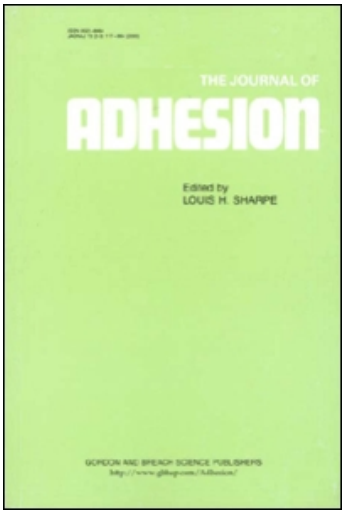

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Virus Concentration and Adhesion Measured by Laser Tracking

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At present there are few methods available for observing the adhesion of viruses. Also, it is difficult to determine virus concentrations on-line. This paper describes the "NanoSight," a microscope instrument which counts nanoparticles directly from scattered laser light and then determines their diameter by laser tracking the Brownian movement and applying the Stokes-Einstein theory to the random walk pathways. By applying this instrument to preparations of adenovirus, the concentration of viruses has been measured and compared with polystyrene latex spheres. Then, the instrument has been used to detect aggregates of viruses in the suspension. Taking the number of aggregates as a measure of the interparticle adhesion for equal spheres, the self-adhesion of the virus particles has been estimated as a function of two parameters, the adhesion energy and the range of the interaction. The results showed that the virus adhesion was similar to the self-adhesion of polystyrene.

Keywords: Aggregation; Laser tracking; Nanoparticles; Particle adhesion; Polystyrene latex; Virus

INTRODUCTION

There is a significant problem in understanding the adhesion of viruses and other nanoparticles such as polystyrene latex. Electron microscopy has been the major technique for observing virus particles

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in contact with cells and other surfaces, ever since the first transmission electron microscope (TEM) images from 1939 [1] revealed tobacco mosaic virus particles. A typical micrograph showing virus self-adhesion [2] to give doublets and triplets is given in Fig. 1. More recently, atomic force microscopy has been used to detect adhesion and aggregation of the nanoparticles [3].

Stanley [2] first observed virus particles adhering to form regular crystalline arrays of mosaic virus (TMV) and received the Nobel Prize in 1946. He had interpreted the patterns as protein but they also contained nucleic acid and were actually virus crystals. The rod-like virus particles could readily be seen like log-rafts in the TEM, having turned from dispersed particles into solid regular aggregates adhering together.

The crystallisation of satellite tobacco mosaic virus (sTMV) particles, which are spherical, can now be studied using atomic force microscopy and the growth of the ordered layers measured under various conditions of supersaturation and temperature [4]. Clearly, the self-adhesion of the virus particles is very small, around $3kT/2$ the thermal energy, otherwise the particles would aggregate into random structures as the particles adhered instantly. It was estimated from the crystal growth behaviour of the sTMVs that the energy of the crystal edge was $0.26\,mJ\,m^{-2}$, 15 times less than typical hydrocarbon rubber spheres adhering in water [5].

Many types of virus particles have been crystallised since the 1950s enabling much information to be gathered about the structures and

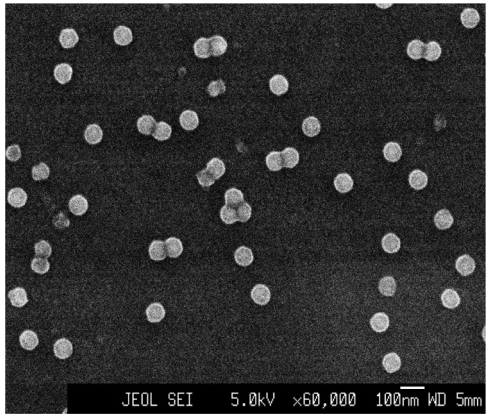


FIGURE 1 FESEM micrograph of adenovirus particles showing 7 doublets and 1 triplet [3] with permission from Liebert publishers.

the chemistry of crystalline layers. An interesting study was by Casselyn *et al.* on Brome mosaic virus [6]. A dispersion of brome mosaic virus (BMV) particles was purified and then rapidly mixed with poly(ethylene glycol) (PEG) and buffer in about 10 ms. Crystallisation was observed by small angle X-ray scattering (SAXS) to see the nucleation and growth of structure. Low concentrations of virus and PEG did not nucleate crystals, but crystals appeared at higher PEG, with high molecular weight being more effective. The virus crystals were face centred cubic (FCC) with a unit cell size of 39.1 nm. There was a sharp transition in the phase diagram from solution phase virus to crystal phase, then to amorphous at high PEG additions as shown in Fig. 2.

