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Full Length Research Paper

Ultramicroscopic observation of recombinant adeno-associated virus type 2 on the surface of formvar-carbon coated copper grids under different relative humidity and incubation time using negative stain transmission electron microscopy

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The purpose of this investigation was to compare the effects of different relative humidity (RH) on the microcosmic conformation of the recombinant AAV-2 virion at 22°C. rAAV-2 virions prepared on copper grid were placed in a high, middle or low RH cabinet and incubated for 72, 48 and 24 h, respectively. The rAAV-2 virions were observed by transmission electron microscope and the values of major axis length, minor axis length and ellipticity of the rAAV-2 virions were obtained using the IMS cell image analysis system. After incubation for 48 and 72 h, the major axis length and minor axis length of the rAAV-2 virion started to rapidly decrease in high RH. Conversely, the axis lengths rapidly increased in low RH. Then, the ellipticity of the rAAV-2 virion would almost tend to approach the identical value of 0.9 for 48 and 72 h incubations in high RH. The results suggest that the rAAV-2 virion tended to favor a smaller, round, more stable conformation in high RH compared to low RH which implied that the rAAV-2 virion was probably prone to living in high relative humidity conditions.

Key words: rAAV-2 virion, ultramicroscopic morphology, relative humidity, formvar-carbon, transmission electron microscopy.

INTRODUCTION

Viability and infectivity of airborne viruses are affected by many environmental factors such as temperature, relative humidity (RH), high-energy radials, organic and inorganic chemicals, negative oxygen ions, ozone and surface qualities of holders. Certainly, viability and infectivity are also affected by the virus' own structure and character, which can be modified by the temperature and the RH (Cox, 1989; Benbough, 1971). In general, viruses are classified into two groups.

One group has a lipid envelope wrapping the capsid of the virus, and the other group has no lipid envelope outside the capsid. In previous studies, most investigators focused on researching the macroscopic effect of environmental temperature and RH on the viability and infectivity of airborne virus (Harper, 1963; Sattar et al., 1984; Ijaz, 1985; Ijaz and Satter, 1987). It was reported in these previous investigations that the effect of environmental RH on the airborne virus with a lipid envelope and on virus without a lipid envelope under an appropriate constant temperature such as room temperature (25°C) was opposite (Akers, 1973; Dejong et al., 1973). The generally accepted concept was that the phage and the virus without a lipid envelope were able to acclimatize to

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high RH (RH>50%) at room temperature and thereby maintained high viability and infectivity, while viruses with a lipid envelope could be viable and infectious at lower RH (RH>30%) at room temperature (25℃). It was inferred that the lipid envelope of a virus was more unstable and denatured in a high RH (RH > 50%) environment comparable to a low RH (RH>30%) environment (Hemmes et al., 1960; Harper, 1961; Akers and Hatch, 1968; Veen et al., 1972; Cox, 1987). On the contrary, the capsid protein of the virus without a lipid envelope was more unstable and denatured in a low RH (RH > 30%) environment comparable to a high RH (RH > 50%) environment (Cox, 1987; Rosebury, 1947; Webb et al., 1963; Harris and Horne, 1986). This showed that the structure and character of the virus envelope and capsid played a key role in the effect of RH on viability and infectivity of airborne virus. Additionally, the surface qualities of different materials holding the virus played an important role in the effect of RH on viability and infectivity of airborne virus (Sattar et al., 1986; Mbithi et al., 1991; Xavier et al., 1994). The previous results were obtained on the basis of macroscopic observation and statistical analysis of the RH effect on viability and infectivity of airborne virus. Hitherto, information based on microscopic observation and statistical analysis of the effect of RH on conformation of airborne virus was unknown. In order to examine the RH effect on the conformation of the virus by microscopic observation and statistical analysis, we built a simple RH and constant temperature incubation system. Microscopic observation and statistical analysis of the virus conformation under different RH effect was carried out using transmission electron microscopy (TEM) and IMS cell image analysis system. In this investigation, the recombinant virus adeno-associated virus type 2 (rAAV-2) was selected as the experimental virus.

Adeno-associated virus (AAV) is a small replication-defective parvovirus, which cannot replicate on its own and requires a cell to be co-infected with adenovirus and other relative viruses in order to replicate. In the absence of co-infection with adenovirus, the AAV goes into a latent phase. It then integrates its DNA into the genome of the host cell at a specific site on the nineteenth chromosome. This latent phase makes AAV attractive for gene therapy applications since a gene of interest inserted into AAV can persist in the host cell genome for a long period.

