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EFFECTS OF SAMPLE IMPURITIES ON THE ANALYSIS OF MS2 BACTERIOPHAGE BY SMALL-ANGLE NEUTRON SCATTERING

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Small angle neutron scattering (SANS) has been used to extend the structural characterization of the MS2 phage by examining its physical characteristics in solution. The impact of small molecular weight impurities of the resolution of structural data obtained by SANS of the bacteriophage MS2 distorts the resolution and sharpness of contrast variation peaks. However, the position of the visible peaks is unchanged contaminated samples when compared to pure MS2 samples.

15. SUBJECT TERMS

Virus	Virus detection	Integrated Virus Detection System (IVDS)
Purification	Detection	Small Angle Neutron Scattering (SANS)
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PREFACE

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1. INTRODUCTION

Bacteriophage MS2 is a 275Å RNA virus that infects male *Escherichia coli* (Stockley *et al.*, 1994).[1] Because of its small size, relatively simple composition, and ease of growth, MS2 is used as a model organism for a number of macromolecular processes, including viral replication, translation, infection, and assembly (Peabody and Al-Bitar, 2001;[2] Stockley *et al.*, 1994).[1] Increasingly due to its ease of purification, harmlessness to man, and durability, MS2 is also used as a quantitative marker for the effectiveness of antiviral and antiseptic agents, and the efficiency of water treatment plants and filtration devices (Jolis *et al.*, 1999;[3] Oppenheimer *et al.*, 1997;[4] Woolwine and Gerberding, 1995;[5] Lykins *et al.*, 1994).[6] In addition, genetically modified forms of MS2 are available for vaccine development and for use as clinical diagnostic tools (Van Meerton *et al.*, 2001;[7] Heal *et al.*, 2000;[8] Stockley and Mastico, 2000;[9] Pasloske *et al.*, 1998;[10] Mastico *et al.*, 1993;[11] Pickett and Peabody, 1993).[12] A great deal is known about the MS2 bacteriophage. Its complete genome has been sequenced (Fiers *et al.*, 1976).[13] The 3,569 nucleotide genome encodes a coat protein, a maturation protein (or A protein), a replicase subunit, and a lysis protein (Atkins *et al.*, 1979;[14] Fiers *et al.*, 1976).[13] The MS2 coat protein is the primary structural component of the MS2 protein shell. In addition to this function, it binds to the MS2 operator site and acts as a translation repressor of transcription of the MS2 replicase cistron. The A protein (or maturation protein) has been shown to be involved in attachment to the bacterial pilus, replication, RNA packing, and infectivity *in vivo*. The replicase and lysis proteins are involved in replication and the lysis of the *E. coli* bacteria, respectively.

The MS2 virion is comprised of three components: the coat protein (relative molecular weight Mr=13,700), the A protein (relative molecular weight Mr=44,000), and a single-stranded RNA molecule. The three dimensional structure of the intact virion has been determined and refined at 2.8Å resolution (Golmohammadi *et al.*, 1993;[15] Valegard *et al.*, 1991;[16] Valegard *et al.*, 1990;[17] and Valegard *et al.*, 1986).[18] From crystallographic analysis, the MS2 virion is thought to be composed of 90 coat protein homo-dimers arranged in a quasi-equivalent T=3 lattice to form the icosahedral capsid shell of the type described by (Caspar and Klug, 1962).[19] In the capsid, coat protein dimers are thought to adopt two possible non-covalent quasi-equivalent arrangements, A/B and C/C. The A and C subunits interact at the quasi 6-fold axes while the B-type subunits interact at the 5-fold axes. Structurally, the primary difference between these conformers lies in the position of the FG loop region of the protein. In the A and C subunits, the FG loop is extended, while in the C subunit it is folded back in the direction of the protein (Valegard *et al.*, 1991;[16] Valegard *et al.*, 1990;[17] Valegard *et al.*, 1986).[18]

