Characterization of virus-like particles by atomic force microscopy in ambient conditions

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Abstract
Recombinant virus-like particles (VLPs) are attractive candidates for vaccine design since they resemble native viroids in size and morphology, but they are non-infectious due to the absence of a viral genome. The visualization of surface morphologies and structures can be used to deepen the understanding of physical, chemical, and biological phenomena. Atomic force microscopy (AFM) is a useful tool for the visualization of soft biological samples in a nanoscale resolution. In this work we have investigated the morphology of recombinant surface antigens of hepatitis B (rHBsAg) VLPs from Cuban vaccine against hepatitis B. The rHBsAg VLPs sizes estimated by AFM between 15 and 30 nm are similar to those reported on previous transmission electron microscopy (TEM) studies.

Keywords: hepatitis B, AFM, rHBsAg, virus-like particles

Classification numbers: 2.04, 2.05, 5.08

1. Introduction
In recent years, virus-like particles (VLPs) have received widespread attention as vaccine candidates [1]. VLPs are formed by proteins derived from viruses. A suitable heterologous expression system allows the production of structural proteins involved in viral capsid formation that self-assemble into VLPs. The assembly process does not require viral components, such as multiple structural or non-structural proteins and viral genome [2, 3]. VLPs cannot cause any infection because they contain no genetic material from the virus itself [1]. Protein–protein interactions in VLPs are relatively strong and can result in the formation of stable structures. In some cases these proteins are embedded within lipid bilayers [2, 3]. The knowledge about the assembly process of the VLP is vital to set the usefulness of such particles to the presentation of their own or foreign epitopes as carriers for proteins transiently expressed as a means of vaccine production. VLPs used as a platform technology to develop vaccines are very effective in causing high immune responses [4, 5].

The hepatitis B virus (HBV) is a member of the hepadnavirus family. It has a circular genome of partially double-stranded DNA (DNA virus). The virus consists of a nucleoprotein core containing the genome surrounded by a protein capsid known as the hepatitis B core antigen (HBcAg) [6]. This nucleocapsid is surrounded itself by an envelope of the hepatitis B surface antigen (HBsAg) monomer and host-derived lipids [7].

The HBsAg is a lipid enveloped VLP expressed by recombinant engineering. It forms non-infectious particles that can elicit the induction of antibodies as during an infection. The HBsAg VLPs are 22 nm spherical lipid–protein particles [8] composed of three envelope proteins [7] that produce a strong protective immune response [9].
The recombinant hepatitis B (rHBsAg) vaccine, developed in the 1980s, was the first commercial vaccine based on VLPs; the VLPs were cloned and expressed in *Saccharomyces cerevisiae* [10].

The current size measurement techniques used in the characterization of VLPs are the transmission electron microscopy (TEM) [11–14], the dynamic light scattering (DLS), the static light scattering [15, 16] and the atomic force microscopy (AFM) [17]. TEM provides information of size, number, and form of vesicles, but vesicles are subject to deformation caused by the high vacuum conditions and the dyeing processes. DLS has become a regular and convenient technique for measurement of vesicle size, but precise and accurate results are obtained only for vesicles having a narrow size distribution. As AFM technology progresses and improves, it has been a promising tool for biological application and it occupies a unique position among the methods of direct visualization available for the biologist today. AFM is a powerful technique for measuring the vesicle size and size distribution in ambient conditions. Single particles can be imaged without a high cost for sample preparation [16]. AFM relies on the local interaction between a probe tip, which acts as a force sensor and the surface of a sample, yielding images that can reflect both the topography and the physical characteristics of surfaces.

In our study, AFM was used to characterize the rHBsAg VLPs in ambient conditions. The particle sizes were estimated by two methods: one based on the measured height and another based on the measured width of the particles. The changes induced on the VLPs by the measuring time are also discussed.

### 2. Materials and methods

The recombinant surface antigens of hepatitis B (rHBsAg) particles were supplied by the Center for Genetic Engineering and Biotechnology (Havana, Cuba) from C203S batch (0.61 mg ml$^{-1}$) according to Muzzio et al [14]. The original solution was diluted 1 : 300 with redistilled water. An aliquot of the diluted rHBsAg was deposited on a freshly cleaved mica surface for 2 h at room temperature to allow passive adsorption. Then the sample was rinsed three times with redistilled water to remove salts and unattached molecules, and dried in a dry chamber at room temperature for 20 min.

