Characterization of the size distribution and aggregation of virus-like nanoparticles used as active ingredients of the HeberNasvac therapeutic vaccine against chronic hepatitis B*

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Abstract
The use of virus-like particles (VLPs) as antigens constitutes a well established strategy in preventive vaccination. These non-infective particles have a composition, size, and structure favoring their interaction and processing by the immune system. Recombinant viral nucleocapsids encapsulating bacterial nucleic acids result in potent Th1-driving immunogens. Several antigens have been coadministered with VLPs or conjugated to them to further increase their immunogenicity. In the present work we characterize the size distribution of two different recombinant VLPs obtained as components of HeberNasvac, a novel therapeutic vaccine recently registered to treat chronic hepatitis B. The vaccine ingredients, hepatitis B virus surface and nucleocapsid antigens (HBsAg and HBCAg, respectively) and the vaccine formulation, were evaluated using dynamic light scattering (DLS), transmission electron microscopy (TEM) and light obscuration technology. The results demonstrate that both antigens are nanoparticles with sizes ranging between 20–30nm, in line with reports in the literature. In addition, DLS studies evidenced the capacity of both antigens to form homologous and heterologous aggregates, both as active ingredients as well as being part of the final product. The evaluation of subvisible particles in HeberNasvac formulation fulfills the requirements in terms of quantity and size established for parenteral pharmaceutical compositions.

Keywords: virus-like particle, nanovaccines, HBsAg, HBCAg
Classification numbers: 2.04, 4.02, 5.08
1. Introduction

Virus-like particles (VLPs) are self-assembling multi-protein nanoparticles that mimic the structural organization and conformation of viruses. VLPs are useful in medicine and nanotechnology. Their repetitive molecularly-defined architecture is attractive for engineering multivalency, notably for vaccination. VLPs enable their antigenic and carrier function both by surface display as well as a delivery system for encapsulation, providing versatility as vaccines and in general as a biomedical research tool [1].

Proteins that assemble into VLPs derive from a variety of viruses, including HIV-1 [2–4], rubella virus [5], human papillomavirus [6, 7], Semliki forest virus [8], RNA phages [9], and hepatitis B virus (HBV) [10, 11]. Due to the highly repetitive structure of such VLPs they are very immunogenic for B cells and induce strong and long-lasting IgG responses in the absence of adjuvants [12, 13]. The 20–200 nm size of VLPs facilitates direct drainage to lymph nodes, increasing uptake by antigen-presenting cells and cross-presentation [14, 15]. VLPs also efficiently reach the MHC class I pathway in vivo. Also, the RNA or DNA packaged in VLPs triggers costimulatory signals through Toll-like receptors TLR3/7/8/9 [15, 16]. Considering all the above information, display on VLPs greatly enhances the response to otherwise poorly immunogenic epitopes [14, 17].

Chronic hepatitis B (CHB) infection remains a health problem worldwide with more than 250 million people in this condition. Persistent infection causes serious liver complications like cirrhosis and hepatocellular carcinoma [18]. Taking into account the limitations of current treatments for CHB, the development of a therapeutic vaccine has been considered a promising immuno-therapeutic approach [19]. In this sense, HeberNasvac was designed as a formulation based on the HBV surface and nucleocapsid antigens (HBsAg and HBcAg, respectively) [20]. This formulation is highly immunogenic after mucosal and/or parenteral administration [21, 22]. HeberNasvac have already completed several clinical trials in CHB patients with successful results [23, 24] The active ingredients HBsAg and HBcAg antigens were obtained as VLPs by recombinant methods in Pichia pastoris [25]. The HBcAg was expressed in E. coli strain W3110 transformed by a recombinant expression vector under the control of a tryptophan promoter. The resulting HBcAg had a purity of >95%. HeberNasvac formulation containing both antigens was obtained by simple mixture in phosphate saline buffer [20].

The HBsAg samples of active pharmaceutical ingredient (API) were selected among batches obtained between 2009 and 2014. The HBcAg samples were selected from batches obtained between 2005 and 2012. The evaluation of HeberNasvac formulation was conducted in batches produced from 2007 to 2015 (table 1). All batches were produced and released at CIGB and were stored at 4 °C since production. They were kept as witness samples of the product under controlled conditions.