The AAV genome consists of a single molecule of linear negative-sense, or positive-sense single-stranded DNA. The complete wildtype AAV genome is 4.7 kb nucleotides long and has the accession number, but the upper limit for its use in gene therapy applications is about 4.4 kb. The single stranded AAV DNA genome is encased in a 20-25 nm diameter protein capsid with icosahedral symmetry (Concalves, 2005; Timpe et al., 2005). There are five distinct AAV serotypes among which the AAV-type 2 is often used as a gene therapy vector. In general, the recombinant adeno-associated virus type 2 (rAAV-2) containing the interest gene has a simple structure and consists of a capsid with 60 capsomers, which is round and exhibits icosahedral symmetry. The capsid is isometric and has a diameter of 18-22 nm or 20-26 nm. Surface projections of the capsid are small and the surface appears rough. The capsid is not enveloped with a lipid membrane (Srivastava et al., 1983; Berns et al., 1987; Ralf et al., 1999). In our study, the rAAV-2 containing an EGFP (enhanced green fluorescence protein) gene was used to examine the RH effect on the ultramicroscopic morphology of the virus without a lipid envelope. We found that RH might play an important role in the morphology of the virus without lipid envelope and thereby affect the viability and infectivity of the virus.

MATERIALS AND METHODS

Virus sample and key reagent

rAAV-2 (concentration 5.0 × 10^{11}v.g/mL) was bought from Vector Gene Technology Company Limited (VGTC) of China. There was an EGFP (enhanced green fluorescence protein) gene inserted in the genome of rAAV. Commonly, the rAAV-2- EGFP is stocked in phosphate-buffered saline (1×PBS) at 4℃. Two percent phosphortungstic acid (PTA, pH 6.8) was used as a negative stain for TEM. Polyformaldehyde (0.2%) was used as a fixation reagent. BSA solution (0.1%) was used to wash the formvar-carbon coated copper grid.

Key instruments

The transmission electron microscope was a HITACH H-7000 TEM made by Toshiba Co. (Japan). IMS cell image analysis system containing the Panasonic MV-CP410 vidicon and the Olympus BH2 microscope was made in Japan. The adjusted electronic dampproof cabinets for virus incubation to different relative humidity (RH) were made by Bossmen Inc. (Taiwan). The model SMR 95 L specialty dampproof cabinet had RH control range: 1% RH ~ 40% RH, Variance: ±2% RH; the model PQ 95 L dampproof cabinet had RH control range: 20% RH ~ 60% RH, Variance: ±2% RH; the model PQ 95 L specialty dampproof cabinet with humidifier had RH control range: 40% RH ~ 100% RH, Variance: ±2% RH.

Methods and experimental procedures

The three different models of dampproof cabinets were placed in the 22℃ constant temperature room. The interior relative humidity of the three dampproof cabinets was adjusted to 10% RH (low RH), 50% RH (middle RH) or 90% RH (high RH) respectively. After the interior relative humidity and temperature of the dampproof cabinets was equilibrated, the clean plates covered with Parafilm were quickly placed in each dampproof cabinet. Moreover, the Parafilm in the 10% RH cabinet was marked with A1,A2 and A3 at different locations, while the Parafilm in the 50% RH cabinet was marked with B1,B2 and B3 and the Parafilm in the 90% RH cabinet was marked with C1,C2 and C3. With this set up, the incubations of rAAV virus at different RH could be analyzed.

In a biological safety cabinet, the formvar-carbon coated copper grid was washed with a 0.1% BSA solution, then distilled deionized water and air-dried. A 3 µl suspension of rAAV sample diluted 2×10^4 times with 1×PBS was put on six of the washed formvar-carbon coated copper grids. After the rAAV sample was adsorbed

Table 1. rAAV incubation to different humidity during different incubation times at 22°C.

Incubation time (h)	Relative humidity (RH)		
	Low RH: (10%)	Middle RH: (50%)	High RH: (90%)
24	A3	B3	C3
48	A2	B2	C2
72	A1	B1	C1

Control experiments without incubation in RH were also performed.

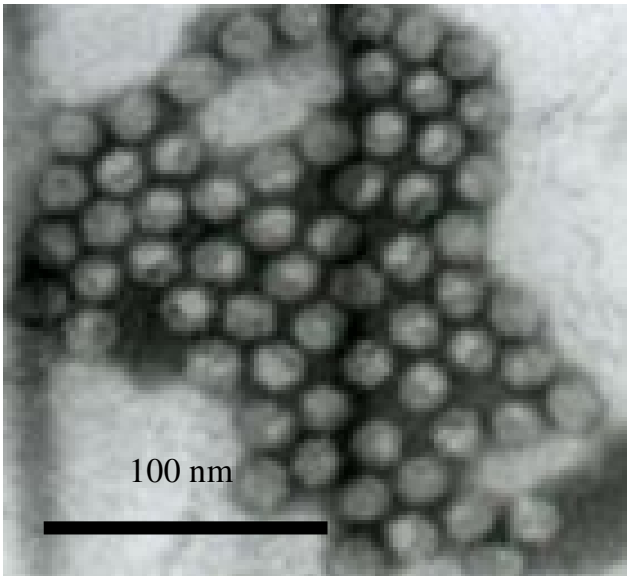

Figure 1. TEM image of rAAV in PBS buffer with no incubation in RH at 22℃ (magnification 150 K).

