Dyed viruses and metal particles for advanced separation studies

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ABSTRACT

Single nanometer sized particles are poorly retained by conventional water treatment methods and are hardly detectable in water samples. The particles present a separate class of pollutants and their transport and fate cannot be studied by tracking bacteria, turbidity, free dyes or ions. Dyed bacteriophages and gold nanoparticles are two novel tools that facilitate the studies of virus transport, adsorption and inactivation. The approach is exemplified in studies of slow and rapid sand filtration, ultrafiltration, chlorination and UV disinfection, performed over the last decade. An analysis of general retention trends points to the interrelation between macroscopic particle characteristics and its retention. A better retention, a higher zeta potential and a shorter residence time are associated with larger viruses. A ratio of virus size to its surface area highlights the importance of diffusion as the transport step and electrostatic interactions as the attachment step.

Key words | MS2, phi X 174, T4, Vaccinia virus

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INTRODUCTION

Viruses are a separate class of emerging nanoparticles responsible for 8% of acute human gastroenteritis (Leclerc *et al.* 2002). Table 1 presents typical retention efficiencies of bacteria, protozoa and viruses by filtration and disinfection (WHO 2004). The retention of viruses by any means of filtration is lower than the retention of bacteria and protozoa. Only the disinfection of protozoa with ozone and chlorine requires more disinfectant than the dose needed to inactivate viruses to the same level. In addition to the poor retention, the detection of viruses in water samples is complicated, time-consuming and labor-intensive. The latter deficiency is especially troubling. Although the first indications for the presence of viruses in treated water are obtained between 12 and 48 hours, a detailed analysis can often take 1 or even 2 weeks. That time is significantly longer than the average residence time of treated drinking water in the distribution system. In a realistic scenario contaminated water reaches a consumer before the analysis of water for the presence of viruses is completed. An additional concern arises from the gap between detectable and real concentrations of viruses in traditional microbiological methods.

The retention of viruses is often compared to the retention of other solutes that are either constantly present in water or added before the assessment. The intention is to understand the virus retention data with quick and simple tests of retention of other impurities that might resemble the virus. The retention of mineral particles detected by turbidity and particle size analysis is a classic example for that type of a surrogate assessment. Other popular virus surrogates include chemical (anions, surfactants, soluble dyes) tracers, fluorescent latex microspheres and attenuated viruses (Gitis *et al.* 2002a). Bacteriophages, a class of viruses that attack only the bacterial genome, are probably the most reliable method widely used in both laboratory research and in practice. Bacteriophages are closest in size, shape, and surface properties to enteric viruses and contain an RNA genome of similar size to the picorna-, calici- and astroviruses. Yet the studies of virus retention suffer from complicated and time consuming plaque forming unit (pfu) enumeration; inability to distinguish between a single virus and a cluster; a need for a careful sample handling and preservation; and a significant delay in delivery of test results.

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Table 1 | Efficiency of different treatments in the retention of waterborne pathogenic microorganisms

Treatment process	Retention of bacteria	Retention of protozoa	Retention of viruses
Slow sand filtration	50%	50%	20%
Microfiltration	99.9–99.99%	99.9–99.99%	<90%
Ultrafiltration	Complete	Complete	99.9–99.99%
Nanofiltration, RO	Complete	Complete	Complete
Chlorine (mg-min/L), pH 7–7.5	Ct_{99}: 0.08 at 1 °C	*Giardia* Ct_{99}: 41 at 25 °C, *Cryptosporidium* not killed	Ct_{99}: 8 at 10 °C
Ozone (mg-min/L), pH 6–9	Ct_{99}: 0.02 at 5 °C	*Giardia* Ct_{99}: 0.63 at 15 °C, *Cryptosporidium* Ct_{99}: 4.4 at 22 °C	Ct_{99}: 0.3 at 15 °C
UV irradiation, 99% inactivation	7 mJ/cm^2	*Giardia* 5 mJ/cm^2; *Cryptosporidium* 10 mJ/cm^2	59 mJ/cm^2

