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A novel application of small-angle scattering techniques: Quality assurance testing of virus quantification technology

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Abstract

Small-angle scattering (SAS) techniques, like small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS), were used to measure and thus to validate the accuracy of a novel technology for virus sizing and concentration determination. These studies demonstrate the utility of SAS techniques for use in quality assurance measurements and as novel technology for the physical characterization of viruses.
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1. Introduction

Emerging viruses associated with disease, such as severe acute respiratory syndrome (SARS), which cannot be easily or safely grown in the laboratory, as well as viruses constructed in the laboratory from sequence information alone and bioengineered viruses for clinical diagnostic research have highlighted the need for technology that can rapidly measure virus size and concentration in solution without knowledge of any other physical properties (Crowther, 2004; Curry et al., 2006; Ksiazek et al., 2003; Pasloske et al., 1998; Smith et al., 2003).

The basic properties necessary for the initial characterization and study of any previously unidentified or unknown virus samples (be they naturally occurring or artificial) for basic, biomedical, or environmental research are the determination of (a) the virus host, (b) virus size and (c) the concentration or number of virus particles per milliliter.

In general, identification of the virus host is trivial because viruses are usually isolated in conjunction with their host (or food source). Analysis of virus size (and shape) is not so easily measured but provides vital clues to the identification of an unknown or uncharacterized virus sample. This is primarily due to the fact that viruses of a particular type (or family) are often in the same general size and shape range. Knowledge of the virus size and host range can help to exclude a large number of possible virus types during the early stages of the identification and characterization process, saving valuable time, labor and resources.

Typically, virus size is measured using microscopic methods such as cryo-electron microscopy, transmission electron microscopy (TEM) or scanning electron microscopy (SEM) (Crowther, 2004; Curry et al., 2006). The use of electron microscopy (EM) for virus sizing is well established but has the drawback that it requires technical expertise and some knowledge of the growth and fixation

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conditions needed for the virus of interest. Furthermore, it is time and labor intensive (Fedorko and Hijazi, 1996). Despite these drawbacks, EM techniques are the primary method used to measure the size and shape of viruses (Crowther, 2004; Curry et al., 2006).

Virus concentration determination is not often directly measured on a routine basis due to the small size of viruses. Instead, traditionally, virus concentration is deduced as a function of the ability of the virus to infect and to form plaques on its host cell per unit volume. Therefore, the working concentration of a given virus stock can roughly be defined in terms of its infectivity, as the number of plaque-forming units (PFUs) mL^{-1} (Sambrook and Russell, 2001). This indirect measure of concentration assumes that every virus in the solution is equally infectious with equal access to the viral host of interest. However, in the case of the bacterial virus MS2, as well as many other viruses, it has been shown that virus infectivity varies and that, in some cases, as little as only 30% of the virus population in solution is infectious. Therefore, PFUs may often be an underestimate of virus concentration (Davis and Sinsheimer, 1963).

Alternatively, for pure samples of well-studied viruses whose molar (or extinction) coefficient and molecular weight are known, optical density (OD) or absorbance using conventional spectrophotometry has been shown to be a sensitive measure of virus concentration (Eisenberg, 1979; Mazzone, 1998). Additionally, scattering methods such as classical light scattering, small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) have also been employed to measure the concentration of samples when the molecular weight of the virus is known (Guinier, 1939; Guinier and Fournet, 1955; Jacrot and Zaccai, 1981; Koch et al., 2003). Most virus samples (be they naturally occurring or recombinant) are typically not sufficiently pure and/or available in large enough quantities for accurate concentration determination using these methods. When an accurate measure of virus concentration (or particle number) is required, a specialized method of EM, quantitative EM, has also been successfully employed, but is technically challenging for routine use (Zheng et al., 1996).

Currently, there is no single established method for simultaneous virus sizing and concentration determination. The development of instrumentation capable of such a feat would additionally require rigorous quality assurance testing methods to validate its measurements and to evaluate its feasibility in a variety of laboratory and clinical settings. The primary goal of this study is to explore the utility of using small-angle scattering (SAS) techniques, such as SAXS and SANS, as a general approach to the evaluation and quality assurance testing of virus characterization technology, using the integrated virus detection system (IVDS) instrument as a test technology.

In general, SAS involves the passage of particles (in this case X-rays or neutrons) through the sample. The resulting scattering pattern reveals information about the average size, shape and orientation of the sample (Krueger, 1998). When the concentration of the sample is known, then the average molecular weight of the sample can be determined by SAS. Similarly, if the total molecular weight of the sample is known, then the concentration of the sample can be determined using SAS techniques.