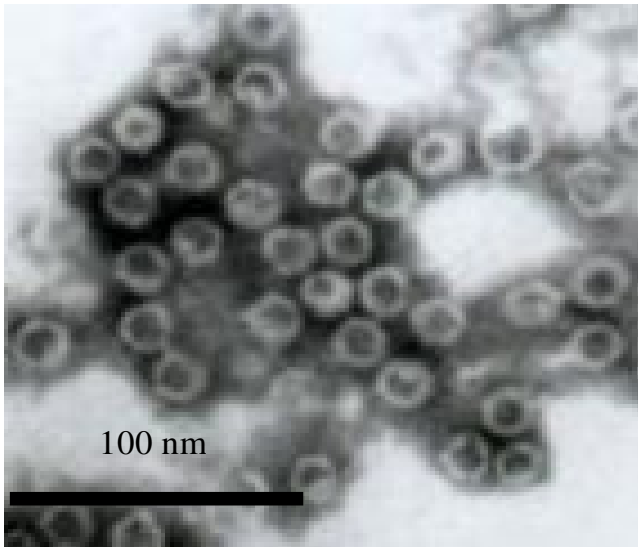

Figure 2. TEM image of rAAV under 10% ± 2% RH, 22℃ for 24 h (magnification 150 K).

to the copper grid for ten minutes, excess liquid was drained off by touching edge of the grid to a piece of clean filter paper. The grids adsorbed with rAAV sample were placed at locations of A1, B1or C1 on the Parafilm in different dampproof cabinets in duplicate for 72 h. One day later, the same preparation of rAAV sample was applied to the formvar-carbon coated copper grids and the grids were placed at locations of A2, B2 or C2 of the Parafilm in different dampproof cabinets (two grids in each location) and left for 48 h. Two days after the initial rAAV preparation, the rAAV sample was spotted on the formvar-carbon coated copper grids and the grids were put at locations of A3, B3 or C3 of the Parafilm in different dampproof cabinets (two grids in each location) and left for 24 h. On the third day, all grids in different dampproof cabinet were removed and negative stained for TEM detection. This experimental procedure design could be shown by Table 1.

After the grids were removed from the dampproof cabinets, the grids were touched (filmed side down) to a drop of 0.2% polyformaldehyde for 3 minutes to fix the rAAV. Excess polyformaldehyde was drained and the grids were subsequently touched (filmed side down) to a drop of 2% phosphotungstic acid (PTA ,PH6.8) for about 3-5 minutes, and drained as above. Grids were allowed to dry for a few minutes, before photographing from five different angles on the grid using a Toshiba HITACH H-7000 transmission electron microscopy with an acceleration voltage of 90 kV. At the same time, two control experiments in which rAAV samples were diluted the same as above with 1×PBS and were immediately analyzed according to the above protocol. The pictures of 50 rAAV virions in each RH and corresponding incubation time were measured and analyzed using the IMS cell image analysis system. The measured parameters of the rAAV virus included length of the minor axis, length of the major axis and ellipticity (the ratio of the length of the minor axis to the length of the major axis).

The t-test for assessing a statistical difference between two means was applied in statistical analysis to compare the conformation parameters of rAAV viruses measured under different relative humidity and incubation time.

RESULTS

TEM of rAAV in different relative humidity (RH) and incubation time

The rAAV sample grids were taken out of the cabinets observed and photographed using the HITACH H-7000 TEM. The TEM pictures of rAAV are shown in Figures 1 - 10.