2. Materials and methods

2.1. Samples

HBsAg was produced to more than 95% purity at the Center for Genetic Engineering and Biotechnology production facilities (CIGB, Havana, Cuba) as a component of the commercial HBV prophylactic vaccine, Heberbiovac-HB. HBsAg for this vaccine is expressed and purified from the yeast P. pastoris. HBcAg was expressed in E. coli strain W3110 transformed with a plasmid containing the entire core antigen gene under the control of a tryptophan promoter. The resulting HBcAg had a purity of >95%. HeberNasvac formulation containing both antigens was obtained by simple mixture in phosphate saline buffer [20].

| Sample       | Batch number | Average particle size (nm) | Manufacturing date |
|--------------|--------------|---------------------------|--------------------|
| HBsAg        | 02.IFA.C927S | 47.9                      | 09/2009            |
|              | 02.IFA.C008  | 60.7                      | 04/2010            |
|              | 02.IFA.C207S | 57.7                      | 04/2012            |
|              | 02.IFA.C208S | 66.4                      | 05/2012            |
|              | 02.IFA.C221S | 54.0                      | 09/2012            |
|              | 02.IFA.C315S | 50.4                      | 09/2013            |
|              | 02.IFA.C322S | 54.3                      | 01/2014            |
| HBcAg        | BM5055       | 40.2                      | 2005               |
|              | BN1104       | 41.1                      | 12/2011            |
|              | BN1106       | 41.4                      | 12/2011            |
|              | 12.IFA.1201  | 42.1                      | 09/2012            |
|              | 12.IFA.1204  | 39.5                      | 09/2012            |
| HeberNasvac  | HC071202     | 51.0                      | 12/2007            |
|              | HC080801     | 45.2                      | 08/2008            |
|              | HC080902     | 43.9                      | 09/2008            |
|              | HC090901     | 46.7                      | 09/2009            |
|              | 2N3HC01      | 53.0                      | 07/2012            |
|              | 2N3HC02      | 58.4                      | 12/2012            |
|              | 3N3HC01      | 54.4                      | 02/2013            |
|              | 3P2HC01      | 61.6                      | 02/2013            |
|              | 3P2HC02      | 50.12                     | 02/2013            |
|              | 5NN0201      | 55.7                      | 02/2015            |
|              | 5NN0401      | 57.4                      | 02/2015            |

2.2. Particle analyzer

A Delsa Nano C Particle Analyzer (Beckman Coulter, USA) was used to measure the particle size distribution by DLS to different liquid formulations at 25 °C. The intensity of scattered light was detected at an angle of 165°.

2.3. Mathematic and statistic analysis

Mathematic and statistic analysis were used in Delsa Nano C Particle Analyzer software. The principle of the method is based on the fact that the diffusion rate of particles is determined by their size; information about their size is contained
in the rate of fluctuation of the scattered light. So, by correlating the fluctuation, we can determine the particle size distribution of the population present. The diffusion coefficient, $D$, is inversely proportional to the particle size according to the Stokes–Einstein equation

$$D = \frac{k_B T}{3\pi \eta d},$$

where $D$ is the diffusion coefficient, $k_B$ is Boltzmann’s constant, $T$ is absolute temperature, $\eta$ is viscosity and $d$ is the hydrodynamic diameter. The results were compiled and the mean value of size was determined using the peak value as provided by the equipment.

2.4. Transmission electron microscopy

This technique was used for the characterization of the formulation and each individual antigen. Briefly, 25 $\mu$l of each sample (0.2 mg ml$^{-1}$) were placed on a copper grids (400 mesh), previously coated with formvar-carbon membrane, in the vacuum evaporator (JEOL-JEE-4X, Japan). Later on, the grids were incubated during 30 s with 2% uranyl acetate solution and subsequently washed with bi-distilled water and dried. The samples were visualized using a TEM (JEOL 1400X, Japan) with accelerating voltage of 80–100 kV and magnification between 50000 and 100000 times. For each sample, three random fields of each grid were photographed and analyzed. The particle size was estimated by digitalized measurement using the Image J software (Maryland, USA). The average particle size was calculated after analyzing at least 100 nanoparticles.

2.5. Subvisible particle measurement

The light obscuration method was employed to evaluate the presence of subvisible particles in the HeberNasvac vaccine formulation. A KL-04A liquid-borne particle counter (Rion, Japan) was used. The number of particles over 10 and 25 $\mu$m were quantified. The USP and EP pharmacopeia acceptance criteria were considered.

3. Results

3.1. Particle size distribution by DLS

The source of recombinant HBsAg nanoparticle is the CIGB’s industrial facility for Heberbiovac, the vaccine for anti-Hepatitis B prophylaxis during more than 25 years [25]. The HBCAg nanoparticle is also an antigen produced under GMP at the CIGB facilities. The particle size distribution of both VLPs was evaluated by DLS using several batches. Figure 1 shows the DLS results obtained for three individual batches of HBsAg, HBCAg and HeberNasvac; representatives of the batches in table 1.