The IVDS is a virus characterization instrument for virus size and concentration determination (Wick, 2002a, b; Wick and Anderson, 2000). In general, IVDS is a single instrument that consists of two modules and a computer system. The first module contains a detachable ultrafiltration unit which can be used for sample purification and/or concentrating the virus into smaller volumes. This aspect of the technology was not analyzed in these studies (Wick, 2002a; Wick and McCubbin, 1999c: Wick, 1999, p. 49). The detachable ultrafiltration module is connected to the central IVDS module, which contains a gas-phase electrophoretic mobility analyzer (GEMMA), differential mobility analyzer (DMA) and a condensation particle counter. The GEMMA aerolizes the liquid virus sample and then the DMA separates the virus particles by size for subsequent presentation to the condensation particle counter which ultimately sizes and counts the virus particles. Finally, the data are collected and analyzed by a separate computer system. The GEMMA, DMA and condensation particle counter in combination with the IVDS computer system is collectively referred to herein as the IVDS. Although similar technology such as charged reduced electrospray size spectrometry (CRESS) (Bacher et al., 2001; Hogan et al., 2006, 2005; Kaufman et al., 1998) has been described in the literature, only this instrument was specifically designed, patented and available for commercial use for the analysis of viruses (Wick, 2002a, b; Wick and Anderson, 2000). In principal, the IVDS instrument has been shown to size and to determine the concentration of virus particles in the 10–100 nm size range (Wick, 2002a, b; Wick and McCubbin, 1999a). However, the accuracy and reliability of this technology and its counterparts have not been specifically described.

The IVDS instrument has a number of advantages over the other virus analysis methods previously mentioned. Namely, virus size and concentration can be determined simultaneously without prior knowledge of any other physical sample features, or the need for the addition of sizing standards to the sample. Furthermore, virus-containing samples need not be homogenous. Samples containing a variety of different viruses can be assayed in solution and their size and concentration determined individually under physiological conditions. Also, sample analysis is rapid. Typical measurements take about 5 min (Wick, 2002a, b; Wick and Anderson, 2000; Wick and McCubbin, 1999a). Since the accuracy of the IVDS technology has not been reported, IVDS presents an ideal test system to determine if SAS techniques are suitable for quantitative quality assurance measurements necessary for the validation of novel virus characterization technology.

Table 1
Summary of size analysis of 40 nm size standard microspheres

Label (nm)	Source	Lot number	Mean diameter (nm)	Sizing method	(Microspheres cm^{-3})	Reference
40	Interfacial Dynamics Corp.	C-100.1	43 ± 8	TEM	2.7×10^{14}	Interfacial Dynamics Corp. (2001)
40	Interfacial Dynamics Corp.	C-100.1	43 ± 9	IVDS	6.25×10^{12}	This study

To this end, a two-step approach to the analysis of the IVDS instrument has been undertaken. First, IVDS virus sizing ability was measured using synthetic (commercially available latex microspheres) and biological (bacterial virus MS2) size standards. Specifically, National Institute of Standards and Technology (NIST)-traceable synthetic size standard microspheres were chosen as size standard controls (or standard reference material (SRM)) because they are generally accepted as the state of the art in the area of size standard particles and they are commercially available. The bacterial virus MS2 was used as a biomarker and size standard to control for the possibility that the IVDS instrument might analyze synthetic microspheres differently compared to naturally occurring biological material. In addition, MS2 is a model internal control particle because its size, molecular weight and molecular coefficient have been reported in detail by a variety of groups using numerous methods (Kuzmanovic et al., 2003; Overby et al., 1966; Strauss and Sinsheimer, 1963).

Second, for the analysis of concentration determination ability, the MS2 virus was used as an internal control for the comparison of IVDS with the more established OD concentration determination method. Specifically, the concentration of a purified stock of MS2 virus was measured using IVDS and OD techniques, and the validity of both methods was evaluated using two complementary SAS techniques, SAXS and SANS.

2. Materials and methods[3]

2.1. Instrumentation

The IVDS has been described in detail previously and is described in brief here. IVDS is a multi-component system consisting of (a) a detachable ultrafiltration unit; (b) the central instrument unit which houses a GEMMA detector and a DMA, which is connected to a condensation particle counter; and (c) the computer system. The ultrafiltration unit which concentrates and purifies virus samples in solution has been previously described (Wick, 2002a, b; Wick and Anderson, 2000; Wick and McCubbin, 1999a–c: Wick, 1999, p. 49) and was not utilized in these studies. Instead this study focused on the quantitative abilities of the GEMMA detection unit and the DMA, which have been specifically configured for the analysis of viruses (Wick, 2002a, b; Wick and Anderson, 2000). The IVDS GEMMA detector consists of an electrospray that aerosolizes the sample for subsequent passage through charged air to dry the particles. In the process, most of the charges from the particles are removed, leaving them either charge neutral or singly ionized. Singly ionized particles are then introduced into the detector, a DMA, to separate the sample by size. The individual particles (transmitted by the DMA) are counted by the condensation particle counter (Wick, 2002a, b). The instrument is controlled and the data are recorded and displayed by the computer system. Only the IVDS beta unit was tested herein. Models containing upgrades or modifications were not specifically evaluated. These results are only valid for the calibrated IVDS beta instrument at the time of the study using the calibration curves described herein. The specific IVDS instrument examined in this study was beta model instrument, number GEMMA-WP, serial number 103 (TSI Incorporated, Shoreview, MN).