The statistical results of rAAV conformation parameters

The pictures of the rAVV were measured and counted

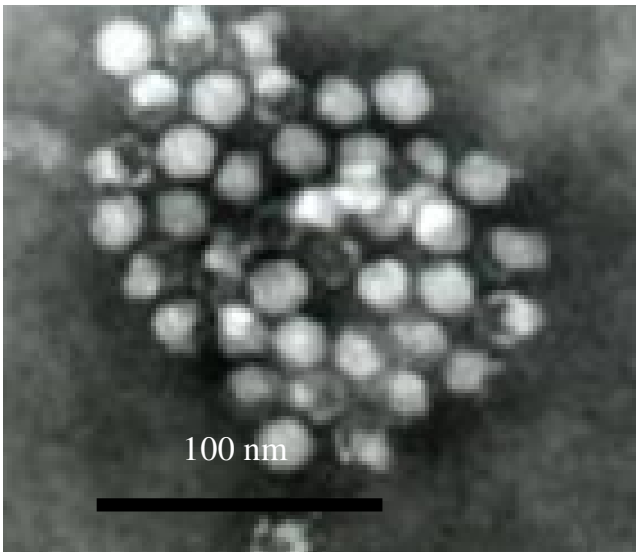

Figure 3. TEM image of rAAV under 10% ± 2% RH, 22℃ for 48 h (magnification 150 K).

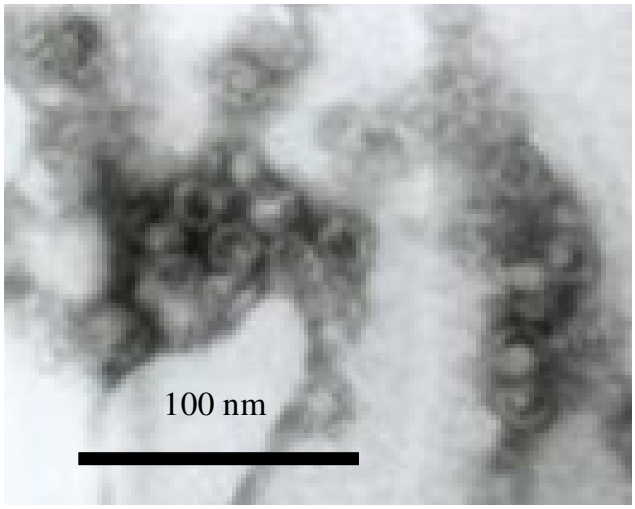

Figure 4. TEM image of rAAV under 10% ± 2% RH, 22℃ for 72 h (magnification 150 K).

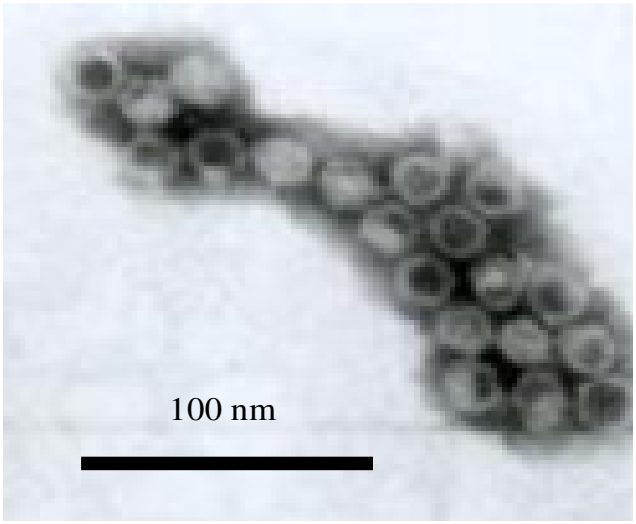

Figure 5. TEM image of rAAV under 50% ± 2% RH, 22℃ for 24 h (magnification 150 K).

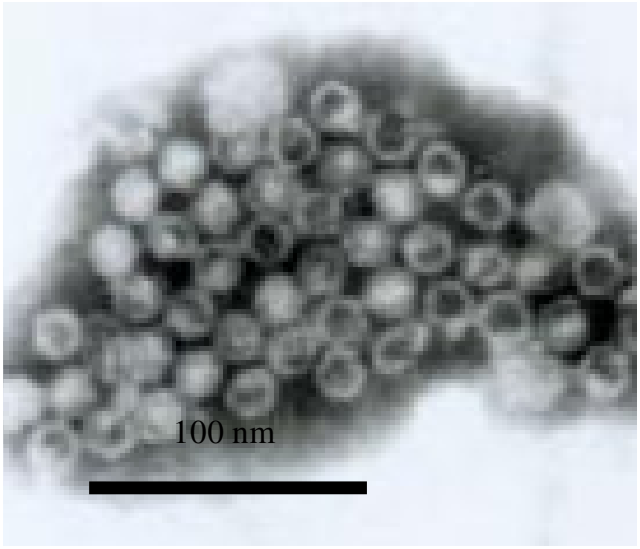

Figure 6. TEM image of rAAV under 50% ± 2% RH, 22℃ for 48 h (magnification 150 K).