The critical nucleus size was 36 virus particles and the critical activation energy 65 kT. It is evident from these experiments that the virus particles were all exactly the same diameter and grew into crystals like polystyrene latex which also gives face centred cubic (FCC) crystals at the phase boundary. The PEG molecules were acting to increase the adhesion attraction between the virus particles by causing a depletion effect between the viruses.

A more complex phase diagram [7] was discovered for tomato bushy stunt virus (TBSV) which was encouraged to crystallise by adding either ammonium sulphate or poly(ethylene glycol) (PEG8000). Three morphologies of crystal were seen, the most common being the body centered (BCC) structure with 38.3 nm unit cell parameter. TBSV was the first icosahedral virus to be crystallised [8] and those early X-ray investigations had led to the understanding of the virus architecture by 1970 [9].

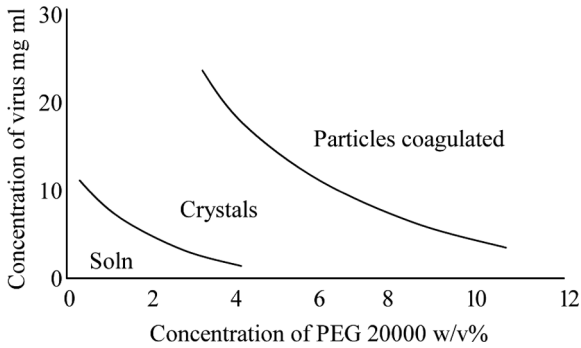


FIGURE 2 Phase diagram [6] of virus structure in buffer as a function of BMV concentration and PEG 20,000.

The problem with both X-ray and electron microscope results is that, although the virus particles can be seen adhering to themselves and to cells, there is little information on the adhesion energy. A more direct method of virus observation is, therefore, required. This paper shows that tracking the nanoparticle random walk using scattered laser light can be used to calculate adhesion from the theory of aggregate formation.

THEORY

Consider an ensemble of dispersed uniform spheres in a kinetic model as shown in Fig. 3a. The spheres move with a Boltzmann distribution of velocities and interact through a square well potential as shown in Fig. 3b such that reversible adhesion is observed. Such a square well has been much used in modelling the structure of particle dispersions [10–14].

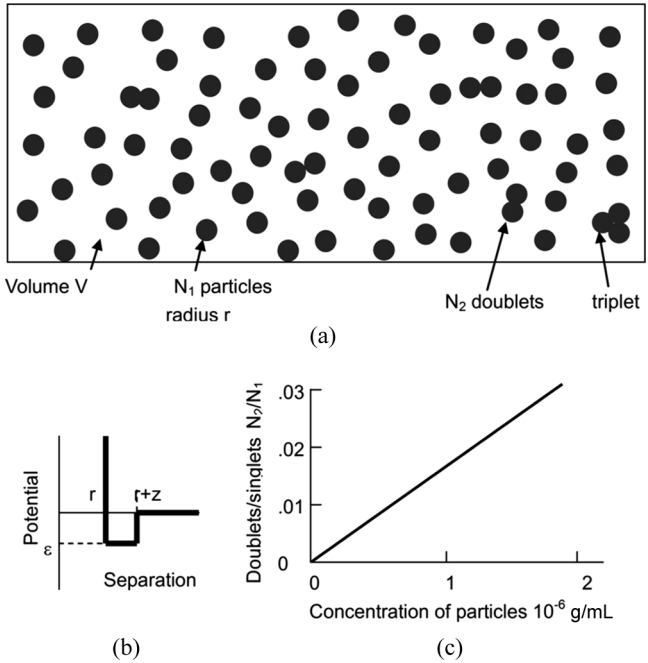


FIGURE 3 (a) N_1 uniform spheres in a dispersion containing N_2 doublets. (b) Square well potential defined by the adhesion energy, ε, and the range, $r + z$. (c) Adhesion characteristic curve obtained by plotting N_2/N_1 *vs* concentration.