In addition to the coat protein dimers, the MS2 capsid contains a single copy of the A protein. Although the exact location of the A protein in the MS2 virion is not clear. Antibody binding experiments indicate that the A protein is exposed on the

capsid surface, which supports the idea that the A protein comprises one of the vertices of the MS2 icosahedral shell (Curtiss, [20] 1974 #44; O'Callaghan,[21] 1973 #62). The A protein also has been shown to be tightly associated with the MS2 genomic RNA (Shiba and Suzuki, 1981),[22] which is important for RNA packing *in vitro* (Argetsinger,[23] 1966 #63; Heisenberg,[24] 1966 #64). In addition to its role in RNA packing, the A protein is important for host recognition, attachment and subsequent transfer of phage genomic RNA into its host (Stockley *et al.*, 1994).[1]

The purpose of this study is to extend the structural characterization of the MS2 phage by examining its physical characteristics in solution by using small angle neutron scattering (SANS). In general, SANS is a process where a neutron beam is passed through a sample and the resulting scattering pattern reveals information about the average size, shape, and orientation of the sample (Svergun and Koch,[25] 2002; Glinka *et al.*, 1998).[26] The use of neutron scattering for structural analysis of biological macromolecules has a number of advantages. It is not sensitive to errors due to contamination by dust particles, like classical light scattering, or to assumptions about the partial specific volume of the particles, like sedimentation gradient and does not require the use of quantitative standards, like quantitative electron microscopic techniques (Glinka *et al.*, 1998;[26] Mazzone, 1998).[27] Furthermore, neutron scattering experiments do not cause radiation damage to the sample, and typical experiments can be performed under physiological conditions in solution. Also, when the concentration (or particle number) of the sample is known, then the molecular weight of the sample can be determined by SANS since the data are obtained on an absolute scale (usually in cm^{-1}). Similarly, if the total molecular weight of the sample is known, then the concentration of the particles in the sample can be determined (Mazzone, 1998).[27] A number of phage and viral molecular weights [e.g., frog virus 3 (Cuillel *et al.*, 1979),[28] influenza (Cusack *et al.*, 1985),[29] pf1 phage (Torbet, 1979),[30] and Semiliki Forest virus (Freeman and Leonard, 1980)][31] have been successfully determined by this method.

SANS is a powerful tool for structural analysis. But, when combined with the contrast variation method, SANS also permits additional structural information to be obtained about the individual components in a macromolecular complex. In the case of MS2, the contrast variation technique involves varying the solvent water to deuterated water ratio so that structural information about the protein and nucleic acid components can be obtained separately (Glinka *et al.*, 1998;[26] Struhrmann and Miller, 1978).[32]

This study focuses on the effects of sample preparation on the analysis of the MS2 virion by SANS.

2. EXPERIMENTAL PROCEDURES*

2.1 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). *E. coli* strain 15597 was grown on MS2 broth. MS2 broth contains, per liter: 10 g tryptone, 8 g NaCl, and 1 g Bacto-yeast. After autoclaving: 10 mL of sterile 10% glucose, 2 mL of 1 mol/l (M) $CaCl_2$ and 10 mg/ml of thiamine hydrochloride were added per liter (Davis and Sinsheimer, 1963).[33] MS2 was stored in Tris-Salt-Magnesium (TSM) buffer unless otherwise stated. TSM buffer contains 10mM Tris (ph 7.0), 100 mM NaCl, and 1 mM $MgCl_2$.