using the IMS cell image analysis system. Through the statistical analysis, the rAAV main conformation parameters were: mean length of the major axis, standard deviation of the major axis, mean length of the minor axis, standard deviation of the minor axis, mean ellipticity and standard deviation of the ellipticity under different relative humidity (RH) and incubation time. These parameters are shown in Table 2. The comparison of rAAV mean ellipticity in different RH and incubation time at 22℃ was performed using the most common t-test for testing a distinct difference between two sample means. The results of the comparison analysis are show in Table 3. As indicated by Table 3, the RH had an evident effect on the conformation (mean ellipticity) of the rAAV virion. With longer incubation time, especially after 48 h incubation time, the conformation (mean ellipticity) of the rAAV virion in low RH for 72 h incubation was significantly different from those in middle RH and high RH for 48 h and 72 h incubation respectively. The results above suggested that different RH took a different effect on the conformation of the rAAV virion.

The variation of rAAV conformation parameters

Using the data in Table 2, the variation of mean length of

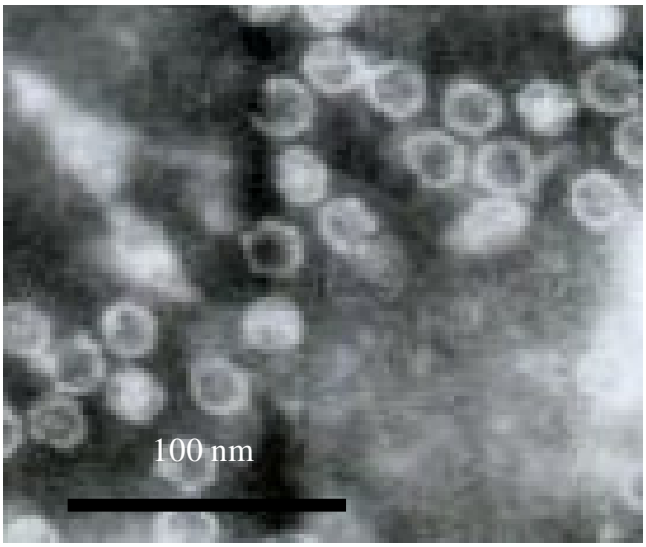

Figure 7. TEM image of rAAV under 50% ± 2% RH, 22°C for 72 h (magnification 150 K).

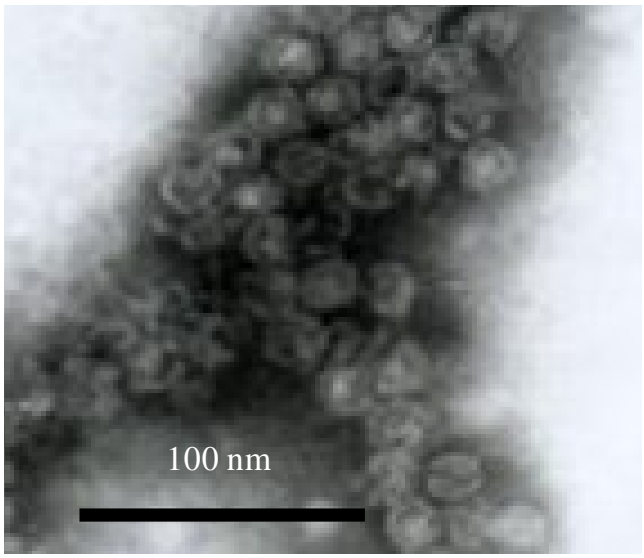

Figure 8. TEM image of rAAV under 90% ± 2% RH, 22°C for 24 h (magnification 150 K).

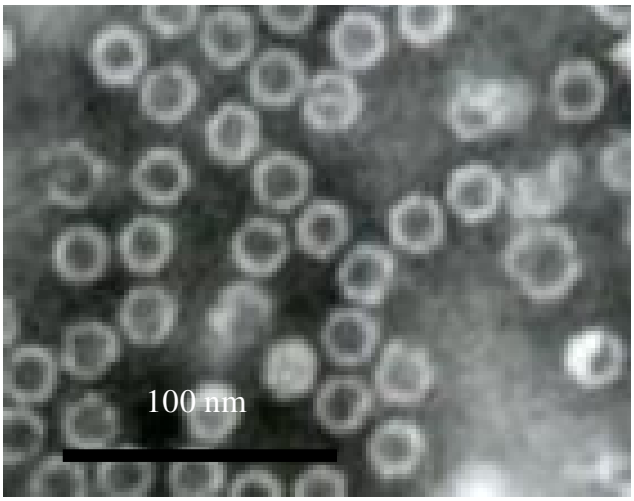

Figure 9. TEM image of rAAV under 90% ± 2% RH, 22°C for 48 h (magnification 150 K).