2.2. Standard reference microspheres

NIST-traceable standard 40 nm standard reference microspheres were purchased from Interfacial Dynamics Corporation (Portland, OR). The microspheres were calibrated by the manufacturer using quantitative TEM using the 300 nm NIST SRM 1691. A full description of NIST SRM 1691 has been described, as well as its routine use as an internal calibration standard (Duke and Layendecker, 2003; Letteri and Hembree, 1988; Mulholland and Bauer, 2000). All microspheres are composed of sulfate white polystyrene latex and their technical information is summarized in Table 1.

The 40 nm NIST-traceable microspheres were serially diluted twofold from 2.7×10^{13} to $2.44 \times 10^{10}\,cm^{-3}$. The linear equation which best describes all of the data including the standard error is $Y = 9.5 \times 10^{-11}(X) + B$, where Y is the raw IVDS count, X the concentration (particles cm^{-3}) and B any constant between -24 and $+65$. For concentration determination, the above equation was solved for concentration, X, with $B = -21.2$. Higher

[3]Certain commercial materials, instruments and equipment are identified in this paper in order to specify the experimental procedure as completely as possible. In no case does such an identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials, instruments or equipment identified are necessarily the best available for the purpose.

concentrations of the 40 nm standard microspheres were not available for purchase during this study. Similarly, 40 nm standard stocks with concentrations below $2.4\times10^{10}\,\text{cm}^{-3}$ are not described here because they cannot be accurately measured under the conditions described herein.

2.3. Bacteriophage, hosts and medium

MS2 bacteriophage strain 15597-B1 and its *Escherichia coli* (*E. coli*) host 15597 were purchased from the American Type Culture Center (Manassas, VA). The purified concentrated stock of MS2 bacteriophage was generated as described in Kuzmanovic et al. (2003) and then was serially diluted twofold in $0.2\,\text{mol}\,\text{L}^{-1}$ (M) ammonium acetate at pH 8.0 and measured by IVDS or diluted in water for OD readings.

2.4. Concentration determinations

2.4.1. OD concentration determinations

OD at 260 nm wavelength (OD_{260}) measurements yielded the virus concentration (c) for each sample, which was used to calculated the number of virus particles per sample volume, n, using the equation $n = cN_A/\text{Mw}$, where N_A is Avogadro's number, expressed as particles per mole, and Mw is the total molecular weight of the MS2 particle, in $\text{g}\,\text{mol}^{-1}$. Since n is expressed as particle number per sample volume, it has units of cm^{-3}. Therefore, c must be expressed in units of $\text{g}\,\text{cm}^{-3}$. Sample concentrations were measured after the SANS experiments and before the SAXS experiments by measuring the OD absorbance at 260 nm and using Beer's Law:

$$c = A_{260}/\varepsilon \cdot L, \tag{1}$$

where ε is the molar coefficient in $\text{cm}^2\,\text{g}^{-1}$ and L is the pathlength of the light in cm, to calculate the concentration (Eisenberg, 1979). Since the molar coefficient is also dependent upon the total Mw of the particle, this method of determining the numerical particle density is only useful if the total Mw of the particle is known (Eisenberg, 1979). Sample concentrations were measured in duplicate using a Hewlett-Packard model 8450A spectrophotometer. The spectrophotometer was calibrated using NIST transmittance and wavelength SRM numbers 930, 2031 and 2034.

2.4.2. IVDS concentration determinations

For these experiments, the IVDS instrument was set for 60 s scans. Each sample was scanned four times on average. Note that this IVDS instrument does not reliably measure the concentration of synthetic microspheres of concentration greater than 1×10^{13} or below 1×10^{13} particles mL^{-1} under the conditions described herein. The former samples clog the instrument, thus limiting the number of scans, and the latter are just simply too dilute to be accurately detected. The issue of clogging appears to be unique to synthetic material not biological material, in our hands. Although these extreme measurements are out of the optimal detection range of this model of the IVDS instrument, all of the data are included here to fairly represent the technology. The total number of scans was averaged and the standard variance, s^2, was calculated using the following equation:

$$s^2 = \sum (X - M)^2/N - 1, \tag{2}$$

where X is the total count per scan, M the average total count per scan and N equal to the total number of scans per sample. The standard deviation, s, is defined as $s = \sqrt{s^2/N}$. The percent standard error is defined as $s/M \times 100$. Calibration curves were generated and used as standard curves for microsphere sizing and concentration determination purposes. All data were analyzed using the IGOR Pro scientific graphing and data analysis software (WaveMetrics, Inc., Lake Oswego, OR).