A similar size range and relatively similar particle size distribution was observed in all cases; however, a slightly wider particle size distribution was detected in the HBsAg preparations due to the normal polydispersity of samples. Table 1 summarizes the average particle size of all the studied samples. In the case of recombinant HBCAg, HBsAg and HeberNasvac, the results for the three batches are shown in figures 1(a)–(c), respectively. In general, for all the evaluated samples, the particle size distribution was in the range between 20 and 180 nanometers (nm). The average size values...
were similar between each independent API and the final vaccine formulation (table 1).

3.2. Morphology and VLP size estimation by TEM

TEM was used to analyze the morphological aspect of the VLPs and measure the individual particle size per sample. In this study we didn’t carry out the measurement of the intrinsic agglomerates in each preparation. More than five batches of HBsAg, HBcAg and HeberNasvac were evaluated. Figure 2 comprises representative results for each active ingredient and for the final formulation in three replicates each.

The HBsAg preparations microphotographs show spherical nanoparticles of approximately 20–30 nm. The presence of dimeric or multiple valence particle agglomerates were observed. The HBcAg nanoparticles are similar to HBsAg in shape and size; however they can be identified by the presence of a high electronic density in the HBcAg central region. The HBcAg preparations also show agglomerated structures. In the HeberNasvac samples we observed that the antigens comprised in the formulation are able to agglomerate although the individual antigens conserve their specific morphology.

3.3. Subvisible particle evaluation

The presence of subvisible particles was evaluated in sixteen HeberNasvac and two placebo batches by the method of light obscuration using a liquid-borne particle counter KL-04A (Rion, Japan). The data obtained is presented in table 2. All the measures fulfilled the USP and EP pharmacopeia acceptance criteria for vaccine preparations (6000 particles or less in the range of less than 25 nm and 600 particles or less between 25 and 50 nm range).

4. Discussion

The use of VLPs in vaccination is based on their favorable interaction with immune system cells [1, 14]. The present study characterizes by TEM and DLS techniques the physical aspect and size distribution of the VLPs and protein agglomerates found in the active ingredient preparations as well as in HeberNasvac, and quantifies the agglomerates of VLPs that reach the subvisible range of size using the light obscuration method.

DLS was an important tool for characterizing the size of the nanoparticles and the particle agglomerates of the HeberNasvac drug product and the active ingredients (HBsAg...
confirmed by TEM analysis. A similar result has been reported
depend on the 200 nm filter pore size at the sterilizing filtration
final product. The final size of the agglomerates in all cases may
protein agglomerates in the active ingredients as well as in the
arations (20 and 180 nm) can be justified by the formation of
HBsAg.
(52.49 nm) detected by DLS was similar to that obtained for
as expected. However, the final product average particle size
similar results to the antigens measured as active ingredients
vidual antigen within the HeberNasvac formulation rendered
HBcAg. The HBcAg individual nanoparticle size calculated by the
The measurement by TEM of the mean size of each indi-
agglomerates. Such agglomerates were also visualized by
The measurement by TEM of the mean size of each indi-
individual antigen within the HeberNasvac formulation rendered
similar results to the antigens measured as active ingredients
expected. However, the final product average particle size
In general, the DLS size distribution found in all prep-
maximum peak of size. The agglomerates of both VLPs can be
the final product, in both cases with a similar size range and
samples analyzed are polydisperse with polydispersity index between 0.15 and 0.25, in all cases less than 0.3.
The average particle size found for HBsAg (55.91 nm)
by DLS could be explained by the preferential formation of agglomerates in the preparation. The measurement of the size
for independent HBsAg nanoparticles carried out using TEM photographs and Imagen J software rendered a mean size of
23.09 nm, consistent with the previously reported HBsAg size [26, 27]. The association between two or more individual par-
References