Two additional tools that have proven useful for studies of virus transport and fate in water and wastewater treatment systems are described below. Dyeing bacteriophages with fluorescent dyes overcomes the abovementioned shortcomings. Dyeing does not change the size, the surface charge and the degree of hydrophobicity of native viruses (Gitis *et al.* 2002a, b). The only change is a non-viability of dyed phages. The latter can be seen as an additional advantage that removes all limits to the use of the probe in water samples. The concentration of dyed viruses can conveniently be detected by fluorescence using linear calibration curves. The curves connect the concentration of native viruses before dyeing with a fluorescence intensity after washing away free remaining molecules of the dye. The retention of dyed bacteriophages in slow and rapid sand filtration is close to the retention of native viruses (Gitis *et al.* 2002a, b). The inactivation of viruses by chlorination and UV linearly correlates with the degradation of dyes (Asseraf-Snir & Gitis 2011). Separation and inactivation of viruses in these processes include a transport step where viruses are approaching the treatment site, and accumulation or inactivation of viruses at the site (Gitis *et al.* 2010). The degree of retention of native phages is a superposition of all three steps. The retention of dyed viruses includes the transport and accumulation stages so the degree of inactivation can be deducted from the degree of retention of native phages. Performing spiking experiments with the synthesized nanoparticles and with native viruses to distinguish between transport, accumulation and inactivation mechanisms was exemplified and detailed in Gitis *et al.* (2011a). A solid link found between macroscopic virus parameters and its removal is logical in filtration and surprising in disinfection. The retention of viruses by low pressure membrane filtration is more straightforward and can be explained by the size exclusion. Thus the size similarity is the main requirement to probe the retention of viruses by a membrane. Gold nanoparticles with tunable size and surface charge synthesized in the laboratory showed similar retention of viruses and gold nanoparticles by ultrafiltration (UF) membranes (Gitis *et al.* 2006a, b).

MATERIALS AND METHODS

Bacteriophages

The phi X174 (ATCC 13706-B1), MS2 (ATCC 15597-B1) and T4 (ATCC 11303) bacteriophages and their *Escherichia coli* host cells were purchased from Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (DSMZ, Germany). Vaccinia viruses (VV) were received from the department of virology at Ben Gurion University of the Negev. Table 2 details the main phage characteristics.

The viruses differ in size from 22 to 360 nm, morphology is icosahedral and tailed, and isoelectric point is from pH 3.2 to 6.6. Sizes and isoelectric points of the phages were detected in previous studies (Gitis *et al.* 2002a, 2011a and references therein). All bacteriophages were cultivable in different strains of *E. coli* as the host bacterium. A fresh stock was prepared for each experiment. Phages were propagated by inoculation of infected *E. coli* cells into Lysogeny Broth (LB) medium followed by 24 h incubation at 37 °C. During this time, the cells were at the exponential growth phase (suspension turbidity from 0.2 to 0.3 optical density, OD). The cell lysis was later performed with chloroform. The resulted suspension was centrifuged at 37,000 rpm for 40 min, the

Table 2 | Main characteristics of studied viruses

Virus	Family/group	Particle morphology	Nucleic acid[a]	Size (nm)	Isoelectric point, pH
phi X174	Microviridae	Icosahedral	Circular ss DNA	25	6.6
MS2	Leviviridae	Sym. Icosahedral	Linear ss RNA	28	3.9
T4	Myoviridae	Tailed phage	Linear ds DNA	Head 65×80, tail 120×20	3.2
VV	Poxviridae	Icosahedral	Linear ds DNA	$360 \times 270 \times 250$	4.8

[a]ss = single-strained; ds = double-strained.

pellet was discarded, and the bacteriophage-containing supernatant was stored at 4 °C. Bacteriophage concentration was determined by a pfu assay, using a double-layer overlay method. The initial phage concentration in different studies varied between 2.3×10^7 and 5×10^9 pfu/mL.

Phages were labeled with four different dyes such as rhodamine B (9-(o-Carboxyphenyl)-6-(diethylamino)-3H-xanthen-3-ylidene) diethylammonium chloride), fluorescein, FITC (fluorescein-5-isothiocyanate) and 5-DTAF (5-(4,6-dichlorotriazinyl)aminofluorescein). The labeling reaction was performed in the presence of a coupling agent DEC (1-[3-(Dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride), which was needed to attain a permanent attachment. The suspension was placed into a dialysis membrane (molecular weight cut-off (MWCO) = 6,000–8,000 Da). A gradual diffusion through the membrane into distilled water resulted in washing out of free dye molecules. The dialysis was performed for approximately 1 week at 4 °C under a continuous stirring until no fluorescence was observed in the rinsing water. The purified labeled bacteriophages were stored at 4 °C in a dark room and warmed to room temperature 2 h before an experiment. Fluorescence was measured with TECAN Infinite M200 plate reader equipped with a monochromator. The fluorescence intensity was reported in the instrument's relative fluorescence units.

