities that confound virus size, electrophoretic mobility, and surface energy measurements. Specifically, agar-propagated MS2 stock purified by PEG precipitation or centrifugal diafiltration (100 kDa) included large (~ 0.4 to 0.5 μm) aggregates that persisted through multiple cycles of post-filtration with a 0.1 μm nominal pore size membrane. Further, the isoelectric point of MS2 in this preparation could not be determined as the bacteriophage remained electronegative even at pH 1.4. CsCl density gradient centrifugation could remove most impurities but still gave a relatively broad MS2 size distribution. Regardless of the purification method, such interferences were not observed for MS2 propagated in E. coli broth.
Accordingly, the degree of bacteriophage purity achievable with each of the three purification methods was compared based on the data for MS2 and P22 propagated only in broth media.

CsCl density gradient centrifugation produced virus suspensions of highest quality. The impurities remaining in the virus suspension after PEG precipitation and centrifugal diafiltration broadened the size distribution and interfered with either electrophoretic mobility measurements or hydrophobicity characterization or both. The impact on the free energy of virus-virus interfacial interaction determination was especially dramatic with P22 bacteriophage appearing hydrophilic in the presence of impurities and hydrophobic in better purified stocks. The three purification methods yielded similar recovery of infectious MS2 (60% to 70%). PEG precipitation, a solubility-based method, recovered P22 with a lower efficiency of ~ 36%, which was attributed to differences in PEG interactions with MS2 and P22. Comparison of total and infections P22 recoveries showed that CsCl density gradient centrifugation selected for intact P22 virions over damaged one. Ultimately, the choice of the optimal purification procedure is a matter of tradeoff and should be made based on the intended application. In making the selection, the demands for product quantity and quality (e.g. virus concentration and infectivity, tolerance for residual impurities) should be weighed against practical considerations such as the time and cost of virus purification.

Acknowledgements

This material is based upon work supported by the National Science Foundation Partnerships for International Education and Research program under grant IIA-1243433. We would like to thank Dr. Merlin Bruening (Chemical and Biomolecular Engineering, University of Notre Dame), Dr. Wei Zhang (Plant, Soil and Microbial Sciences, Michigan State University) and Dr. Kristin Parent (Biochemistry and Molecular Biology, Michigan State University) for reading the Hang Shi’s dissertation chapter that formed the basis of this manuscript and for providing very helpful feedback. We are grateful to Dr. Kristin Parent for granting access to the equipment used to purify MS2 and P22 as well as to Dr. Irene Xagoraraki (Civil and Environmental Engineering, Michigan State University) who made the qPCR instrument in her laboratory available for P22 quantification.
References