2.5. SAS methods

The SAXS and SANS measurements, as well as details of the data analysis, have been described in detail elsewhere (Kuzmanovic et al., 2006, 2003). The Mw of the MS2 was obtained from the forward scattering intensity, $I(Q=0)$, with $Q = 4\pi\sin(\theta)/\lambda$, where λ is the neutron wavelength and 2θ is the scattering angle. Both the Guinier approximation, $I(Q) = I(0)\exp(-Q^2\text{Rg}^2/3)$, used on the low-$Q$ portions of the data, and the GNOM program (Semenyuk and Svergun, 1991), which makes use of all of the data, were used to obtain values for the radius of gyration, Rg, and the forward scattering intensity, $I(0)$, of the samples. The total Mw of the MS2 was then calculated from $I(0)$ using the equation

$$I(0) = n(\Delta\rho V)^2, \tag{3}$$

where $\Delta\rho = (\rho - \rho_s)$ is the contrast or the difference between the scattering length density of the molecule (ρ) and the solvent (ρ_s), n is the number density of MS2 and V is the volume of the MS2 particles. The number density can be written as $n = cN_A/\text{Mw}$, where c is the concentration and N_A is Avogadro's number. The volume can be written as $V = \text{Mw}/(N_A d)$, where d is the mass density. Now, Eq. (3) can be rewritten as

$$\frac{I(0)}{c} = \frac{(\Delta\rho)^2}{N_A d^2}\text{Mw}. \tag{4}$$

The only unknown parameter in Eq. (4) is the Mw, since all other parameters can be measured or calculated. The $I(0)$ value is generally taken from the GNOM (Semenyuk and Svergun, 1991) analysis of the data. Both c and d can be directly measured during sample preparation and $\Delta\rho$ can be calculated from the chemical composition of the sample and solvent.

It is important to note that $I(0)$ must be on an absolute scale, usually in cm^{-1}, in order to obtain accurate Mw values from Eq. (3) or (4). The SANS data were placed on

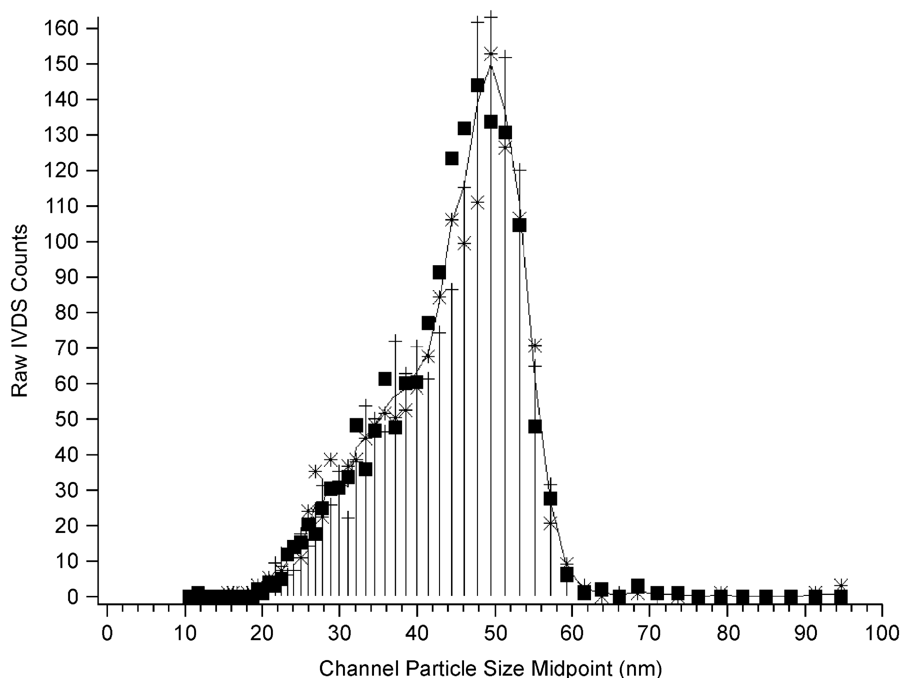

Fig. 1. Forty nanometer size standard curve. Scans 1–3 and the average fit curve for all of the data are denoted by the symbols (+), (■), (*) and (—), respectively.

an absolute scale, in cm^{-1}, by normalizing the scattered intensity to the incident beam flux, which is independent of prior knowledge of the Mw of MS2 or the concentration of the sample. On the other hand, the SAXS data were put on an absolute scale by comparing the scattered intensity of the MS2 sample at $Q = 0$ to that calculated using the known Mw, scattering length density and volume of the RNA and coat protein components, as verified by earlier SANS studies (Kuzmanovic et al., 2003). Since this method is not independent of prior knowledge of the Mw of the MS2 sample, the SAXS data could not be used to obtain an unbiased determination of the Mw of MS2.