It has been demonstrated that the number, N_2, of doublet aggregates observed in the dispersion is a measure of the interaction potential as shown in the statistical mechanics theory originally described in 1998 [10–14], *i.e.*,

$$N_2/N_1 = 4\phi\{([z + r]/r)^3 - 1\} \exp(\varepsilon/kT). \quad (1)$$

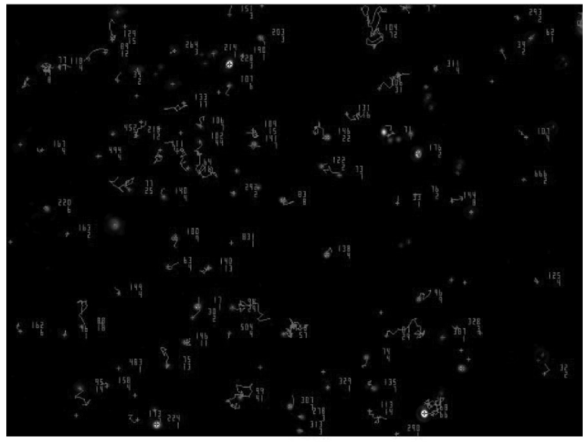
This equation is the only analytical result known to describe the aggregate distribution of particles based on an attractive potential. It assumes rigid identical spherical particles with a square well particle interaction potential where N_2 is the number of doublets, N_1 the number of singlets, ϕ is the volume fraction of particles, ε is the adhesion energy, and z is the width of the square well added to particle radius r.

By measuring the ratio of doublets to singlets in a dilute system as a function of volume fraction (Fig. 3c), it is therefore possible to estimate the adhesion parameters. The purpose of the experiments was to achieve this for virus to compare with other nanoparticles.

EXPERIMENTAL

A new method was developed for quantifying concentration and adhesion of viruses using the "NanoSight" instrument (NanoSight, Salisbury, UK), a particle tracking device developed originally by Malloy and Carr [15] in 2006. This instrument is now being used for the detection of different types of nanoparticles, and also to measure very small adhesion forces [16]. The method is based on the Brownian motion of nanoparticles, which can be observed easily by an optical microscope fitted with a movie camera to monitor small particles individually. Several tracks are counted and a histogram of nanoparticle sizes plotted. A typical still from the movie is shown in Fig. 4a and the histogram of 95.6-nm polystyrene spheres dispersed in water is given in Fig. 4b. It may be seen that the measured distribution is not symmetrical, suggesting that there is a small number of larger particles, which we interpret as aggregates resulting from adhesion. Because the aggregates are tracked over several frames in the movie, such larger particles must be truly adherent and cannot be explained by coincidences or overlaps.

Uniform polystyrene nanoparticles have been used as standards to check the instrument and the methods used, although the method itself is absolute and does not require calibration. Polystyrene latex suspensions were obtained in aqueous dispersions from Polysciences



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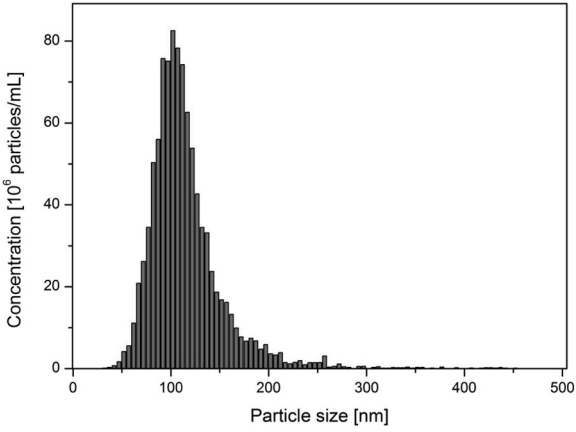


(b)

FIGURE 4 (a) Single frame of movie with random walk tracks shown. (b) Histogram of 95.6 nm polystyrene particles obtained from software analysis of tracks.