and HBcAg) in solution. The samples analyzed are polydisperse with polydispersity index between 0.15 and 0.25, in all cases less than 0.3.
The average particle size found for HBsAg (55.91 nm) by DLS could be explained by the preferential formation of agglomerates in the preparation. The measurement of the size for independent HBsAg nanoparticles carried out using TEM photographs and Imagen J software rendered a mean size of 23.09 nm, consistent with the previously reported HBsAg size [26, 27]. The association between two or more individual particles was detected by TEM.
The HBcAg individual nanoparticle size calculated by the TEM technique (28.84 nm) was consistent with the reported value for this antigen [28]. The DLS analysis of the active ingredient (40.86 nm), suggested the formation of HBcAg agglomerates. Such agglomerates were also visualized by TEM. The HBsAg particle size is greater than HBcAg determined by DLS.
The measurement by TEM of the mean size of each individual antigen within the HeberNasvac formulation rendered similar results to the antigens measured as active ingredients as expected. However, the final product average particle size (52.49 nm) detected by DLS was similar to that obtained for HBsAg.
In general, the DLS size distribution found in all preparations (20 and 180 nm) can be justified by the formation of protein agglomerates in the active ingredients as well as in the final product. The final size of the agglomerates in all cases may depend on the 200 nm filter pore size at the sterilizing filtration final production step. The agglomeration of both antigens was confirmed by TEM analysis. A similar result has been reported for the HBsAg preparation [29], and was also preliminary reported for the HBcAg and the final formulation [20].
On the other hand, the morphological structure observed by TEM for each VLP correlates with data previously reported by other authors using similar antigen preparations [30, 31], including blood isolated HBV antigens [32, 33]. The presence of a central region of higher electronic density in the HBcAg nanoparticles indicates the presence of encapsulated nucleic acids. This feature of the HBcAg is relevant for vaccine purposes, considering the potent Th1 adjuvant effect that confers the DNA/RNA produced in bacteria [34, 35].
The formation of micrometric aggregates in the final product also constitutes an advantage for this vaccine preparation. It is reported that aggregated structures of sizes ranging between 20–200 nm are highly immunogenic in animals and humans. The presence of aggregates favors the uptake by antigen-presenting cells and the subsequent development of the immune response [14, 15, 36]. The above-mentioned characteristic of the final product is consistent with the promising results obtained for this formulation in the clinical evaluation [23, 24]
Analysis of subvisible particles by the light obscuration method indicates that the final product fulfilled the USP and EP pharmacopeia acceptance criteria for parenteral vaccine preparations. As expected from the size distribution found at the nanometric scale, the higher number of nanoparticles present in the vaccine formulation are between 10 and 25 μm. Although there is a variable amount of particles in both ranges of quantification, the numbers were in the range of acceptability for particles in parenteral formulations. Several batches, representative of a long period of time were analyzed, and the results were reproducible, demonstrating the robustness of the production processes in relation to the studied variables.
In conclusion, the characterization of the particle size distribution in the API and final vaccine formulation is consistent with the reported mean size of recombinant HBsAg and HBcAg particles. The agglomerates of VLPs are detected both by DLS and TEM in the active ingredients as well as in the final product, in both cases with a similar size range and maximum peak of size. The agglomerates of both VLPs can be quantified also by light obscuration and resulted in quantities below the range of acceptability according to major international pharmacopoeia.

Table 2. Average number of particles between 10 and 25 micrometers and between 25 and 50 μm measured by the light obscuration method.

| HeberNasvac batches | Number of particles between 10 and 25 μm per dose (1 ml) | Number of particles between 25 and 50 μm per dose (1 ml) |
|---------------------|----------------------------------------------------------|--------------------------------------------------------|
| 5NN0101             | 2084.1                                                   | 15.7                                                   |
| 5NN0401             | 1190.2                                                   | 8.3                                                    |
| 5NN0201             | 897.3                                                    | 5.5                                                    |
| 5NN0301             | 1699.6                                                   | 27.6                                                   |
| 4NN0501             | 1336.6                                                   | 15.1                                                   |
| 4NN0601             | 1324.3                                                   | 14.9                                                   |
| 4NN0701             | 764.7                                                    | 30.4                                                   |
| 4MM0301             | 137.5                                                    | 37.3                                                   |
| 4MM0401             | 176.5                                                    | 44.7                                                   |
| 4MM0501             | 116.7                                                    | 9.7                                                    |
| 3N3HC01             | 1256.4                                                   | 23.5                                                   |
| 3N3HC02             | 1231.7                                                   | 27.9                                                   |
| 3P2HC01             | 1632.3                                                   | 34.7                                                   |
| 3P2HC02             | 616.9                                                    | 6.5                                                    |
| 2N3HC02             | 1194.2                                                   | 11.0                                                   |
| 2N3HC01             | 2306.9                                                   |                                                         |
| 3P0HC01 (Placebo)   | 34.1                                                     | 0.3                                                    |
| 3N0HC01 (Placebo)   | 60.9                                                     | 0.3                                                    |
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