At the resolution level of the SAS measurements, MS2 has been shown to be approximated very well by a spherical shell, with inner radius, $R1$, outer radius, $R2$, and shell thickness, $t = R2 - R1$ (Kuzmanovic et al., 2003). Thus, the SAXS and SANS data were fit to a core-shell sphere model in order to obtain the radii of the protein shell and the solvent core (Hayter, 1983). The core-shell model fits take into account the resolution function of the SANS and SAXS instruments. The outer radius, $R2$, was taken as the total size of the MS2 particles and is used for comparison to size parameters obtained from other techniques.

3. Results and discussion

3.1. Particle sizing determination

A commercially available synthetic size standard and a purified stock of the bacterial virus (MS2) were used to evaluate particle size determination using the IVDS. NIST-traceable polystyrene 40 nm size standard microspheres were chosen as size standards because they have been previously described in detail and their use as size standards is widely accepted. First, the size standard microsphere stock was diluted as in the Materials and methods and then analyzed by IVDS. The technical information for the size standard microspheres as well as a summary of the sizing data using IVDS is shown in Table 1 and Fig. 1. The size of the size standard microspheres as measured by IVDS is 43 ± 9 nm which is in good agreement with its reported particle size of 43 ± 8 nm (Interfacial, 2001).Therefore, the IVDS instrument is an accurate measure of the size of these synthetic microspheres.

Next, the virus sizing ability using IVDS was analyzed to determine if synthetic and biological particles are sized with equal efficiency. The size of the MS2 bacterial virus was measured by IVDS, SAXS and SANS and compared to published results as shown in Fig. 2 and summarized in Table 2. The IVDS MS2 virus size measurement is 23.3 ± 1 nm. The size of MS2 virus is 26.8 ± 0.4 nm, as measured by SAXS (Kuzmanovic et al., 2006), and 27.2 ± 0.4 nm, as measured by SANS (Kuzmanovic et al., 2003). These MS2 virus size measurements compare favorably with the TEM size measurement of the MS2 virus of between 24 and 26 nm as well as the size measurements from X-ray crystallography, 27 nm (Golmohammadi et al., 1993). Furthermore, IVDS sizing of MS2 is also in good agreement with sizing results of 23.3 ± 1 and 24.13 ± 0.06 nm reported by Wick and McCubbin (1999a) and Hogan et al. (2006) using IVDS and charged reduced electrospray spectroscopy, respectively.

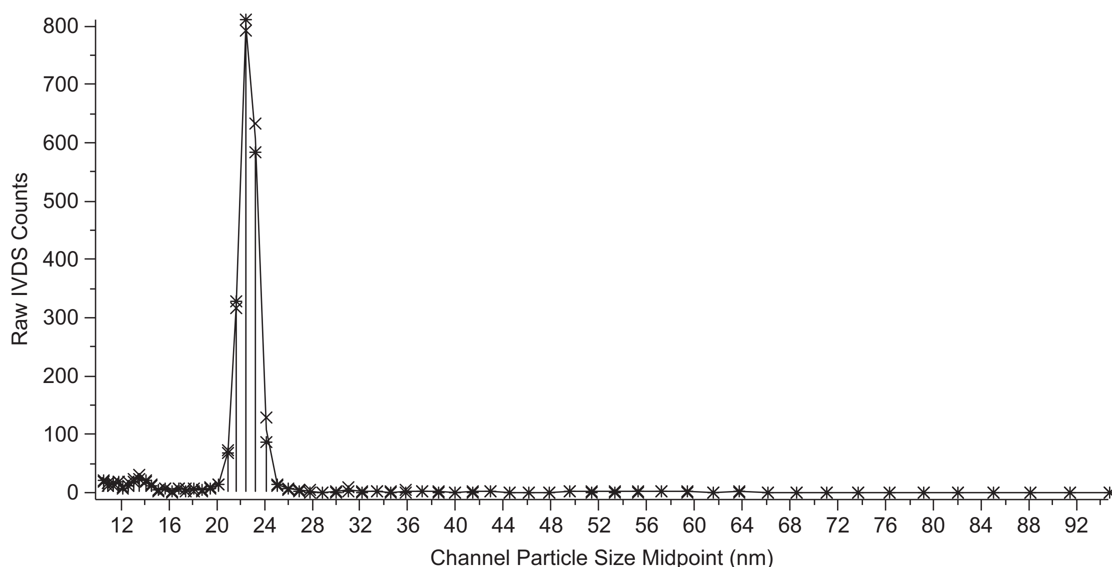

Fig. 2. IVDS analysis of MS2 virus size. Scans 1 and 2 and the average fit curve for all of the data are denoted by the symbols (×), (*) and (—), respectively.