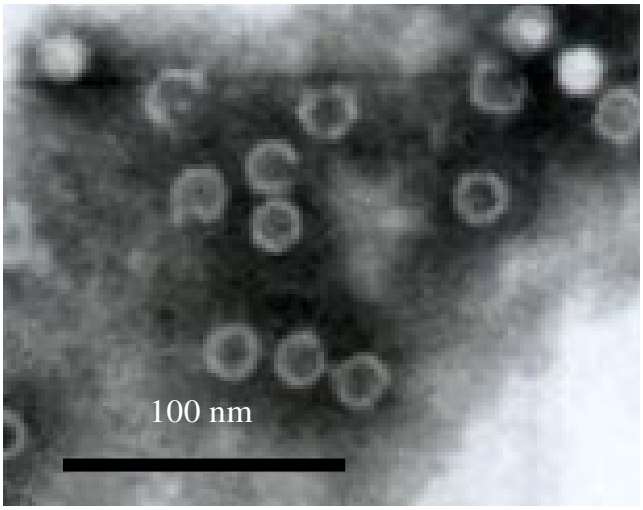

Figure 10. TEM image of rAAV under 90% ± 2% RH, 22°C for 72 h (magnification 150 K).

the rAAV virion minor and major axis was plotted with incubation time in the three different RH conditions (Figure 11). Figure 11 indicates that the mean length of the minor and major axis gradually increased along with the incubation time (24-72 h) in low RH. This implies that the volume of the rAAV virion constantly increases with the incubation time in low RH so that the rAAV virion was easily affected by outside environment and became unstable. On the contrary, the mean length of the minor and major axis rapidly decreased with the incubation time extending from 48-72 h in high RH, which implied that the volume of rAAV virion would start to decrease with the incubation time. Therefore, in high RH the rAAV virion was not easily affected by outside environment and became more stable in high RH comparable to low RH. In middle RH condition, the mean length of the minor and major axis also gradually increased along with the incubation time from 48 - 72 h, but the final mean length of the minor and major axis in middle RH for 72 h incubation time fell between these parameters measured for low and high RH.

To compare the conformational (ellipticity) changes of the rAAV virion with incubation time at the three different RH conditions, a plot of ellipticity versus time was constructed using the data in Table 2 (Figure 12). Figure 12 indicates that the conformation of the rAAV virion almost approaches a round shape under the three different RH

Table 2. Mean length and standard deviation of rAAV virion major and minor axis, mean ellipticity and standard deviation at room, low, middle and high RH at 22°C for 0, 24, 48 and 72 h incubation time, respectively.

rAAV virion condition	Mean length of major axis (nm)	Standard deviation of major axis	Mean length of minor axis (nm)	Standard deviation of minor axis	Mean ellipticity	Standard deviation of ellipticity
rAAV in room RH for 0 h	24.77	1.78	21.84	1.57	0.8835	0.05654
rAAV in low RH for 24 h	20.44	1.24	18.97	1.05	0.9302	0.05295
rAAV in low RH for 48 h	21.08	2.18	19.21	2.06	0.9122	0.05104
rAAV in low RH for 72 h	23.05	1.24	21.32	1.08	0.9266	0.04988
rAAV in middle RH for 24 h	20.71	0.96	19.16	1.23	0.9254	0.04731
rAAV in middle RH for 48 h	20.09	0.94	18.99	0.96	0.9461	0.04138
rAAV in middle RH for 72 h	21.50	1.41	19.27	0.98	0.8988	0.05761
rAAV in high RH for 24 h	21.47	1.24	18.92	1.22	0.8829	0.05859
rAAV in high RH for 48 h	21.87	1.11	19.81	1.15	0.9069	0.05254
rAAV in high RH for 72 h	20.34	1.28	18.41	1.21	0.9065	0.05640

Table 3. T-test results for the comparison of rAAV mean ellipticity at different RH and incubation time at 22°C.

Comparison (t-test)	rAAV in low RH for 24 h	rAAV in low RH for 48 h	rAAV in low RH for 72 h	rAAV in mid RH for 24 h	rAAV in mid RH for 48 h	rAAV in mid RH for 72 h	rAAV in high RH for 24 h	rAAV in high RH for 48 h
rAAV in low RH for 24 h								
rAAV in low RH for 48 h	NSD							
rAAV in low RH for 72 h	NSD	NSD						
rAAV in mid RH for 24 h	NSD	NSD	NSD					
rAAV in mid RH for 48 h	NSD	NSD	SD	SD				
rAAV in mid RH for 72 h	SD	NSD	SD	SD	SD			
rAAV in high RH for 24 h	SD	SD	SD	SD	SD	NSD		
rAAV in high RH for 48 h	SD	NSD	SD	NSD	SD	NSD	SD	
rAAV in high RH for 72 h	SD	NSD	SD	NSD	SD	NSD	SD	NSD