Procedure for preparing and characterizing the nanoparticles

Seven samples of monodispersed gold aquasols were prepared by reduction of hydrogen tetrachloroaurate with sodium citrate and tannic acid (Turkevich *et al.* 1951; Slot & Geuze 1985). For instance, 4.0 mL of a 1% solution of trisodium citrate and 5.0 mL of tannic acid were added to 40.0 mL of a 0.01% (w/v) solution of $HAuCl_4$. All chemicals were purchased from Sigma-Aldrich. The mixture was stirred for 5 min under gentle boiling, cooled to room temperature, and stored at 4 °C. The initial gold concentration was 52 mg/L. Obtained suspensions were characterized by JEM-1230 (JEOL) transmission electron microscopy operated at 120 kV. The microscope was equipped with a TemCam-F214 (TVIPS) camera. Size distribution and zeta potential were measured with a ZetaPlus (Brookhaven Instruments) zeta potential and particle size analyzer equipped with 30 mW 657 nm laser (Hamamatsu Photonics). The measurements were performed at a pH of 6, and at a flex angle of 90°. Each measurement was a composite of 10 runs of 10 s each; runs with high baseline levels were disregarded. To reduce interference from multiple scattering, size distribution and zeta potential were measured at different concentrations.

Experimental set-up

UF experiments were performed with new polyethersulphone PES-30, polyvinylidene fluoride PVDF-30, polycarbonate PC-30 and PC-100 membranes (Sterlitech Corporation). The digits in polyethersulfone (PES) and polyvinylidene difluoride (PVDF) membranes indicate their nominal MWCO in kDa, and in PC membranes the pore size in nm. Prior to filtration, the membranes were soaked in NaOH solution (pH 9) and vibrated at 55 °C for 2 h. The efficiency of pre-cleaning was assessed by the filtration of deionized water (reverse osmosis (RO) quality). Similar feed and permeate total organic carbon levels were obtained. The filtration was performed in a 150 mL autoclaved stirred cell equipped with a back-pressure TMP controller (Kuzmenko *et al.* 2005). The transmembrane pressure (TMP) was set with a precision regulator IR2000-FO2 (SMC Corporation).

Two sets of sand filtration experiments were performed. Laboratory experiments were performed in 23 cm long

columns with an internal diameter of 5.5 cm each. Columns were wet-packed with uniformly sized quartz sand (Haifa Bay) with a geometric mean diameter of 1.06 mm and a uniformity coefficient (UC) of 1.55. Experiments were performed at approach velocities of 0.12, 0.085, 0.05, and 0.012 m/h. The hydraulic residence time was calculated by dividing the volume of the entire column by the water flux. For the approach velocity of 0.12 m/h the calculated hydraulic residence time was 25.6 min. The filter porosity ϕ of 0.235 was measured volumetrically. The entire set-up included a 5-L feed reservoir, peristaltic pumps, sand columns, a 10-L waste drum, and an FC 203B fraction collector (Gilson Inc.). The experiments were performed with tap water (Beer Sheva quality).

A set of rapid sand filtration experiments was performed at Beer-Sheva wastewater treatment plant. The experiments were performed with secondary effluents or tap water filtered through two 10 cm diameter acrylic filtration columns. The columns were filled with 1.1 m of uniformly sized quartz sand (Haifa Bay) with an effective size (d_{10}) of 0.89 mm and a UC of 1.35. The measured filter porosity was 0.44. Filtration was performed at constant approach velocities of 5, 7.5, and 10 m/h. Details of the experimental set-up can be found in Aronino *et al.* (2009).