Table 2
Summary of MS2 size determinations

Sizing method	Size (nm)	References
SAXS	26.8 ± 0.4	This study and Kuzmanovic et al. (2006)
SANS	27.2 ± 0.4	This study and Kuzmanovic et al. (2003)
IVDS	23.3 ± 1	This study
CRESS[a]	24.13 ± 0.06	Hogan et al. (2006)
IVDS	23.3 ± 1	Wick and McCubbin (1999a)
X-ray crystallography	27	Golmohammadi et al. (1993)
TEM	25	Overby et al. (1966)
TEM	24	Sugiyama et al. (1967)
TEM	26	Strauss and Sinsheimer (1963)

[a]Charged reduced electrospray size spectrometry.

In summary, SAS methods confirm that the IVDS instrument can accurately size synthetic microspheres. Furthermore, the IVDS technology measures the average size of a naturally occurring biomarker, the bacterial virus MS2, to a similar level of accuracy to other well-established methods such as TEM, SANS, SAX and X-ray crystallography.

3.2. Concentration determinations

The IVDS instrument measures or "counts" the total number of particles in 40 nL increments, after which the total concentration of the sample particles is calculated using a software algorithm. Therefore, only the raw IVDS count number is a reliable measure of particle number since the algorithm calculated concentration could be modified or optimized for the specific particles under study. Furthermore, the deduced concentration of a given particle may be affected by the composition, size or shape of the material under study. For this reason, we first sought to determine the relationship between the raw IVDS particle count number (in IVDS counts per 40 nL) and the concentration of a sample whose concentration is known (in particles cm^{-3}), from which a concentration reference curve could be generated. The concentration reference curve would serve two purposes: (a) it would directly describe the relationship between raw counts and concentration of a sample (in particles cm^{-3}). (b) If that relationship is linear, then the resulting equation could be used to determine the concentration of unknown virus samples.

The generation of a concentration reference curve requires the use of a sample of known concentration. To date, no concentration standard particles are commercially available. For this reason, NIST-traceable size standard microspheres were used to generate the concentration reference curve. The rationale being that, although the NIST-traceable size standard microspheres are not officially concentration standards, their physical properties are extremely well known and represent the best characterized particles which are widely available. However, since the size standard particles are not established for use as concentration standards, the validity of the relationship between raw IVDS counts and concentration particle number as described by the concentration reference curve had to be validated by measuring some other concentration-dependent physical property, as described in the next section.

To generate the concentration reference curve, the 40 nm size standard stock solution was serially diluted twofold from 2.5×10^{13} to $2.44 \times 10^{10} cm^{-3}$ and then analyzed by IVDS. Table 3 contains a summary of the concentration reference curve data. Fig. 3 shows the

Table 3
Concentration reference curve data

Concentration (particles cm^{-3})	Scan 1	Scan 2	Scan 3	Scan 4	Average	% Standard error
2.5×10^{13}	2042	2635			2339 ± 296.5	12.7
6.25×10^{12}	594	606	649	570	605 ± 16.54	2.7
3.13×10^{12}	266	291	257	214	257 ± 16.04	6.2
7.81×10^{11}	27	38	35	35	34 ± 2.39	7.0
33.91×10^{11}	10	17	16	17	15 ± 1.68	11.2
1.95×10^{11}	4	4	8	7	6 ± 1.04	17.3
9.77×10^{10}	1	3	1		2 ± 0.71	35.5
4.88×10^{10}	2	0	1		1 ± 0.58	58.0
2.44×10^{10}	0	0	1		0.33 ± 0.41	124.0