SD: Significant difference ($p < 0.05$); NSD: no significant difference ($p > 0.05$).

conditions. Moreover, the mean ellipticity of the rAAV virion would almost tend to approach the identical value of 0.9 for 48 and 72 h incubations respectively in high RH, while the mean ellipticity values of the rAAV virion varied during the same incubation time in low and middle RH. The results above illuminated the mean ellipticity of the rAAV virion was virtually invariable from 48 - 72 h incubation at high RH, but obviously variable for the same incubation time at low RH and middle RH respectively. In addition, Figure 12 shows that the conformation of the rAAV virion was closer to round in air than in liquid.

DISCUSSION

For some time, researchers have studied the effect of relative humidity (RH) on the viability and infectivity of airborne virus from a macroscopic point of view. Arundel et al. (1986) and Knight (1980) reported that RH had an important effect on the viability of airborne virus at room

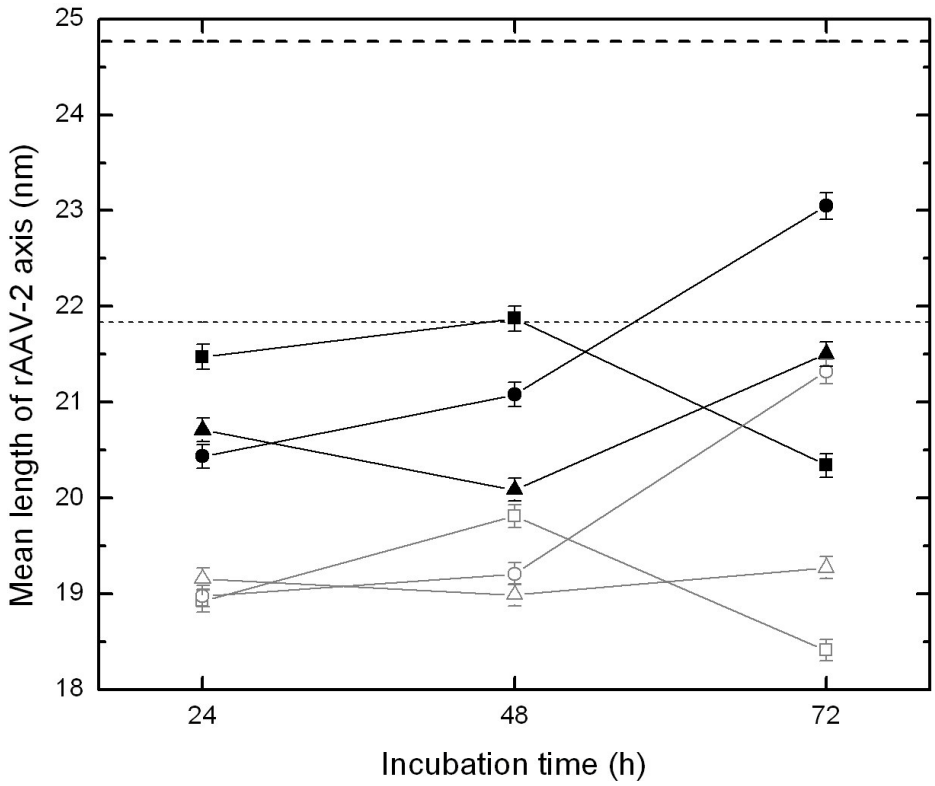

Figure 11. Variation of the mean length of the rAAV virion major and minor axis with incubation time at high, middle and low RH. The variation of the mean length of the rAAV virion major axis (the thick dashed) and the minor axis (the thin dashed) in control experiment (in buffer, no incubation time in RH); the mean length of the rAAV virion major axis (■) and that of the minor axis (□) in high RH (90% ± 2%); the mean length of the rAAV virion major axis (▲) and that of the minor axis (Δ) in middle RH (50% ± 2%); the mean length of the rAAV virion major axis (●) and that of the minor axis (○) in low RH (10% ± 2%).

temperature and that this effect of RH on the virus was primarily due to the virus' molecular conformation. In general, virus consisting of only nucleotide and protein without a lipid envelope can easily survive in high RH conditions (RH > 70%), while virus having a lipid envelope can survive in low RH conditions (RH < 50%). In this investigation, we used a new research approach to explore the RH effect on virus so as to illustrate the effect of RH on the conformation of the virus without lipid envelope from microscopic point of view. In general, the macroscopic behavior of viruses such as viability and infectivity directly result from an accumulation of microscopic conformation changes of the individual virus.