Chlorination experiments were performed in a bench-scale disinfection system consisting of two feed vessels, two peristaltic pumps, a static mixer, a chlorine contact tank (CCT), and a sample collector connected to the CCT outlet. Design of the system was based on common knowledge in disinfection systems and included mixing before CCT and baffling inside the reactor. The CCT was 162 mm long, 40 mm wide, and 40 mm deep and was designed for a theoretical detention time of 10 min. The internal 200 mL volume was divided by 10 baffles to 11 compartments. Chlorination experiments were performed with 2–3 mg/L NaOCl at a constant flow rate of 20 mL/min. The residual chlorine was neutralized immediately at the CCT outlet with sodium thiosulphate, $Na_2S_2O_3$ (final concentration 0.084%).

UV experiments were performed in a collimated beam UV cell. A nearly monochromatic UV radiation at 253.7 nm was generated by a 43-W low-pressure (LP) mercury lamp (Trojan UV, Canada) and focused through a circular opening. The incident irradiance of ~0.25 mW/m^2 UVC output was measured with a radiometer (IL1400; International Light) and UV 254 detector (SEL240) with a filter (NS254). Tested suspensions were slowly magnetically stirred in open 100 × 25 mm Petri dishes at ambient temperature. Exposure times were controlled by shutter. The entire system was enclosed in a wood cabinet having black-painted interior walls. Experiments were performed in a UV dose range between 1 and 110 mJ/cm^2. Control experiments were conducted when a UV lamp was switched off.

The next section displays the obtained trends. Disinfection efficiencies of chlorination and UV irradiation are expressed in log inactivation values (LIV), defined as the base 10 logarithm of the ratio of microorganism concentrations in the feed and in disinfected water. Filtration efficiency is expressed in log removal values (LRV), defined as the base 10 logarithm of the ratio of microorganism concentrations in the influent and the effluent.

RESULTS AND DISCUSSION

Relating the retention of viruses to their characteristics is a superficial approach that nevertheless could be of high importance for water practitioners. With all the criticism that can be raised, it is useful to look on the general trends that relate the size and zeta potential of nanoparticles to their retention. Figure 1 depicts the degree of virus retention or inactivation as a function of its size. A logarithmic increase in LIV and LRV values with an increase in virus size was observed (Aronino *et al.* 2009; Gitis *et al.* 2011a, b). Novel UV and UF treatments were more successful in virus retention. The 6–7 LIV/LRV of VV removal and even 2–3 LIV/LRVs of phi X 174 (Arkhangelsky & Gitis 2008) were much higher than 0.4–2 LIV/LRVs achieved by sand filtration and by chlorination. UV irradiation displayed nonlinearity in inactivation of MS2 and phi X 174 related to a higher UV sensitivity of DNA-based bacteriophages (Rauth 1965). The expected trend was opposite to the received as small viruses with higher relative nucleic acid content were expected to be more vulnerable to UV irradiation. The higher degree of retention of bigger viruses by sand filtration runs counter to the currently accepted filtration theory. The bigger viruses are in the size range of 200–350 nm, dimensions that approach the minimum transport efficiency of 1 μm (Yao *et al.* 1971; O'Melia & Ali 1978).

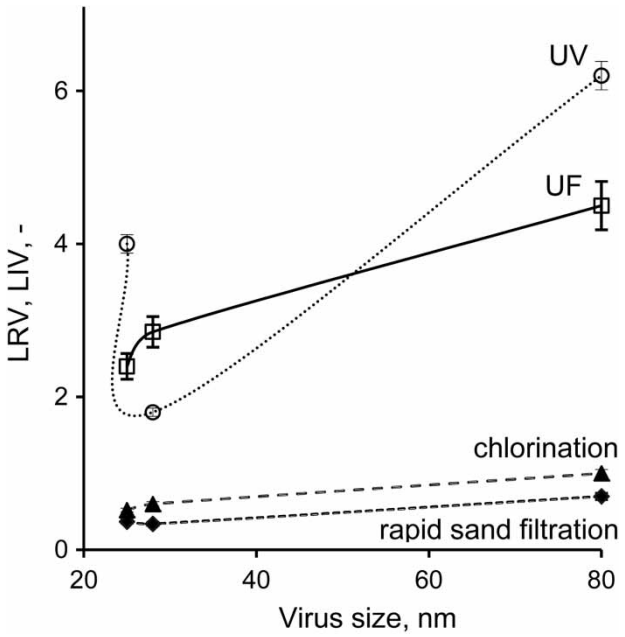

Figure 1 | LRV and LIV values as a function of virus size. Experimental conditions: UV – 30 mJ/cm^2; UF – PES-30, TMP 2 bars; rapid sand filtration – 100 cm depth; chlorination – 2 mg/L free chlorine, contact time 2 min. The UF of VV resulted in 6 LRV (not shown).