The AFM images were collected with an NTEGRA Vita (NT-MDT, Russia) system in air at ambient temperature. The semi-contact mode and silicon cantilevers (NSG10 type, NT-MDT) were employed for imaging in air. The tip radius was obtained by employing test grating TGT1 (NT-MDT). The set point voltage was generally adjusted for optimum image quality (70–80% of the free oscillation amplitude). The images were recorded at 0.5 Hz scan rate, and 512 pixel × 512 pixel.

The images were processed using the Gwyddion 2.30 software. The real diameter $D$ of particles was estimated using the equation

$$D = 2 \left( \sqrt{R^2 - \frac{d^2}{4}} - R \right),$$

where $d$ is the apparent diameter measured at half-height and $R$ is the spherical radius of the tip [17]. Statistical analyses were performed using Origin Pro version 8.0 software.

### 3. Results and discussions

#### 3.1. Estimation of the particle size

For determination of rHBsAg particles morphology by AFM, a solution of around 2 μg ml$^{-1}$ was used in order to obtain satisfactory AFM images. Figures 1(A) and 1(B) show typical AFM images of rHBsAg VLPs in ambient conditions. The topographic image reflects a lower aggregation of these particles at this concentration, and it is possible to perform the size measurements using height profiles (figure 1(C)). In particular, the measured VLPs’ width is larger than the real one as a result of broadening effects of the tip size. The estimated tip radius was $R = 12$ nm. This value was used in equation (1) to determine the particle size. The average diameter value of the rHBsAg VLPs was 21.9 nm with standard deviation of 4.5 nm (figure 1(D)).

On the other hand, the protein shell provides stiffness to this VLP structure. If we assume that the particles are not deformable under the applied force, the vertical height of the particles gives an accurate value for their true diameter [18]. A histogram of the heights of rHBsAg is shown in figure 1(E). The average height value was 19.5 ± 5.4 nm.

It is clear that the estimated particle diameter using equation (1) is similar to the diameter based on measurements of vertical heights. In addition, these results are similar to those obtained by TEM studies [11–14]. Therefore, both pathways can be used for determining the size of rHBsAg VLPs.

#### 3.2. Influence of the measuring time

The adsorption of the particle to the mica surface in ambient conditions could cause deformation of the particle, aggregation, and dehydration. For that reason, we performed an AFM study of the particles 4 h after drying them.

The height images (figures 2(A) and 2(B)) show partially flattened rHBsAg particles. Similar results have been reported for other proteoliposomes [16, 19]. Figure 2(C) shows that the particles are partially flattened. This makes it difficult to determine the particle size.

### 4. Conclusions

AFM was successfully used to characterize the size of the rHBsAg VLPs under ambient conditions. The particles’ diameters were estimated by two methods based on the height images. The measured diameters are the same as those reported in previous TEM studies. Nevertheless, the measurements under ambient conditions must be performed as soon as possible. Images recorded 4 h after drying show clear structure degradation.

It is recommended to perform the experiments under physiological conditions to prevent the VLP’s structure degradation. In addition, it would be necessary to perform a larger number of experiments varying different experimental conditions.
Figure 1. Atomic force images of rHBsAg VLPs on mica surface. (A) Height image; (B) higher resolution height image, vertical scales being 25 nm; (C) height profile of a single rHBsAg VLP; (D) distribution of the estimated particle diameters based on equation (1); and (E) distribution of the estimated particle diameter based on the measured heights.

Figure 2. Atomic force images of VLPs after 4 h adsorption. (A) Height image. The vertical scale is 20 nm. (B) Magnification of a single rHBsAg VLP of (A). The height has diminished and the zone around the particle appears to be melted. This is confirmed in the profile across the solid line shown in (C).
conditions (sample concentration below 2 µg ml\(^{-1}\), ionic strength, pH and temperature) in order to get a wider range of data.

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