2.2 Growth of Bacteriophage.

MS2 phage was grown using protocols modified from (Sambrook and Russell, 2001)[34] and is described below. Fresh MS2 broth (0.30 ml) was inoculated with 400 µl of an overnight culture of *E. coli* strain 15597 (American Type Culture Center, Manassas, VA) at OD_{600} = cells/ml). MS2 bacteriophage was added to the inoculant at a multiplicity of infection of 0.01 to 3 and incubated for 20 min at 37 °C. The mixture was added to 500 mL of prewarmed MS2 broth and incubated 8-12 hr. Cell lysis was induced by adding 20 mL of chloroform followed by shaking for 10 min at 37 °C. Cultures were cooled to room temperature. Then, DNAse I and RNAse were each added to a final 50 mg/mL concentration. The cultures were incubated for 30 min at room temperature and then 29.2 g of NaCL (final concentration, 1M) was added. The mixture was incubated on ice for 1 hr and centrifuged at 107,910 m/s^2 (11,000 x g) for 10 min at 4 °C. To the supernatant, 45.2 g of ammonium sulfate was added to produce a 20% (w/w) saturated solution and incubated at 4 °C for 2 hr. The mixture was centrifuged at 11,000 x g for 30 min at 4 °C. 75.2 g of ammonium sulfate were added to the supernatant to produce a 50% (w/w) saturated solution. Following overnight incubation at 4 °C, the sample was centrifuged at 11,000 x g for 20 min at 4 °C. The pellet was resuspended in 30 mL TSM, and 11.0 g of ammonium sulfate was added. The solution was incubated overnight at 4 °C and then centrifuged at 11,000 x g for 20 min at 4 °C. The pellet was resuspended in 35 mL TSM and centrifuged at 11,000 x g for 30 min. For the final precipitation step, the bacteriophage mixture was incubated at 4 °C for 1 hr or overnight and centrifuged at 11,000 x g for 10 min at 4 °C. The aqueous phase contained the crude phage particles.

*Certain commercial materials, instruments, and equipment are identified in this manuscript to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by NIST, nor does it imply either that the materials, instruments, or equipment identified is necessarily the best available for the purpose.

Purified MS2 phage was isolated by cesium chloride equilibrium gradient. The cesium chloride protocol used was as described by Sambrook and Russell, 2001[34] with the following modifications. Cesium chloride was dissolved in TSM medium. Ultra-centrifugation was performed at 23 °C. Following centrifugation, the samples were transferred to Slide-a Lyzer (Pierce, Rockford, IL) and dialyzed in 500 mL TSM for 24 hr with 2 buffer changes. The measured density of the MS2 particles was 1.38 + 0.01 g/cm^3, which is the same density value reported by Strauss and Sinsheimer, 1963.[35] Samples for SANS measurements were made in TSM buffers containing 0, 10, 65, 85, and 100% D_2O. The samples were dialyzed in the appropriate buffers for 2 hr at room temperature, with two buffer changes, and then transferred to sample holders.

2.3 SDS/Polyacrylamide Gel Electrophoresis.

SDS/polyacrylamide gel electrophoresis was performed according to the method of Laemmli, 1970.[36] Commercially available pre-cast 18% SDS polyacrylamide gels (Tris-Glycine gels) for the Novex gel apparatus system were purchased from Invitrogen (Carlsbad, CA) and used according to the manufacturer's instructions. The Tris-Glycine SDS-PAGE running buffer and sample buffers were either purchased from Invitrogen (Carlsbad, CA) or made according to the manufacturer's instructions. Samples were diluted by 50% (v/v) in 2X Tris-Glycine sample buffer, incubated at 85 °C for 2 min, and then directly loaded on gels. Electrophoresis was carried out for 2-3 hr at 30-40 mA/gel. The gels were stained in Brilliant Blue R solution (Sigma, St. Louis, MO) according to the manufacturer's instructions and de-stained in a 30% methanol:10% acetic acid:60% (v/v) water solution for 8 hr (Maniatis, Fritsch et al., 1982).[37]