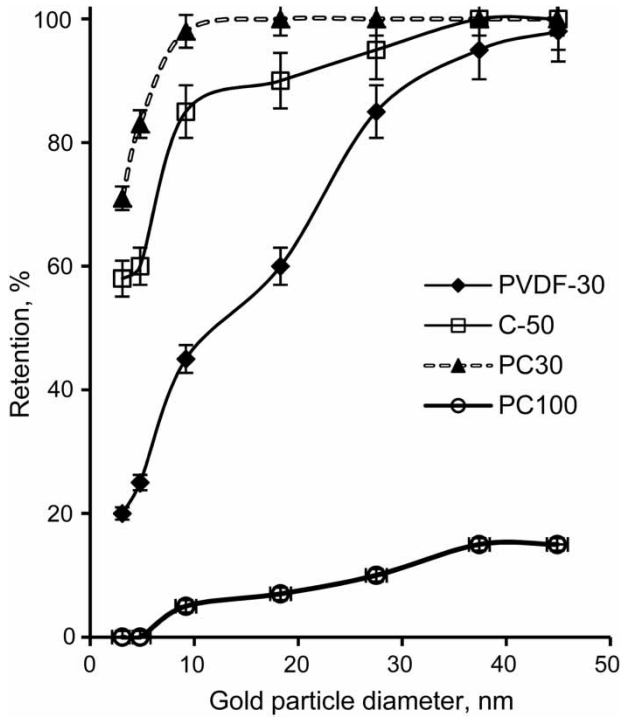

Figure 2 | Percentage of gold nanoparticle rejection by PVDF-30, C-50, PC-30 and PC-100 membranes.

Figure 2 provides a closer look on the virus retention with UF membranes. Although in general the trend of better retention of bigger viruses remains, the presence of absolute pore size of UF membrane suggests similar degree of retention of bigger and smaller viruses. Experiments with seven gold nanoparticles in a close size range displays an almost similar retention mostly governed by a pore size of the membrane. Thus, although in general a higher degree of retention can be associated with higher virus dimensions, some limitations posed by the treatment itself results in equal virus retention due to a size exclusion.

Figure 3 displays virus mean residence time as a function of its size. Higher mean residence time of viruses of smaller dimensions is noticeable in rapid sand filtration and chlorination. Both treatments are performed in reactors, and in both the diffusional transport is usually overlooked. The Stokes–Einstein equation suggests a relation between the size of a particle and its diffusive motion in a form

$$D = \frac{K_B T}{6\pi r \mu} \quad (1)$$

where D is diffusion coefficient (m^2/s), K_B is Boltzmann constant (J/K), T is absolute temperature (K), r is the radius of a spherical particle (m), and μ is water dynamic viscosity (kg/m-sec, Welty *et al.* 2001). Table 3 displays diffusion coefficients for the viruses calculated based on provided estimated values of their radii (Table 2) and assuming their sphericity. The calculations were based on single particles, assuming no aggregation.

There is an order of magnitude difference between the smallest and the biggest viruses, both in size and in diffusion coefficient. There are 2–3 orders of magnitude difference between diffusion coefficients of viruses, bacteria and protozoa (Asseraf-Snir & Gitis 2011). Transport of solutes through a water treatment reactor is governed by convection and diffusion. The evident difference in the diffusion coefficient of investigated solutes suggests a significant diffusion for the small viruses and dominant convection for bigger viruses, bacteria and protozoa. Low diffusion may result in flow through the reactor following a mean path without axial movement to 'dead' compartments. That is also the case for UV where the usual