(New York, NY, USA) in three particle diameters, 95.6, 205.6, and 390.0 nm. In order to make them compatible with the virus particles, they were resuspended in phosphate buffered saline solution (PBS) at pH 7.4 (Sigma-Aldrich, St. Louis, MO, USA) to which protein surfactant HSA had been added to give monolayer coverage (HSA, human serum albumin, Sigma-Aldrich).

To produce suspensions of virus particles, replication incompetent Adenovirus serotype 5 (AdlacZ, E1A deleted, University of Warwick,

Coventry, UK) was grown in human embryonic kidney (HEK) 293 cells, purified by cesium chloride density gradient centrifugation and then desalted by equilibration with 20% glycerol-PBS [17]. The infectivity of purified AdlacZ adenovirus was determined by standard plaque assay in HEK293 cells [18], counting the number of infected regions caused by various dilutions of virus in a number of dishes.

RESULTS

In the first experiments, the objective was to find the concentrations of virus which could readily be measured in the NanoSight instrument. The virus, AdlacZ Adenovirus of TEM particle size of 90 nm was concentrated by centrifuging to a PBS suspension containing 1.0×10^9 pfu (plaque forming units) per 25 μL, then diluted 50 times or more, and examined in the NanoSight giving the plot in Fig. 5. Consequently, the laser tracking device should have found more particles than the sample diluted 100 times, but the numbers were much less than that. The main reason for this problem was the laser tracking difficulty with overlapping tracks. The tracking system could not distinguish overlapping tracks and so could not count the large number of virus present. However, the result did show that the Adenovirus diameter was near 100 nm and that there were few larger aggregates, indicating little self-adhesion between the particles.

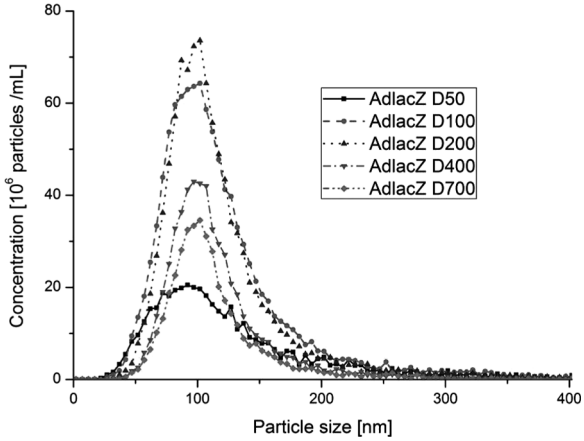


FIGURE 5 NanoSight tracking results for five different dilutions of adenovirus showing that the measurements are only accurate for dilutions higher than 200.

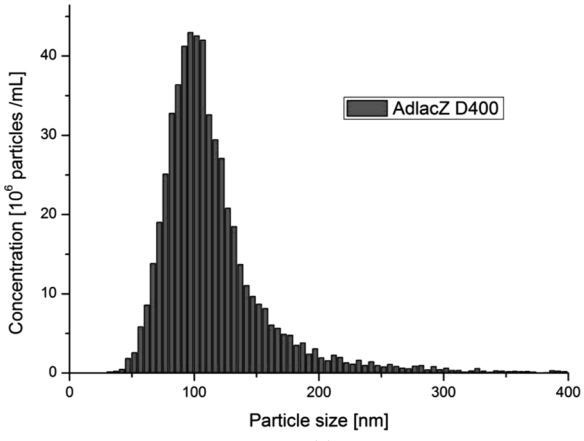
This problem was confirmed when the sample was diluted 100 and 200 times. The concentration measured by the NanoSight instrument seemed to rise as the concentration was reduced, because more independent tracks were being detected as the particles became separated on dilution. At dilutions of 400 and 700, the instrument seemed to be giving sensible concentration results, showing that the NanoSight concentration decreased as dilution proceeded. However, below 10^8 particles per mL, the number of tracks was limited and statistics were insufficient. The conclusion was that the NanoSight could only be used to give reliable results in the range of 10^8 to 10^{10} particles per mL, corresponding to volume fractions of AdlacZ around 10^{-7} to 10^{-5}, for adenovirus near 100 nm diameter.