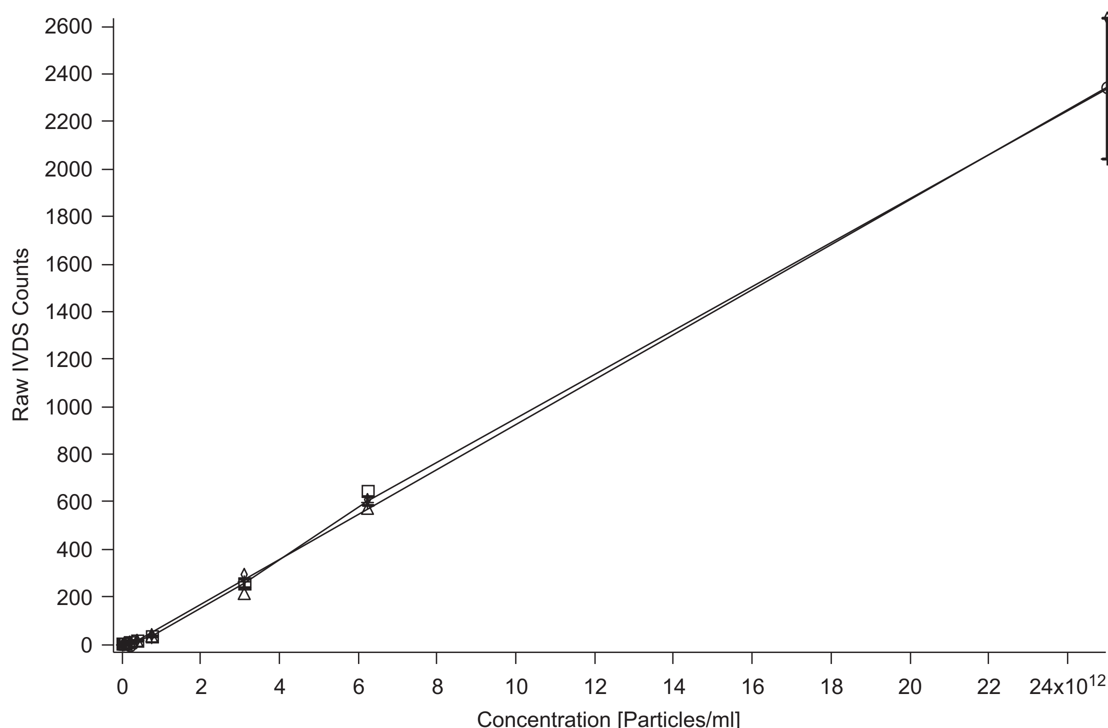

Fig. 3. Concentration standards curve using 40 nm NIST-traceable microspheres. Scans 1–4 as well as the average fit curve for all of the data are denoted by the symbols (+), (◇), (■), (△) and (—), respectively.

concentration reference curve. Notice that the 40 nm standard curve is linear over all data points from 2.4×10^{10} to $2.5 \times 10^{13}\,cm^{-3}$. In most cases the error bars are too small to be easily seen except in the case of the most concentrated sample. This large error is not due to instrument resolution but to the small number of sample particles counted, two instead of the usual four. The linear equation which best describes all of the data including the standard error is $Y = 9.5 \times 10^{-11}X + B$, where Y is the raw IVDS count, X the concentration (particles cm^{-3}) and B any constant between -24 and $+65$. For concentration determination, the above equation was solved for concentration (X). Therefore, now exists an equation which describes the relationship between raw IVDS count number and concentration, expressed in particles per cm^3, in the form of the concentration reference curve for these experiments. However, the validity of concentration reference curve had yet to be established.

3.3. Validation of concentration determination ability using SAS

Validation of the concentration ability of this new virus characterization technology would entail using the concentration of a sample measured by IVDS to measure a different known concentration-dependent physical property. Thus, the molecular weight was measured using SAXS and SANS, which, similar to IVDS, measures physical properties in solution. Recall that in SAS techniques, such as SAXS and SANS, X-rays or neutrons are passed through the sample. The resulting scattering pattern reveals information about the average size, shape

Table 4
MS2 concentration determinations for SANS analysis

Sample	SANS data			References
	c (mg mL^{-1}) from OD	$n \times 10^{14}$ (cm^{-3}) expected from c	$n \times 10^{14}$ (cm^{-3}) from IVDS	
0% D_2O	1.8±0.1	3.0±0.2	–	This study and Kuzmanovic et al. (2003)
10% D_2O	1.8±0.1	3.1±0.2	3.8±0.1	This study and Kuzmanovic et al. (2003)
65% D_2O	2.1±0.1	3.5±0.3	3.8±0.2	This study and Kuzmanovic et al. (2003)
85% D_2O	2.3±0.1	3.8±0.3	3.0±0.5	This study and Kuzmanovic et al. (2003)

Table 5
MS2 molecular weight determinations

Method	$Mw_{TOTAL} \times 10^6$ (Da)	Concentration determination method	References
SANS	3.7±0.2	OD	This study and Kuzmanovic et al. (2003)
SANS	3.5±0.5	IVDS	This study and Kuzmanovic et al. (2003)
Light scattering	3.6	OD	Overby et al. (1966)
Light scattering	3.6	OD	Strauss and Sinsheimer (1963)
Sedimentation velocity	3.87	N/A	Overby et al. (1966)
Sedimentation velocity	3.8	N/A	Strauss and Sinsheimer (1963)
Sedimentation velocity	5.3±0.6	N/A	Moller (1964)

and orientation of the sample (Krueger, 1998). Also, when the concentration of the sample is known, then the molecular weight of the sample can be determined by SAXS or SANS with absolute scaling (Mazzone, 1998).