2.4 SANS Measurements.

SANS measurements were performed on the 30-m SANS instruments at the NIST Center for Neutron Research (Gaithersburg, MD) (Glinka, Barker et al., 1998).[26] The neutron wavelength, λ, was 6Å, with a wavelength spread, $\Delta\lambda/\lambda$, of 0.15. Scattered neutrons were detected with a 64 cm x 64 cm 2-dimensional position sensitive detector with 128 x 128 pixels. Raw counts were normalized to a common monitor count and corrected for empty cell counts, ambient room background counts, and non-uniform detector response. Data were placed on an absolute scale by normalizing the scattered intensity to the incident beam flux. Finally, the data were radially averaged to produce scattering intensity, I(Q), versus Q curves, where $Q = 4\pi\sin(\theta)/\lambda$ and 2θ is the scattering angle. Sample-to-detector distances of 12 m and 2.5 m were used to cover the range $0.005\ Å^{-1} \leq Q \leq 0.17Å^{-1}$. The scattered intensities from the samples were then further corrected for buffer scattering and incoherent scattering from hydrogen in the samples.

2.5 SANS Data Analysis.

The Guinier approximation, $I(Q) = I(0)\exp(-Q^2Rg^2/3)$, was used on the low-Q portions of the data to obtain initial values for the radius of gyration, Rg, and the

forward scattering intensity, I(0), of the samples. This analysis is valid only in the region where QRg ~ 1. The GNOM program (Semenyuk and Svergun, 1991),[38] which makes use of all of the data, rather than a limited data set at small Q values, was used to determine the distance distribution function, P(r), the radius of gyration, Rg, the forward scattering intensity, I(0), and the maximum dimension, D_{max}. Since all of the data are used, this approach typically leads to more accurate determinations of Rg and I(0) that are less influenced by possible aggregation effects.

Since MS2 can be approximated very well by a spherical shell at the resolution level of the SANS measurements, the data were also fit to a core-shell sphere model (Semenyuk and Svergun, 1991)[38] to obtain the radius of the protein shell and RNA core. The neutron scattering length density of the RNA core was an additional fitting parameter that allowed the amount of water, versus RNA, in the core to be calculated using the relation in eq 1:

$$\rho_{CORE} = X\rho_{RNA} + (1\text{-}X)\,\rho_{SOLVENT} \qquad (1)$$

where X is the fraction of RNA in the core, ρ_{CORE} is the fitted scattering length density of the core portion of the core-shell model, and ρ_{RNA} and $\rho_{SOLVENT}$ are the known scattering length densities of the RNA and the solvent, respectively. The core-shell model fits take into account the resolution function of the SANS instruments.

The scattered intensities from the MS2 protein/RNA complex were decomposed into the scattering from their components, $I_{PROT}(Q)$ and $I_{RNA}(Q)$ using eq 2:

$$I(Q) = \Delta\rho_{PROT}{}^2 I_{PROT}(Q) + \Delta\rho_{PROT}\Delta\rho_{RNA} I_{PROTRNA}(Q) + \Delta\rho_{RNA}{}^2 I_{RNA}(Q) \qquad (2)$$

where $\Delta\rho = (\rho\text{-}\rho_s)$ is the contrast or the difference between the scattering length density of the molecule (ρ) and the solvent (ρ_s). The cross-term, $I_{PROTRNA}(Q)$, represents the interference function between the protein and RNA components. The known quantities in eq 1 are $\Delta\rho_{PROT}$ and $\Delta\rho_{RNA}$, and the unknowns are $I_{PROT}(Q)$, $I_{RNA}(Q)$, and $I_{PROTRNA}(Q)$. Since measurements were made at five different contrasts, or D_2O/H_2O buffer conditions, there is sufficient information to solve for the three unknown component intensities from the set of simultaneous equations for I(Q) at each contrast.