COMPARISON USING POLYSTYRENE

It was then important to compare the virus with well-characterised polystyrene particles, first to check the concentration results and, secondly, to test results from standard virus assays.

To check the concentrations, the sample of adenovirus described above, diluted 400 times, was compared directly with a standard polystyrene nanoparticle dispersion measured separately after diluting 8000 times, shown in Fig. 6. The standard polystyrene sample concentration was originally 2.08×10^{13} particles/mL, and after dilution was 2.6×10^9 particles/mL. The two samples behaved comparably in the separate experiments, indicating that the concentrations, the particle size, and the states of aggregation, (*i.e.*, self-adhesion) were similar in the two cases. Because both samples were tested in the same NanoSight instrument with the same measured volume, it was possible to compare the peak areas for the single particle Gaussian fits. In this way, we calculated the concentration of the original AdLacZ sample to be 6.97×10^{11} particles/mL [viral particles (VP) per mL].

Secondly, the new laser tracking method was compared with standard virus assay results from the PFA (plaque forming assay) test which is most commonly used to measure virus concentrations. The number of virus particles measured by the NanoSight was found to be considerably higher than the infective particle numbers measured by plaque forming assay. Usually the explanation for this is the presence of viral particles which are not infective because of damage or lack of reproductive material. The particle infectivity ratio, that is, the total number of particles divided by the infective particles (VP/pfu) for adenovirus, was calculated to be 17.9 from this experiment. This is a reasonable result for such virus preparations [18].



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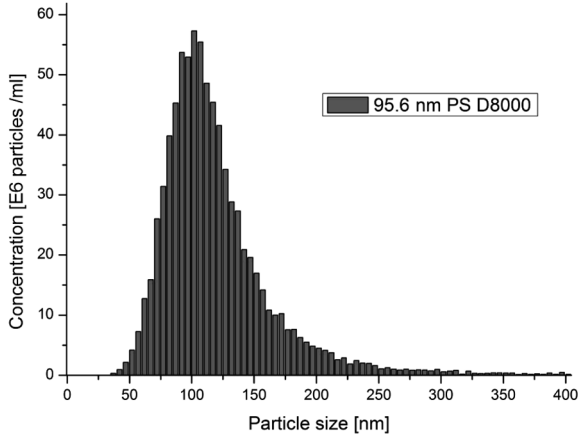

(b)

FIGURE 6 (a) Histogram of virus AdLacZ Adenovirus sample diluted by PBS 400 times. (b) Histogram of 95.6 nm PS aqueous suspension with a concentration of 2.6×10^9 particles/mL.

ADHESION MEASUREMENT

Having verified the particle concentrations measured by laser tracking within defined parameters of size and track overlapping, it was then possible to use the NanoSight to count the number of doublets as a measure of self-adhesion. To estimate self-adhesion of the nanoparticles, the number of doublet aggregates was obtained from the histogram by fitting two Gaussian curves to the results, one for single particles

assumed to be uniformly distributed and a smaller curve for doublets and large aggregates. Figure 7a shows the computer fit for 95.6 nm polystyrene nanoparticles coated with HSA in PBS, the doublet peak at 1.49 times the primary particle diameter and large aggregates to give the measured result for particle size distribution. The ratio of doublets to singlets was then calculated and plotted against virus concentration to give the adhesion characteristic curve fitting Eq. (1).