Therefore, if IVDS can accurately measure virus particle concentration, then using the IVDS concentration measurements (derived using the concentration reference curve) one should be able to confirm the molecular weight of a known sample using SAXS and SANS.

The use of commercially available microspheres as standards for the molecular weight determinations was initially considered. Since the IVDS instrument simultaneously measures both the concentration and size of particles, the size distribution of the size standard microspheres was analyzed using IVDS. Fig. 1 shows the size distribution of the 40 nm size standard microspheres as measured by IVDS. Although the average size of these microspheres is 43±8 nm, note that the microspheres comprise a wide distribution of size ranges from 10 nm, the limit of detection of the instrument, to 90 nm. Therefore, as a general practice, the use of microspheres as a standard for determining the average Mw is not directly useful because the average Mw of the microspheres may vary from batch to batch while the average microsphere size and concentration may remain relatively unchanged. Furthermore, the focus of this work is to determine if the IVDS technology can accurately measure the concentration of virus particles, specifically.

For these reasons, the suitability of using the bacterial virus MS2 as a test sample was explored. MS2 is an ideal biomarker for molecular weight determination for a number of reasons: (a) its molecular weight has been determined using other SAS techniques such as classical light scattering (Overby et al., 1966) as well as non-SAS techniques such as sedimentation gradient velocity (Overby et al., 1966) and (b) the molecular coefficient of MS2 has been reported (Strauss and Sinsheimer, 1963). Therefore, an OD-based concentration determination method would serve as an overall control for molecular weight determination using SAS. The size distribution of the MS2 virus stock was measured using IVDS. Fig. 2 shows the size distribution of the MS2 virus particles using IVDS. Notice that the size of the MS2 virus particles is very discrete. The MS2 virus size distribution spans a narrow size range between approximately 20 and 26 nm. Therefore, its molecular weight should not vary considerably from sample to sample.

To measure the molecular weight of the MS2 virus, the concentration of the sample was measured using two methods: (a) IVDS (using the concentration reference curve to convert the raw IVDS counts to concentration in particles per cm^3) and (b) OD using conventional spectrophotometry. The MS2 virus stock sample was then measured by SAXS and SANS and its molecular weight determined using the concentrations given by both concentration determination methods. Table 4 shows the concentration determination used for the SAXS and SANS analysis, respectively. Table 5 shows the MS2 virus molecular weight determination based on the concentrations given by IVDS and OD, as well as previously reported molecular weight determinations by other groups. The molecular weight of MS2 is generally accepted to be 3.6×10^6 g mol^{-1} (Da) on the basis of classical light scattering methods using OD as a concentration

determination method. The molecular weight determinations described herein and (Kuzmanovic et al., 2003) using SANS, and either the OD or the IVDS concentration determination methods, show good agreement. Specifically, the molecular weight of MS2 using SANS and IVDS concentration determinations is $(3.5 \pm 0.5) \times 10^6$ Da and using SANS and OD concentration determinations is $(3.7 \pm 0.2) \times 10^6$ Da (Kuzmanovic et al., 2003). These results are confirmed by the weighted SAXS analysis of MS2 molecular weight using OD and IVDS concentration determination (Kuzmanovic et al., 2006).

4. Conclusions

For the study of new viruses, or virus samples about which very little is known, rapid measurement of the basic physical properties of the virus, such as size, shape and concentration, is problematic because of the lack of rapid technology for virus characterization. The development of new virus characterization technology brings the added problem of developing new scientific quality assurance approaches and instrumentation to determining the utility of the virus technology.

This study provides a model approach to the evaluation of novel virus characterization technology using small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) methods validated with the use of both naturally occurring and synthetic standards. Specifically, it was determined using SAXS and SANS that the virus sizing and concentration technology, IVDS, can accurately measure the size of synthetic latex microspheres. Additionally, IVDS technology measurements of a biomarker, the bacterial virus MS2, in aqueous solution, are at an accuracy level on par with the traditional size and concentration determination methods such as TEM and OD. Furthermore, it was shown that the data obtained from the use of IVDS technology could be used to accurately measure a third physical property, molecular weight, which was confirmed in this study using small-angle scattering (SAS).

This study is the first, to our knowledge, to describe a SAS-based approach to providing the quality assurance measurements for virus characterization technology designed for basic research, environmental analysis or clinical diagnostic use as well as to establish the feasibility of the use of charged reduced electrospray spectroscopy techniques, like IVDS, for both virus sizing and concentration determinations.

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