The Mw values of the protein and RNA components of MS2 were calculated in a similar manner using the relation in eq 3:

$$I(0) = n(\Delta\rho_{PROT} V_{PROT} + \Delta\rho_{RNA} V_{RNA})^2 \qquad (3)$$

where n is the number density of MS2 particles and V_{PROT} and V_{RNA} are the volumes of the protein and RNA components, respectively. These volumes can be written as $V = M_w/(N_A d)$, where d is the mass density, and N_A is Avogadro's number.
Now, eq 2 can be rewritten as shown in eq 4:

$$\left[\frac{I(0)}{n}\right]^{1/2} = \left(\frac{|\Delta\rho_{PROT}|}{N_A\, d_{PROT}}\right) M_{WPROT} + \left(\frac{|\Delta\rho_{RNA}|}{N_A\, d_{RNA}}\right) M_{WRNA} \qquad (4)$$

where $d_{PROT} = 1.38$ g/cm^3, and $d_{RNA} = 1.89$ g/cm^3. Now, there are only two unknowns, $\Delta\rho_{PROT}$ and $\Delta\rho_{RNA}$. The I(0) values obtained from the GNOM analysis of the data for each D_2O/H_2O buffer are those used with the measured number densities to solve the set of simultaneous equations for these two unknowns to obtain the M_w values for the protein and RNA components separately in the MS2 complex. The total M_w value is then simply the sum of the two component M_w values. It is important to note that I(0) must be on an absolute scale, usually in cm^{-1}, to obtain accurate M_w values from either eq 3 or eq 4.

2.6 Number Density Determinations.

Number density determinations were made using two methods: (1) the concentration was measured by optical density (OD) using a conventional spectrophotometer, and then the number density was estimated using this information, and (2) the number density was obtained directly using the Integrated Virus Detection System (IVDS), which is a particle counting method. Measurements were obtained before and after dialysis and subsequent SANS experiments. However, only the measurements taken after dialysis are used and reported here.

The number densities calculated from the OD_{260} measurements were found from the measured concentration, c, using $n = cN_A/Mw$, where N_A is Avogadro's number, and Mw is the total molecular weight of the MS2 particle. Since n has units of cm^{-3}, c must be converted to units of gcm^{-3}. Sample concentrations were measured after the SANS experiments by measuring the absorbance at 260 nm and using Beer's Law,

$$c = A_{260}/\varepsilon \cdot L, \qquad (5)$$

where ε is the molar coefficient, and L is the pathlength of the light, to calculate the concentration (Eisenberg, 1979).[39] Since the molar coefficient is also dependent upon the total Mw of the particle, this method of determining the number density is only useful if the total Mw of the particle is known (Eisenberg, 1979).[39] Sample concentrations were measured in duplicate using a Hewlett-Packard model 8450A spectrophotometer. The spectrophotometer was calibrated using National Institute of Technology (NIST) transmittance and wavelength standard reference material numbers 930, 2031, and 2034.

The IVDS was used to determine the MS2 particle number directly. The IVDS is a bipartite instrument consisting of (1) an ultra-filtration unit for use in the purification and/or concentration of materials for analysis and (2), a gas-phase electrophoretic mobility analyzer (GEMMA) detector for particle counting and sizing measurements (Wick and McCubbin, 1999).[40] The ultra-filtration unit has been previously described (Wick and McCubbin, 1999)[40] and was not used for these experiments. This work used the GEMMA detector system only. The GEMMA detector consists of an electrospray that sprays the sample into the detector, a differential mobility analyzer to separate the sample by size, and a condensation particle counter for particle counting. These components are in a single module. The complete IVDS instrument has been previously described in detail and was originally designed to detect, quantify, and size

viruses in the 10-100-nm size range (Wick and McCubbin, 1999).[46] The IVDS instrument was calibrated using a NIST-traceable standard reference material. The particle number of samples for the experiments described here was determined using the optimal usage procedures and calibration conditions described in unpublished data.