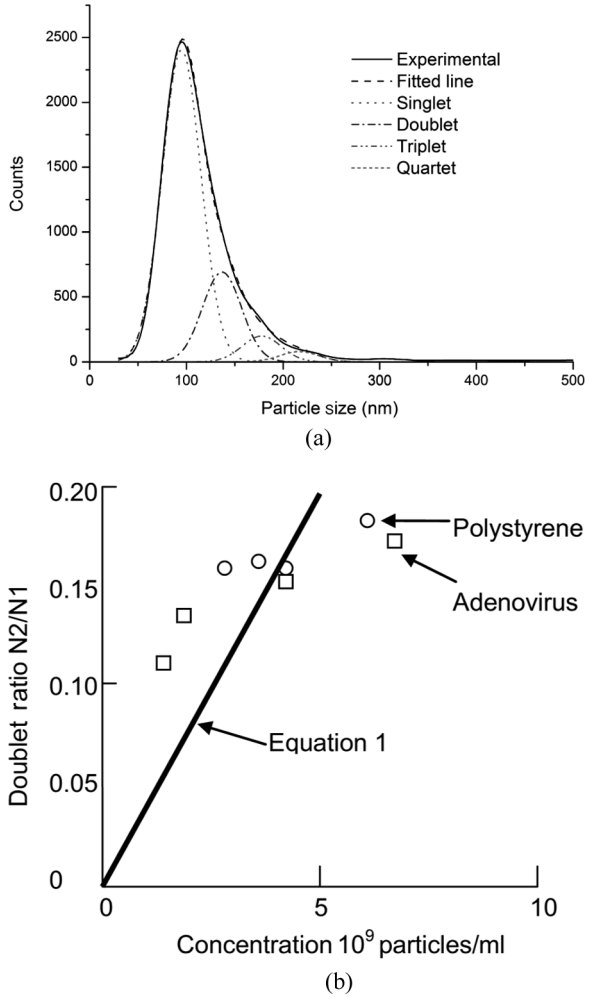

FIGURE 7 (a) Singlet and aggregate peaks fitted to results for 95.6 nm polystyrene coated with HAS in PBS at concentration of 2.5×10^{-7} g/mL. (b) Comparison of doublet results for 95.6 nm polystyrene and adenovirus.

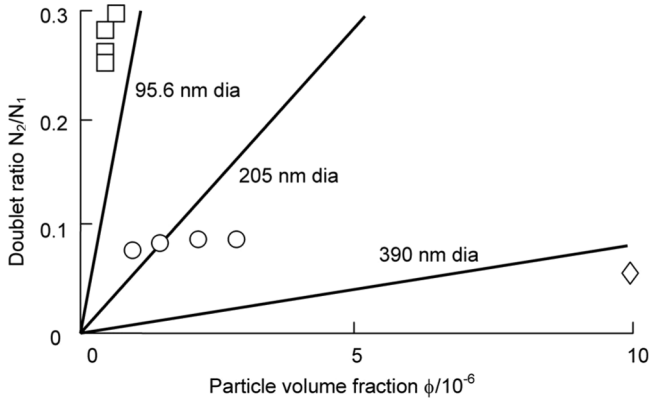

FIGURE 8 Plot of adhesion characteristic curves for polystyrene latexes of several diameters in PBS, showing a fit to the adhesion energy 3.5 kT and range parameter z = 600 nm.

Figure 7b shows the ratios for 95.6 nm polystyrene in aqueous solution and adenovirus at different concentrations. It was evident that the results gave an approximate fit to Eq. (1) but with substantial scatter. The adhesion curve for the virus was a little lower than that for the polystyrene indicating slightly lower adhesion. Using this method, it was not possible to distinguish the energy, ε, and the range, z, to describe the potential curve of Eq. (1). However, for polystyrene, it was possible to use several particle sizes to obtain the two-parameter fit as shown in Fig. 8.

CONCLUSIONS

A new method has been found for measuring both the concentration and the overall adhesion of virus particles, using the NanoSight instrument to detect individual Brownian random walks by scattered laser light and computer tracking.

The method was used to measure concentrations and overall adhesion of adenovirus particles for comparison with polystyrene calibration latexes. Because of cross-over between tracks, it was only possible to measure samples at concentrations between 10^8 and 10^{10} particles per mL (10^{-7} and 10^{-5} volume fraction for 100-nm particles). Also, the method could not clearly distinguish the energy and range effects for the adhesive virus interaction.

By measuring the ratio of doublet aggregates to singlet particles, and plotting this against volume fraction, the characteristic

self-adhesion curve for the virus particles was determined and shown to be comparable with that of polystyrene latex spheres coated with albumin in PBS.

Addition of albumin to the polystyrene particles slightly increased their doublet formation, indicating a small rise in adhesion.

The measurement of several different diameter polystyrene particles showed that the range of the adhesion interaction potential was 600 nm, much larger than that expected from single atom interactions.

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