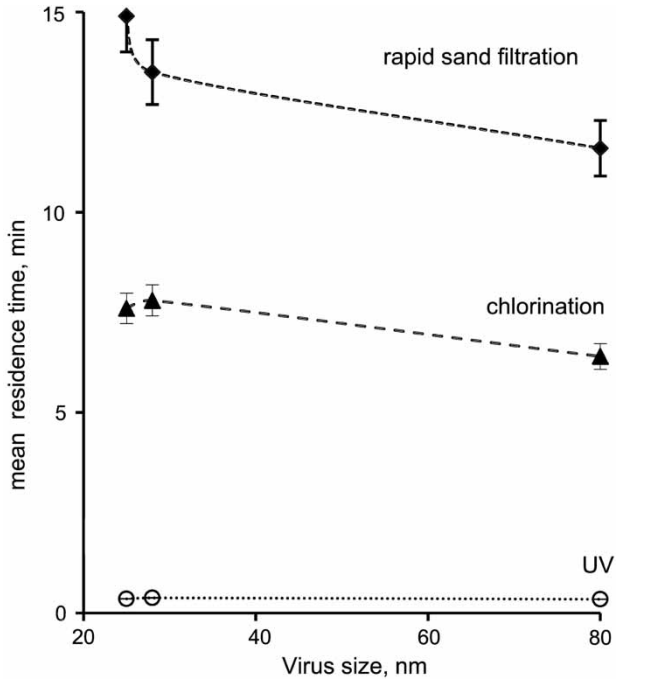

Figure 3 | Mean virus residence time as a function of its size in rapid sand filtration, chlorination and UV treatments.

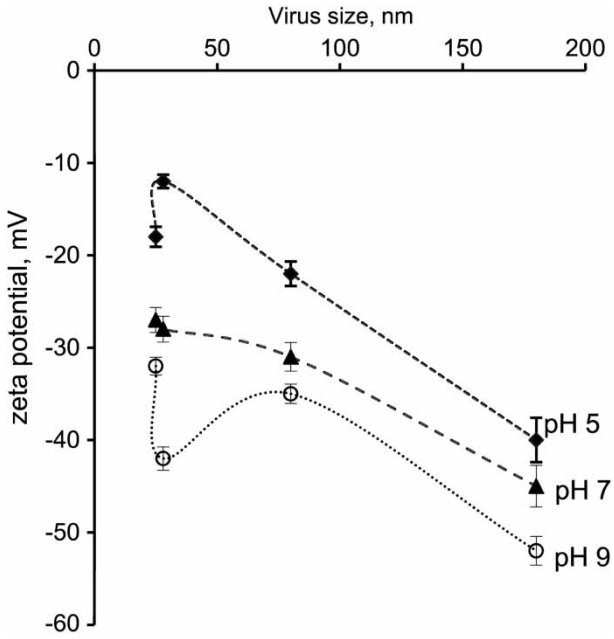

Figure 4 | Zeta potential values as a function of virus size for pH values 5, 7 and 9. Ionic strength 2.6 mM, added NaCl as needed.

Table 3 | Diffusion coefficients[a] of viruses

Virus	*r* (nm)	*D* (m^2/sec)
phi X 174	12.5	1.7×10^{-11}
MS2	14.0	1.5×10^{-11}
T4	40.0	5.3×10^{-12}
VV	180.0	1.1×10^{-12}

[a]At T of 293 K.

residence time is within single seconds and virus trajectories are dominated by the motion of the surrounding fluid.

Figure 4 displays zeta potential values of viruses as a function of their size. Along with some nonlinearity in the zeta potential of small viruses, the general trend is that bigger viruses are charged less than the small ones. That is once again opposite to what was expected as smaller particles were expected to have higher surface expression of negatively charged end groups such as a carboxylic one. Apparently the higher number of amino and carboxylic groups as a function of more amino acids in larger viruses is more important than the size difference. The electrophoretic motility of entities with density close to the density of water could be governed by a total charge and not by dimensions.

CONCLUSIONS

Many previous studies were conducted with a single nanoparticle or with one experimental set-up. The opportunity to connect macroscopic characteristics of viruses and their retention is rare. The reported observations are the result of over a decade research with the same set of nanoparticles and similar treatment systems. The better retention of the large and not the small viruses can probably be linked to their higher zeta potential values. At the same time, the average residence time of larger viruses is smaller, probably due to a less significant diffusion and a prevalent convection. Small MS2 and phi X 174 viruses can give a conservative assessment of transport and fate of bigger viruses. A more realistic assessment can be based on experiments with the novel nanoprobes with tunable macroscopic characteristics.

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