3. RESULTS

Two complete contrast variation series of measurements were performed on two different MS2 sample preparations. The SANS data for both series of measurements are shown, on an absolute scale, in Figure 1. The data shown in Figure 1a are designated as Experiment #1 and those in Figure 1b are designated as Experiment #2. Note that the data from Experiment #1 have sharper features than those in Experiment #2. Lower instrumental resolution, polydispersity and the presence of contaminants can all wash out peaks in the SANS data. Since both sets of data were obtained under identical experimental conditions, differences in data quality are unlikely to be due to instrument resolution. It is possible that the sample used in Experiment #2 is either somewhat more polydisperse or contains trace contaminants compared to Experiment #1. To determine if differences in the sample quality exist between Experiment #1 and Experiment #2, we examined both samples by SDS/polyacrylamide denaturing gel electrophoresis. The results are shown in Figure 2. Experiment #1 and Experiment #2 are labeled a, and b, respectively. The expected bands for the coat protein and A proteins are clearly visible and are labeled. Both samples were purified under similar conditions and experiments were conducted using similar concentrations of phage. However, Experiment #2 has a number of additional protein bands not present in the Experiment #1 sample. Although, from this method, we cannot distinguish between the formation of pure denaturation resistant aggregates (polydispersity) of the MS2 coat proteins at elevated concentrations and the existence of trace amounts of contaminating proteins in Experiment #2, we believe the additional bands are due to polydispersity (data not shown). Polydispersity and the contaminant presence are known to introduce large errors in molecular weight determination (Lindner and Glatter, 2000).[41]

4. DISCUSSION

The MS2 bacteriophage is a model organism for a number of important areas of research, including viral replication, infection, and assembly (Stockley *et al.*, 1994).[1] Recently, noninfectious, genetically modified forms of the MS2 phage that contain varying amounts of RNA (compared to the wild-type phage) have been developed for use as biological standards (Pasloske et al., 1998;[10] Stockley and Mastico, 2000).[9] These commercially available recombinant particles, Armored RNAs, are used as reference material in research assays for the HIV; Ebola; Borna; Hepatitis A, C, and G; Dengue; Enterovirus; West Nile; and Norwalk viruses, among others (Ambion, personal communication, 2003).