In our study, we found that the effect of different RH on the conformation of rAAV virion without a lipid envelope varied with time. The rAAV virion conformation tended to be smaller and more stable in high RH than in low RH. At high RH, the rAAV virion started to gradually become smaller with incubation time (from 48-72 h) thereby reducing surface area and minimizing interaction with the outside environment which suggests that the rAAV virion is more stable at high RH comparable to low RH. Moreover, the ellipticity of rAAV virion tended to the approach the identity value 0.9 with incubation time from 48-72 h in high RH, further more, this trend will be probably kept with incubation time extension. This also seemed to verify the fact that the rAAV virion conformation tended to more stable in high RH, while the ellipticity of rAAV virion tended to be changeable over the entire incubation time from 24 - 72 h at low and middle RH, which suggestes the rAAV virion conformation is more unstable in low and middle RH comparable to high RH. Our findings are basically consistent with the previous results reported by Arundel et al. (1986) and Knight (1980). Furthermore, our results illustrate that the relationship between the microscopic structure and the macroscopic behavior of virus is essential to our completely understanding the effect of RH on the conformation of virus without a lipid envelope. Even with our preliminary result suggestion for the effect of RH on rAAV, many important questions still remain unanswered. For example, at present, very little is known about the mechanism by which the RH affects the conformation and stabilization of virus without lipid envelope and with lipid envelope. Although RH can affect the conformation of rAAV, there is no clear evidence to show that RH can

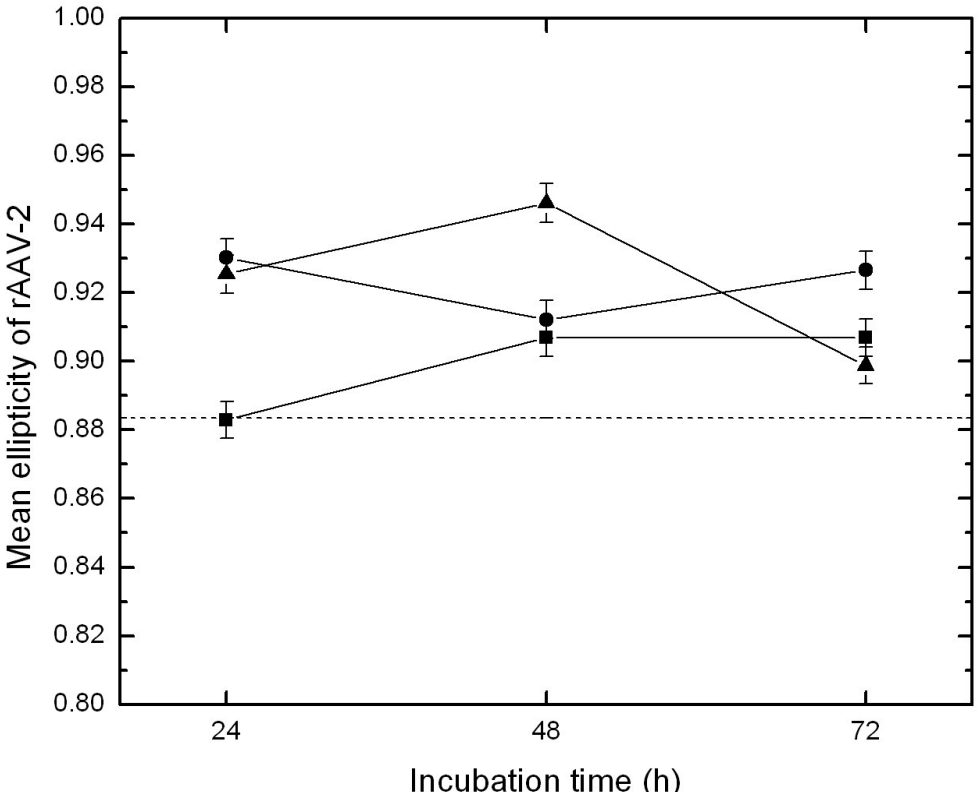

Figure 12. Variation of the mean ellipticity of the rAAV virion with incubation time at high, middle and low RH. The variation of the mean ellipticity of the rAAV virion in control experiement (in buffer, no incubation time in RH) (the dashed); in high RH (90% ± 2%) (■); in middle RH (50% ± 2%) (▲); in low RH (10% ± 2%) (●).

directly affect the stabilization of rAAV. In addition, after all, there is a difference between the wild type AAV and the recombinant AAV, therefore, the effect of RH on the wild type AAV and the recombinant AAV is supposed to be different. Further studies on these questions above are needed.

ACKNOWLEDGMENTS

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