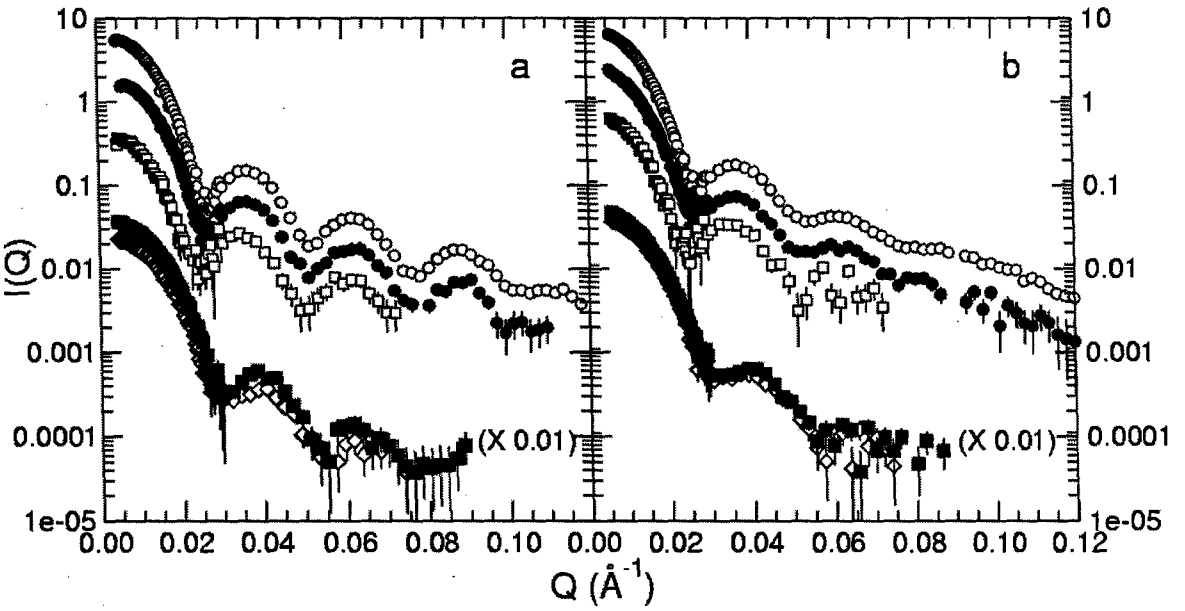

Figure 1. SANS Contrast Variation Data for MS2. MS2 contrast variation series of scattered intensity curves from samples in 100% D_2O (○), 85% D_2O (●), 65% D_2O (□), 10% D_2O (◊), and 0% D_2O (■) for (a) Experiment #1 and (b) Experiment #2. In both cases, the scattered intensity curves for 10% D_2O and 0% D_2O have been multiplied by 0.01 for clarity.

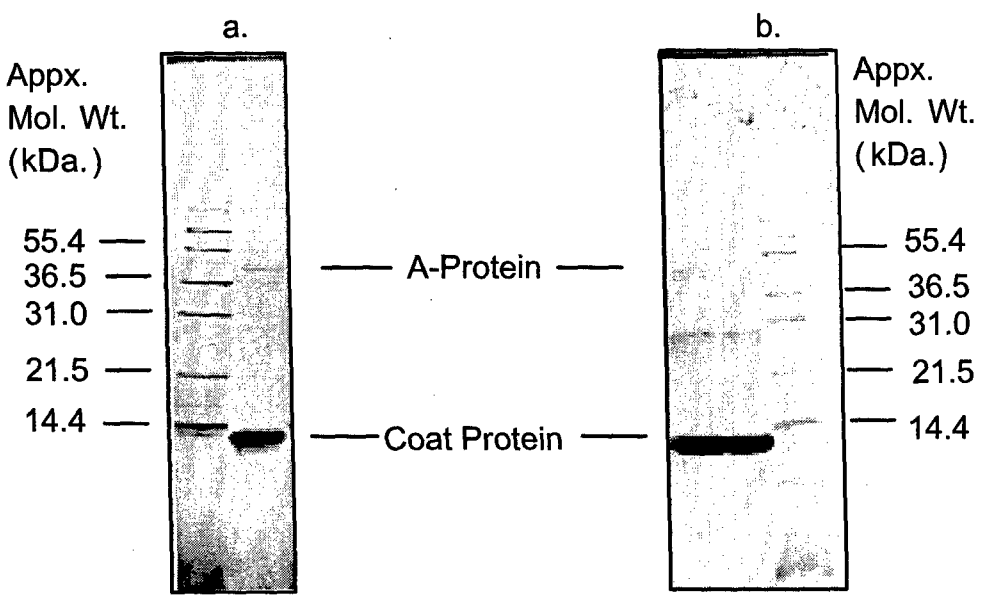

Figure 2. Gel Electrophoresis. Results from SDS/polyacrylamide denaturing gel electrophoresis for samples from (a) Experiment #1 and (b) Experiment #2.

The use of these particles as biological standards in public health screening of humans and livestock has been hampered by the lack of rapid quantitative methods to analyze the physical properties of this family of particles, not found in nature, which cannot be scientifically characterized by traditional methods. These MS2-like biomarkers, because of their small size and the necessity that they be noninfectious, cannot be rapidly or reliably counted. As a result, this new generation of biological reference material cannot be cheaply characterized for general use in public health laboratories. This is solely due to the fact that their physical properties in solution cannot be quantified or confirmed. Thus, there is a need for instrumentation that can count biological particles about which nothing is known and that also can provide structural information about their properties in solution.

This initial study, which is the first of a larger set of completed experiments using recombinant biomarkers, serves as a model for the use of small angle neutron scattering (SANS) and the integrated virus detection system as virus identification and characterization tools.

Our results show that the presence of either aggregates or small contaminates can distort the scattering peaks as seen by SANS. However, the positions of the peaks are still easily distinguishable, and the presence of polydispersity does not seem to affect the fitted parameters.

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